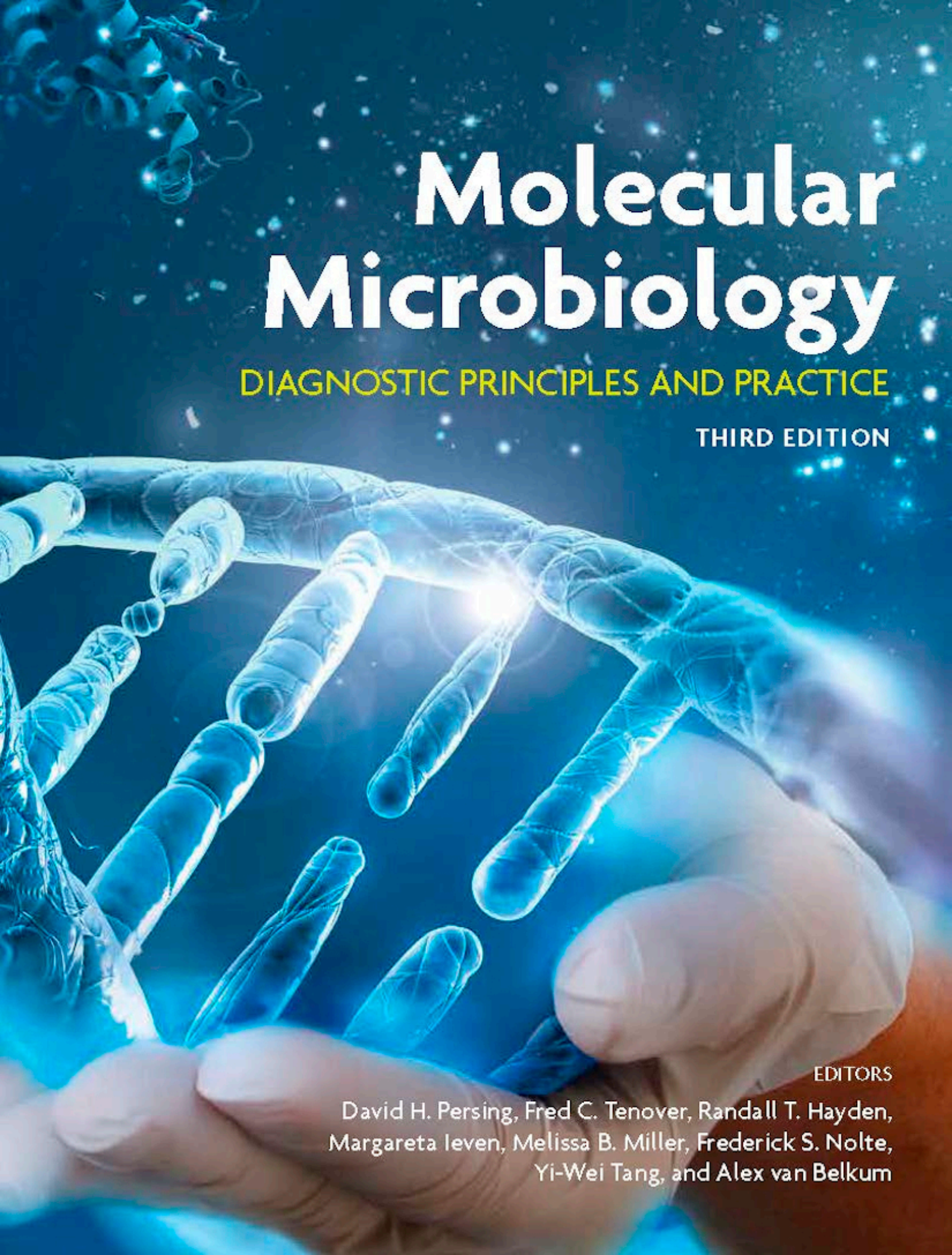
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DIAGNOSTIC PRINCIPLES AND PRACTICE
THIRD EDITION
EDITORS
David H. Persing, Fred C. Tenover, Randall T. Hayden,
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Contents

section IV
Molecular Diagnostics and Public Health

section V
Syndromic Diagnostics

section VI
Virology

section VII
Fungi and Protozoa

section VIII
Point-of-Care/Near-Care Diagnostics

section IX
The Host and Host Response

section X
Information Technology

section XI
Quality Assurance

section XII
The Business of Diagnostics

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Preface

In the 5 years since the 2011 edition of this book, the molecular diagnostics landscape has changed dramatically. In the 1990s, molecular diagnostics was the domain of only a few reference laboratories; it took almost 20 years for these techniques to make their way into about half of the CLIA high-complexity laboratories in the United States. The full potential of this technology was slow to be realized largely because the methods used by these laboratories were not capable of delivering on-demand results or being conducted at the point of care. Over the past year, with the advent of CLIA-waived molecular testing spurred on by the inexorable force of innovation, molecular diagnostics have become increasingly democratized to the extent that physician office laboratories and sexual health clinics are now performing molecular testing on the premises, often delivering results in minutes or a few hours.

Laboratory professionals may at times find themselves a bit bewildered in this rapidly evolving landscape. Adding to this, enter next-generation sequencing (NGS) technology, as described in several chapters in this book (chapters 2, 3, 5, 6, 10–14, and 53). NGS-based analysis of microbial genomes and populations is in some ways similar to where PCR was in 1987: full of opportunities and challenges. For the first time, identification of the full range of pathogens—viruses, bacteria, fungi, and protozoa—can be addressed by using the same core technology. Microbial population analysis can be carried out at unprecedented depth, opening up the field of metagenomics (chapters 10–14). Whole-genome analysis goes beyond organism identification to predict drug resistance and detect pathogenic determinants. As diagnosticians, it seems likely that as this field evolves, so will our job descriptions. Still, much progress remains to be made before NGS can move beyond its current status as a research tool. NGS systems need to become more automated and less expensive to operate. The analysis of complex data sets provided by these systems needs to be simplified; the interpretation of results cannot require a PhD in bioinformatics for delivery of routine results. However, as complex as it is now, NGS too will eventually become democratized by the integration of workflow automation, improvements in sequencing technology, and information technology (IT).

Speaking of which, IT itself is about to play an increasing role in how and to whom our results are delivered (section X). A rapid molecular result is only as good as the downstream action taken in the treatment and management of patients. As we speak, patients in London, along with providers, are getting "push notifications" of results from their sexual health tests, resulting in a dramatically shortened time to therapy. Cloud-based aggregation of molecular test data is providing snapshots of emerging pathogens and drug resistance in real time by collecting de-identified test data directly from testing platforms. From the respiratory cloud to the digital cloud, we are watching the emergence of a new generation of global surveillance capabilities which will be of enormous public health benefit. Rapid detection technologies are also likely to evolve in the direction of on-demand multiplexing for simultaneous detection of treatment-informing targets. The convergence of rapid molecular assays with improvements in IT to deliver actionable information to health care providers is becoming a reality.

In 2015, the White House announced a $20 million prize for innovative diagnostic tests that will lead to more precise antimicrobial therapeutic decisions. In addition, the United Kingdom has announced the Longitude Prize, a challenge with a £10 million award for developing a point-of-care diagnostic test that also will identify when antibiotics are needed and which one to use. Thus, it seems that the importance of molecular diagnostic testing is finally being appreciated at the highest levels, especially to address the global problem of antimicrobial resistance. Let's not disappoint them.

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section I

Novel and Emerging Technologies

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Nucleic Acid Amplification Methods Overview

FREDERICK S. NOLTE AND CARL T. WITTWER

1

The development of the polymerase chain reaction, or PCR, by Saiki et al. (1) was a milestone in biotechnology and heralded the beginning of the modern era of molecular diagnostics. Although PCR is the most widely used nucleic acid amplification strategy, other strategies have been developed, and several have clinical utility. These strategies are based on either signal or target amplification. Examples of each category will be discussed in the sections that follow. These techniques have sensitivity unparalleled in laboratory medicine, have created new opportunities for the clinical laboratory to impact patient care, and have become the new "gold standards" for laboratory diagnosis of many infectious diseases.

SIGNAL AMPLIFICATION TECHNIQUES

In signal amplification methods, the concentration of the probe or target does not increase. The increased analytical sensitivity comes from increasing the concentration of labeled molecules attached to the target nucleic acid. Multiple enzymes, multiple probes, multiple layers of probes, and reduction of background noise have all been used to enhance target detection (2). Target amplification systems generally have greater analytical sensitivity than signal amplification methods, but technological developments, particularly in branched DNA (bDNA) assays, lowered the limits of detection to levels that rivaled those of some earlier target amplification assays (3).

Signal amplification assays have several advantages over target amplification assays. In signal amplification systems, the number of target molecules is not altered, and as a result, the signal is directly proportional to the amount of the target sequence present in the clinical specimen. This reduces concerns about false-positive results due to cross-contamination and simplifies the development of quantitative assays. Since signal amplification systems are not dependent on enzymatic processes to amplify the target sequence, they are not affected by the presence of enzyme inhibitors in clinical specimens. Consequently, less cumbersome nucleic acid extraction methods may be used. Typically, signal amplification systems use either larger probes or more probes than target amplification systems and, consequently, are less susceptible to errors resulting from target sequence heterogeneity. Finally, RNA levels can be measured directly without the synthesis of a cDNA intermediate.

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bDNA

The bDNA signal amplification system is a solid-phase, sandwich hybridization assay incorporating multiple sets of synthetic oligonucleotide probes (4). The key to this technology is the amplifier molecule, a bDNA molecule with 15 identical branches, each of which can bind to three labeled probes.

The bDNA signal amplification system is illustrated in Fig. 1. Multiple target-specific probes are used to capture the target nucleic acid onto the surface of a microtiter well. A second set of target-specific probes also binds to the target and to preamplifier molecules, which in turn bind to up to eight bDNA amplifiers. Three alkaline phosphatase-labeled probes hybridize to each branch of the amplifier. Detection of bound labeled probes is achieved by incubating the complex with dioxetane, an enzyme-triggerable substrate, and measuring the light emission in a luminometer. The resulting signal is directly proportional to the quantity of the target in the sample. The quantity of the target in the sample is determined from an external standard curve.

Nonspecific hybridization of any of the amplification probes and nontarget nucleic acids leads to amplification of the background signal. To reduce potential hybridization to nontarget nucleic acids, isocytidine (isoC) and isoguanosine (isoG) were incorporated into the preamplifier, and labeled probes were used in the third-generation bDNA assays (5). IsoC and isoG form base pairs with each other but not with any of the four naturally occurring bases (6).

The use of isoC- and isoG-containing probes in bDNA assays increases target-specific signal amplification without a concomitant increase in the background signal, thereby greatly enhancing the detection limits without loss of specificity. The detection limit of the third-generation bDNA assay for human immunodeficiency virus type 1 (HIV-1) RNA is 75 copies/ml. bDNA assays for the quantification of hepatitis B virus DNA, hepatitis C virus (HCV) RNA, and HIV-1 RNA are commercially available (Siemens Healthcare Diagnostics, Deerfield, IL). The SiemensVersant 440 analyzer for bDNA assays automates the incubation, washing, reading, and data-processing steps.

Hybrid Capture

The hybrid capture system is a solution hybridization-antibody capture method that uses chemiluminescence

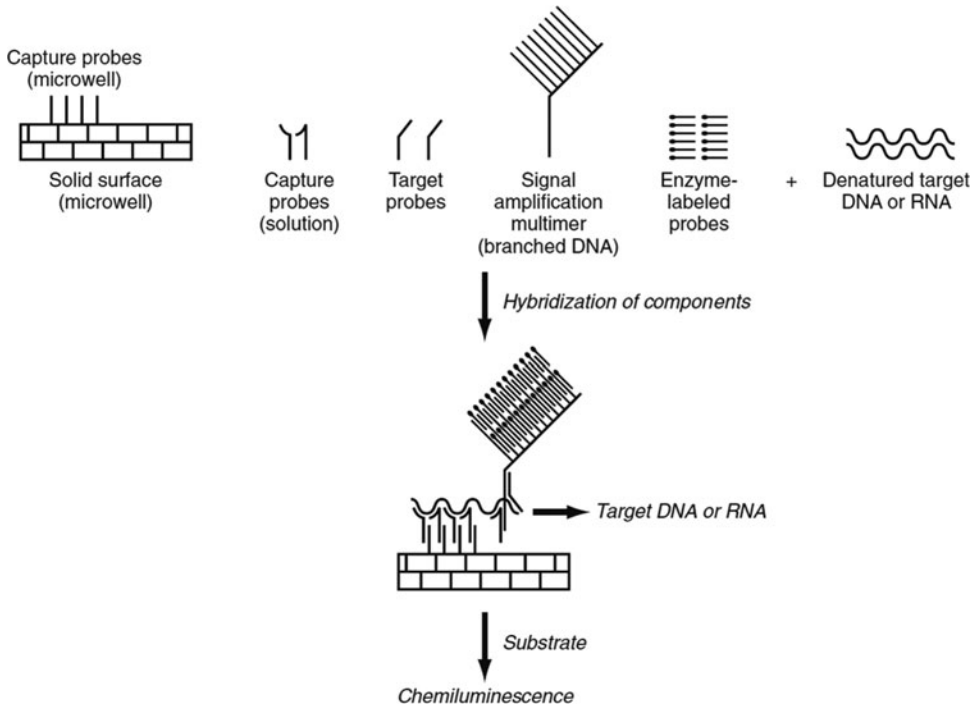

FIGURE 1 Branched DNA signal amplification. Reprinted with permission from reference 70.

detection of hybrid DNA-RNA duplexes (Fig. 2). The target DNA in the specimen is denatured and then hybridized with a specific RNA probe. The DNA-RNA hybrids are captured by antihybrid antibodies that are used to coat the surface of a tube. Alkaline phosphatase-conjugated antihybrid antibodies bind to the immobilized hybrids. The bound antibody conjugate is detected with a chemiluminescent substrate, and the light emitted is measured in a luminometer. Multiple alkaline phosphatase conjugates bind to each hybrid molecule, amplifying the signal. The intensity of the emitted light is proportional to the amount of target DNA in the specimen. Hybrid capture assays for detection of *Neisseria gonorrhoeae*, *Chlamydia trachomatis*, and human papillomavirus in clinical specimens are available from Qiagen, Germantown, MD (7). There are manual and automated (rapid capture system) versions of these assays.

Cleavase-Invader Technology

Invader assays (Hologic/Gen-Probe, San Diego, CA) are based on a signal amplification method that relies upon the specific recognition and cleavage of particular DNA structures by cleavase, a member of the FEN-1 family of DNA polymerases. These polymerases will cleave the 5′ single-stranded flap of a branched base-paired duplex. This enzymatic activity likely plays an essential role in the elimination of the complex nucleic acid structures that arise during DNA replication and repair. Since these structures may occur anywhere in a replicating genome, the enzyme recognizes the molecular structure of the substrate without regard to the sequence of the nucleic acids making up the DNA complex (8, 9).

In the invader assays, two probes are designed which hybridize to the target sequence in an overlapping fashion (Fig. 3). Under the proper annealing conditions, the probe oligonucleotide binds to the target sequence. The invader oligonucleotide probe is designed such that it hybridizes upstream of the probe with a region of overlap between the 3′ end of the invader and the 5′ end of the probe. Cleavase cleaves the 5′ end of the probe and releases it. It is in this way that the target sequence acts as a scaffold upon which the proper DNA structure can form. Since the DNA structure necessary to serve as a cleavase substrate will occur only in the presence of the target sequence, the generation of cleavage products indicates the presence of the target. Use of a thermostable cleavase enzyme allows reactions to be run at temperatures high enough for a primer exchange equilibrium to exist. This allows multiple cleavase products to form off of a single target molecule. FRET probes and a second invasive cleavage reaction are used to detect the target-specific products. FDA-cleared assays for detection of pools of high-risk genotypes and types 16 and 18 of human papillomavirus in cervical samples are available from Hologic/Gen-Probe (10, 11).

TARGET AMPLIFICATION TECHNIQUES

All of the target amplification systems share certain fundamental characteristics. They use enzyme-mediated processes, in which a single enzyme or multiple enzymes synthesize copies of a target nucleic acid. In all of these techniques, amplification is initiated by two oligonucleotide primers that bind to complementary sequences on opposite strands of double-stranded targets. These techniques result in the production of millions to billions of copies of the targeted sequence in a matter of minutes to hours, and in each case, the amplification products can serve as templates for subsequent rounds of amplification. Because of this, these techniques are sensitive to contamination with product molecules that can lead to false-positive reactions. The potential for contamination should be adequately addressed before these techniques are used in the clinical laboratory. However, the occurrence of false-positive reactions

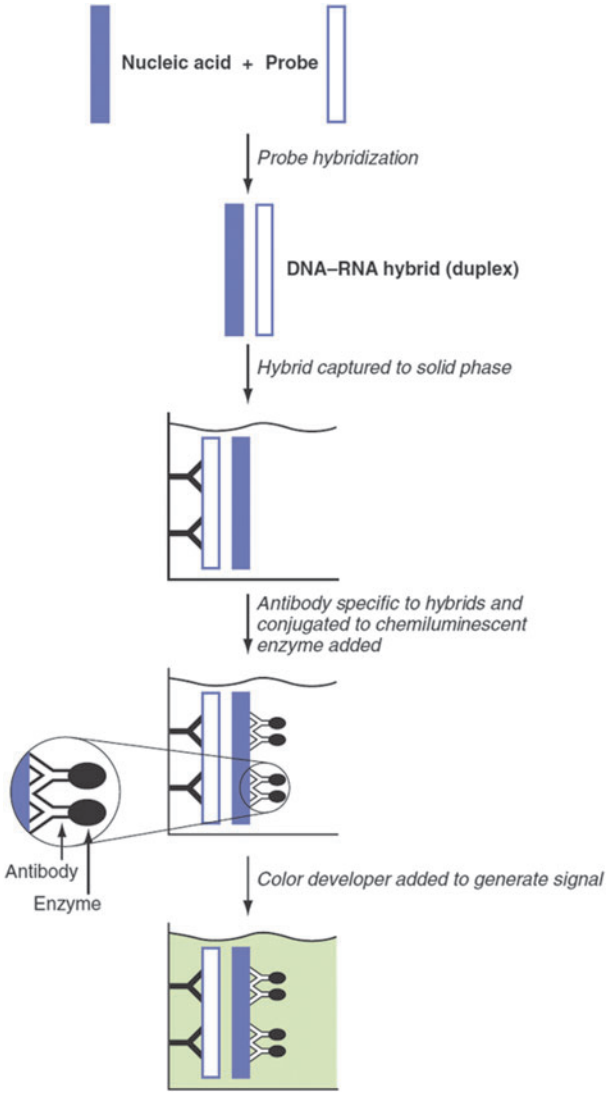

FIGURE 2 Hybrid capture signal amplification. Reprinted with permission from reference 70.

can be reduced through special laboratory design, practices, and workflow (12). In addition, amplification products can be modified by UV light or enzymes into forms that cannot be replicated. For example, if T is replaced with U during the PCR, it can be treated later by an enzyme that degrades U containing carryover products to prevent false-positive reactions (13). The growing use of closed systems where products are not exposed to the environment also helps to greatly reduce the threat of carryover contamination.

PCR

PCR was the first target amplification technique and remains the most popular today, for both research and clinical applications. It deserves such recognition and use because of its simplicity. Kary Mullis received the Nobel Prize in 1993 for its invention. The evolution and development of PCR is covered nicely by many books dedicated to the subject (14–16).

PCR requires a thermostable polymerase, two oligonucleotide primers to select the region to be amplified, a mixture of deoxynucleotide monomers (dNTPs), and template DNA. The polymerase is typically from *Thermus aquaticus*, originally obtained from Yellowstone National Park and later cloned into expression vectors for production. The two primers anneal to opposite DNA strands, typically placed 50 to 1,000 bases apart to select the region to be amplified. Typical reactant concentrations for PCR are shown in Table 1.

PCR is driven by temperature changes. The initial template is denatured or separated by heat (typically 90 to 95°C), lowering the temperature is required for primer annealing (55 to 65°C), and enzyme extension is typically performed at 65 to 75°C. Three-step cycling is performed if all three temperatures are different, although two-step cycling with a combined annealing/extension step is also common in diagnostics. Repeated temperature cycling through denaturation, annealing, and extension accumulates many identical products of defined length (Fig. 4). The products are most commonly detected by agarose gel electrophoresis, hybridization to complementary nucleic acids on solid supports, or probe interaction in solution. For example, if products are sampled during one cycle of PCR and separated on a gel, the process within each cycle can be observed visually (Fig. 5).

The advantages of PCR include simplicity, speed (17), and cost. Basic PCR is off-patent, and most forms of real-time PCR will be off-patent by the time this chapter goes to print. PCR as a process is very similar to bacterial growth. Both processes begin with exponential growth that eventually plateaus (Fig. 6). Growth curves follow a familiar S-curve shape tracking the logistic model of population growth. Although the endpoints of bacterial growth in media and amplification of DNA *in vitro* by PCR are different, they follow the same curve shape. Accurate quantification of the initial template is enabled by controlling denaturation, annealing, and extension by temperature cycling so that each amplification cycle can be measured and overall efficiency calculated.

PCR is clinically used in most laboratory-developed tests and *in vitro* diagnostic tests for infectious diseases. A complete list of all FDA-cleared or -approved nucleic acid amplification tests for detection, quantification, and genotyping of microorganisms can be found at http://www.fda.gov/MedicalDevices/ProductsandMedicalProcedures/InVitroDiagnostics/ucm330711.htm.

Reverse Transcriptase-PCR

When the initial template is RNA instead of DNA, an initial conversion of RNA into DNA is necessary for PCR. This conversion is performed by an RNA-dependent DNA polymerase, and the combined process is called reverse transcriptase PCR or RT-PCR. It can be performed in one or two steps. Two-step RT-PCR is typical of most research studies with two different enzymes and conditions optimized for each. One-step RT-PCR is more common for clinical assays where both the reverse transcription and the PCR are performed in a single tube. RT-PCR enables PCR to amplify common RNA targets, including HIV-1, HCV, enterovirus, and many respiratory viruses. The added complexity does require greater care, especially for viral load and other quantification assays. The MIQE guidelines (Minimum Information for Quantitative PCR Experiments) ensure the integrity of the scientific literature, promote consistency between laboratories, and increase

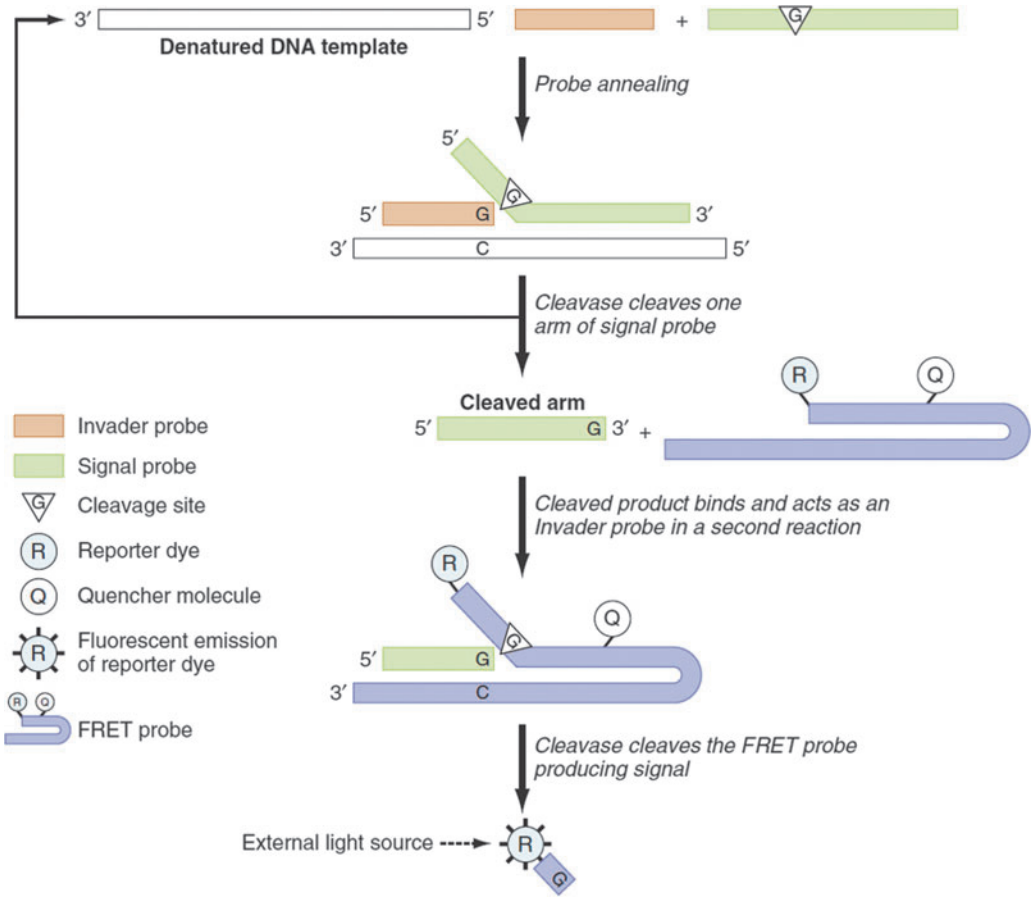

FIGURE 3 Cleavase invader signal amplification. Reprinted with permission from reference 70.

experimental transparency (18). Although written for the research community, these guidelines remain relevant for clinical assays.

Nested PCR

If PCR is followed by a second round of PCR on the products of the first, it is called nested PCR. Typically, both primers in the second PCR are internal to the first, so successful amplification depends on four primers rather than two. However, if one of the primers in the second PCR is the same as the first, it is called "hemi-nested" PCR. The advantage of nested or hemi-nested PCR is a further increase in sensitivity and specificity. The main disadvantage is an increased risk of carryover contamination, and the only nested tests that are FDA-approved are closed-tube real-time systems. The Cepheid MTB/RIF test is hemi-nested and detects *Mycobacterium tuberculosis* and rifampin resistance in <2 h (19). Nested, multiplex panels for respiratory agents (20), positive blood culture bottles (21), and gastrointestinal microbes are also FDA-approved with sample-to-answer results in about an hour and were developed by BioFire Diagnostics, Salt Lake City, UT/bioMérieux, Durham, NC.

Multiplex PCR

When more than one target is amplified by PCR, the process is called "multiplex." Multiplexing can save reagents and sample and is often used when a more complete answer can be obtained by including additional targets. Multiplexing is analyzed by separating products by size on a gel, by spatial separation on a surface or beads, or by probe color in real-time PCR. Real-time PCR is typically limited

TABLE 1 Typical reactant amounts in PCR (10-µl reaction mixture)

Reactant	Type	No. of copies/10 µl
Template DNA	50 ng of human DNA	1.6×10^4
	50 pg of bacterial DNA (3 Mb)	
	0.17 pg of viral DNA (10 kb)	
Polymerase	0.4 U of *Taq*	8.8×10^9
Primers	0.5 µM (each)	3.0×10^{12} (each)
Deoxynucleoside triphosphates	0.2 mM (each)	4.8×10^{15} (total)

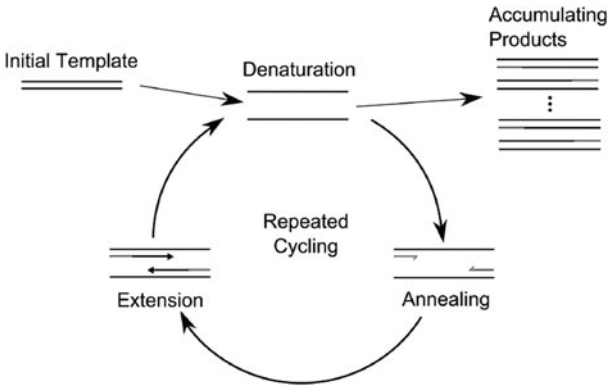

FIGURE 4 The PCR cycle. The initial template DNA is first denatured by heat. The reaction is then cooled to anneal two oligonucleotide primers to opposite strands with their 3′ ends pointed inward. A polymerase then extends each primed template to double the amount of targeted DNA. The cycle is repeated 20 to 40 times through successive steps of denaturation, annealing, and extension, accumulating double-stranded PCR products. Reprinted with permission from reference 16.

to two to six colors, but greater multiplicity is possible by combining color with the melting temperatures of the probes.

One example of multiplexed PCR with clinical utility is for upper respiratory infection. Many viruses and bacteria can cause flu-like illness, and a panel may provide a definitive answer in one multiplexed test. The first multiplexed respiratory panel was FDA-approved in 2008 with 10 viruses (Luminex, Austin, TX). Additional PCR-based respiratory panels are now offered by many companies including Cepheid, Sunnyvale, CA; GenMark Dx, Carlsbad, CA; Nanosphere, Northbrook, IL; Gen-Probe/Hologic, San Diego, CA; and BioFire/biomérieux. BioFire/biomérieux's nested multiplex respiratory panel is most inclusive, with 17 viruses and 3 bacteria (20).

Real-Time PCR

"Real time" implies that data collection and analysis occur as a reaction proceeds. Required reagents for analysis, such as DNA dyes or fluorescent probes, are added to the PCR mixture before amplification. Data are collected during amplification in the same tube and in the same instrument. There are no sample transfers, reagent additions, or gel separations. Real-time PCR is powerful, simple, and rapid and is replacing many conventional techniques in the microbiology laboratory.

Fluorescence is the indicator of choice for real-time PCR. Dyes can be used to monitor double-stranded PCR products, acquiring fluorescence once each cycle (22). If target DNA is present, the fluorescence increases. How soon this rise occurs depends on the initial amount of target DNA. The full power of real-time PCR goes beyond monitoring only once each cycle (23). When fluorescence is monitored as the temperature is changing, melting curves can verify the product amplified and detect sequence variants down to a single base. An example of the data generated from real-time PCR with melting analysis is shown in Fig. 7.

dsDNA Binding Fluorescent Dyes

In research, most real-time PCR is performed with dyes that fluoresce in the presence of double-stranded DNA because of their low cost and convenience (23). However, FDA-approved assays typically use probes instead of dyes. With dyes, any double-stranded product that is formed is detected, including primer dimers and other unintended products. Unless melting analysis of the product is performed, false positives are common (24). Multiplexing is possible by melting temperature discrimination rather than color (25). The mechanism of dye fluorescence during real-time PCR is compared to several probes in Fig. 8.

Hydrolysis (TaqMan) Probes

The most common probes used in FDA-approved real-time PCR assays are hydrolysis probes. If a probe labeled with a

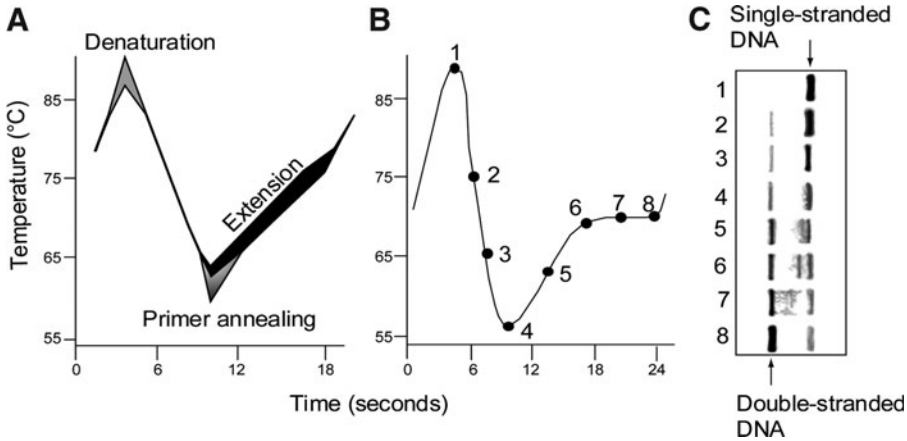

FIGURE 5 Visualization of PCR kinetics. The three phases of PCR (denaturation, annealing, and extension) occur as the temperature is continuously changing (A). Toward the end of PCR the reaction contains single- and double-stranded PCR products. When different points of the cycle are sampled (by snap-cooling the mixture in ice water) (B) and analyzed, the transition from denatured single-stranded DNA to double-stranded DNA is revealed as a continuum (C). Progression of the extension reaction can be followed by additional bands appearing between the single- and double-stranded DNA (time points 5 to 7). Modified with permission from reference 71.

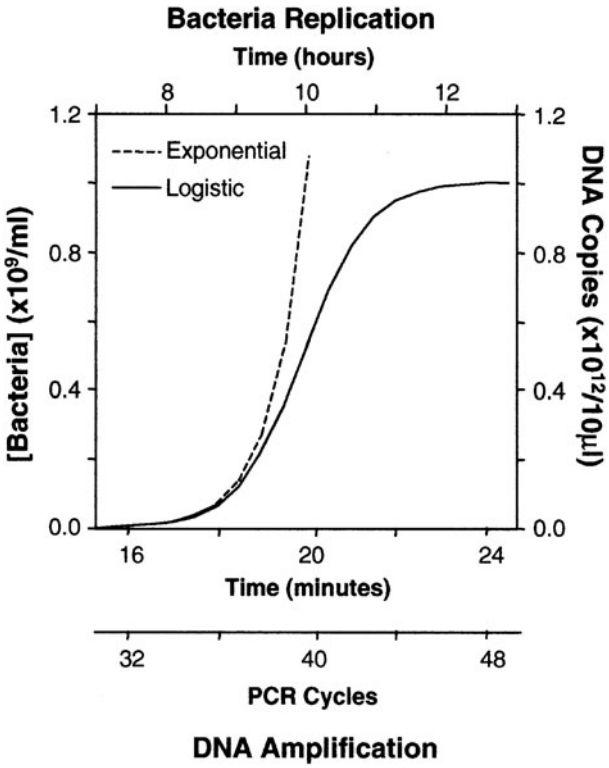

FIGURE 6 Model exponential and logistic curves for bacterial growth and PCR. Doubling times of 20 min and 30 s are assumed for bacteria and PCR, respectively. That is, given the equation $Nt = N_0e^{rt}$, r is 0.0347 min^{-1} for bacteria and 1.386 min^{-1} for PCR. The carrying capacity for bacteria was set at 10^9/ml. Assuming that PCR is primer limited at one-third the primer concentration (Table 1), a carrying capacity of 10^{12} copies of PCR product/10 µl was used. The shapes of the curves for bacteria and DNA are identical, with only the axis scales specific to each method. Starting with a single bacterium, growth plateaus after 11 to 12 h, while PCR takes only 23 min (46 cycles) to amplify a single copy to saturation.

fluorophore and a quencher is hydrolyzed during PCR and the labels are separated, fluorescence will increase. The most frequent implementation uses the 5′-exonuclease activity of a DNA polymerase to hydrolyze the probe and dissociate the labels (26). Another interesting way to hydrolyze fluorescent probes is to produce a DNAzyme during PCR (27). The fluorescence generated by hydrolysis probes is irreversible, and melting analysis is typically not useful. Hydrolysis probes are diagrammed in Fig. 8B.

Dual Hybridization Probes

Hybridization probes change fluorescence on hybridization, usually by fluorescence resonance energy transfer. Two interacting fluorophores are typically placed on adjacent probes (23) so that when they both hybridize, the fluorophores are brought together and energy transfer occurs, changing the color of the emitted fluorescence. Dual hybridization probes were used in the first FDA-approved genetic tests and, along with hydrolysis probes and molecular beacons, are found in many laboratory-developed microbiology tests (28). They are also used in the Roche (Indianapolis, IN) FDA-approved methicillin-resistant *Staphylococcus aureus* (MRSA) test. In contrast to hydrolysis probes, the fluorescence change of hybridization probes is reversible, and melting analysis can be very informative for strain typing and/or antibiotic resistance. Dual hybridization probes are shown in Fig. 8C.

Molecular Beacons

Molecular beacons (hairpin probes) fluoresce when they hybridize to a target (29). A fluorophore and a quencher are present on opposite strands of the stem, typically at the 3′ and 5′ ends of the probe. When the loop hybridizes to the target of interest, the fluorophore and quencher are separated, enhancing fluorescence. Molecular beacons of different colors can be combined with melting temperature for highly multiplexed assays (30). Molecular beacons are used in FDA-approved assays for *M. tuberculosis* and MRSA (Cepheid) and are shown in Fig. 8D.

Scorpion Probes

The fluorescence generated during PCR from self-probing amplicons (31) also depends on separating a fluorophore and a quencher on opposite ends of a hairpin stem. With scorpions, the primer is modified at its 5′ end to include a labeled hairpin similar to a molecular beacon. A blocker prevents copying of the hairpin region during PCR. The hairpin loop is complementary to the primer's extension product, so intramolecular hybridization occurs, replacing one hairpin with another that has a longer stem and is more stable. This separates the fluorophore from the quencher, and fluorescence is increased (Fig. 8E). Scorpion probes are used in FDA-approved assays for group B *Streptococcus* (BD Diagnostics, Franklin Lakes, NJ), *Clostridium difficile* (Focus Diagnostics, Cypress, CA), and some molecular oncology assays.

Dark Quencher Probes

Dark quencher (Pleiades) probes have a minor-groove binder and fluorophore at their 5′ end with a 3′ nonfluorescent quencher. Background fluorescence is very low because hydrophobic attraction between the quencher and minor groove binder ensures efficient quenching, further augmented by the minor groove binder (Fig. 8F). When bound to a target, the fluorophore and quencher are separated, similar to molecular beacons or scorpion primers. The minor groove binder also increases probe stability, making shorter probes possible. Short probes can be an advantage when sequence variation is high. Dark quencher probes are not degraded during PCR and can generate melting curves. Dark quencher probes (ELITech Group, Princeton, NJ) are available as analyte-specific reagents for cytomegalovirus, Epstein-Barr virus, and BK polyomavirus.

Partially Double-Stranded Probes

Partially double-stranded linear probes consist of two complementary oligonucleotides of different length (32). The longer target-specific strand has a 5′ fluorescent label that is effectively quenched by a 3′ quencher on the shorter negative strand (Fig. 8G). When a target is present the longer strand preferentially binds to the target, the shorter strand is displaced, and fluorescence is enhanced. These probes are tolerant to mismatches and are used in FDA-approved assays for HIV-1 and HCV (Abbott Molecular, Des Plaines, IL).

Melting Curve Analysis

Continuous monitoring of PCR (Fig. 9) suggests that hybridization can be followed during temperature cycling

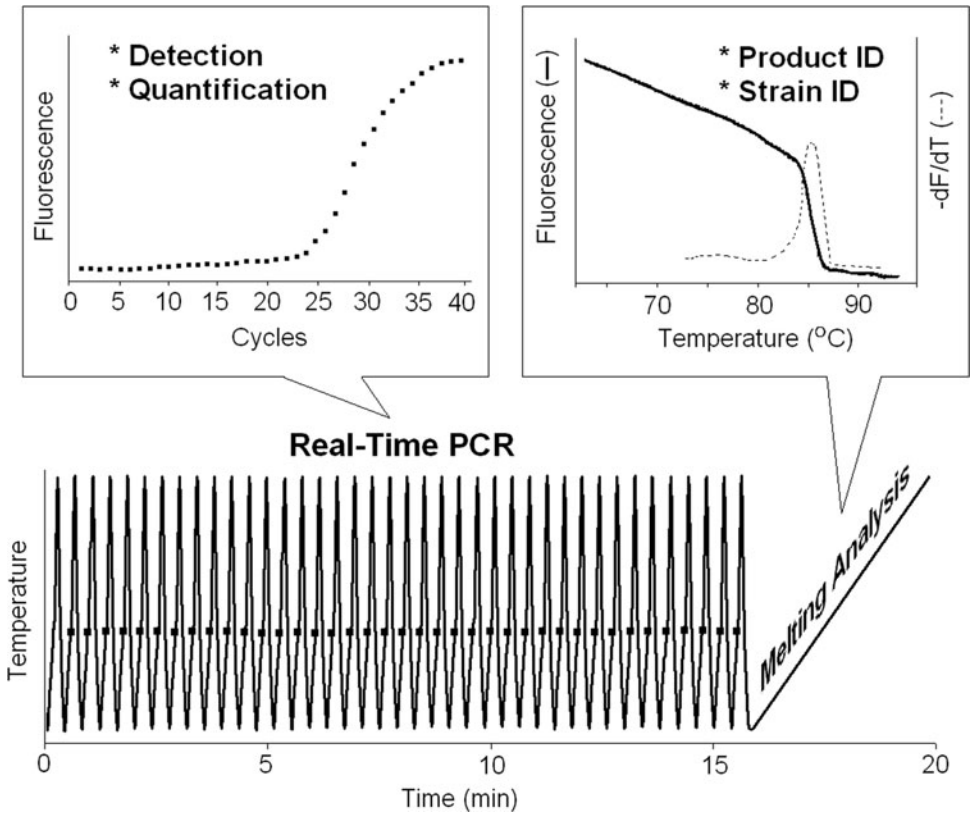

FIGURE 7 Real-time PCR with melting analysis. Detection and quantification are enabled by monitoring fluorescence once each cycle at the end of extension (solid squares). Amplification is immediately followed by melting-curve acquisition. Melting-curve analysis identifies PCR products, microbial strains and sequence alterations by melting temperature. The original melting-curve data (solid line) can also be plotted as a derivative melting curve (dotted line). Reprinted from reference 72 with permission from the American Society of Investigative Pathology and the Association for Molecular Pathology.

with dyes and most probes. Hydrolysis probes are the exception because they are destroyed during signal generation. Instead of monitoring hybridization throughout PCR, a single melting analysis after PCR is typically performed (Fig. 7). The midpoint of melting, called the melting temperature, or T_M, is determined mainly by the GC content and size of the duplex region. DNA melting curve analysis takes advantage of the fluorimeters and temperature control of real-time PCR instruments (17, 23, 24).

Product melting with dyes is useful to confirm PCR specificity by T_M and curve shape. Both T_M and curve shape can be predicted (33). PCR products of >200 bp often have multiple melting domains, and heterozygous products create heteroduplexes, both affecting curve shape. High-resolution melting analysis uses subtle differences in T_M and curve shape for genotyping and mutation scanning (34). Although usually a research technique, high-resolution melting is used in FDA-approved nested, multiplex assays for upper respiratory, blood culture, and gastrointestinal microbes (BioFire/bioMérieux).

Probe melting distinguishes variants only under the probe as opposed to the entire PCR product. For example, single nucleotide variants can be genotyped with hybridization probes because different sequences are revealed by different T_Ms. Irrelevant sequence variants under the probe can be masked by a deletion, mismatch, or universal base (35). Labeled hybridization probes include the dual hybridization probes of Fig. 8C and several single hybridization probes including molecular beacons (Fig. 8D), scorpion primers (Fig. 8E), dark quenchers (Fig. 8F), and partially double-stranded probes (Fig. 8G). Genotyping with labeled hybridization probes is shown in Fig. 10A and B. In parallel to labeled probes, melting and genotyping can also be performed with simple dyes rather than covalent labels. Examples include unlabeled probes (Fig. 10C) and snapback primers (Fig. 10D).

Unlabeled probes have no fluorescent labels but are 3′ blocked with a phosphate or other blocker (36). Unlabeled probes have been used for herpes simplex virus detection and typing (37) and in model studies have distinguished up to 10 variants (34). Similar to scorpion primers, "snapback primers" (Fig. 10D) generate a self-probing amplicon that forms a hairpin (38). Snapback primers achieve probe specificity with only two primers, one of which has a simple 5′ extension without any covalently attached fluorophores. Only amplicon melting is conceptually simpler (Fig. 10E), but the smaller differences between variants usually require high-resolution melting. Melting curves of unlabeled probe and snapback primers show both product and probe melting transitions, providing synergistic information for PCR variant identification (39).

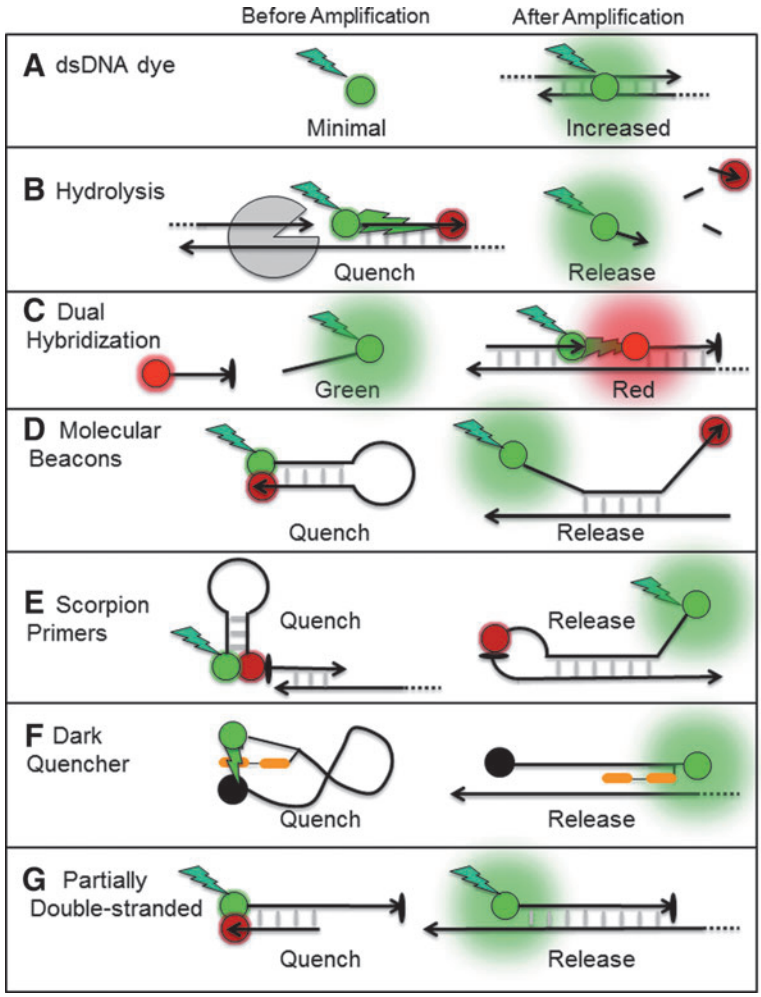

FIGURE 8 Common probes and dyes for real-time PCR. The green lightning bolt is the excitation light. The green circles are fluorophores, the dark red circles are quenchers, and the black circles are dark quenchers. The large hungry gray circle is a polymerase with 5′ to 3′ exonuclease activity. The thin black ovals are blockers, and the orange sausages are minor groove binders. (A) Double-stranded DNA dyes show a significant increase in fluorescence when bound to DNA. (B) Hydrolysis probes are cleaved between a fluorescent reporter and a quencher, resulting in increased fluorescence. (C) Dual hybridization probes change color by resonance energy transfer when hybridized. (D) The molecular beacon hairpin quenches fluorescence until target binding that separates the quencher from the flourophore. (E) Scorpion primers are quenched in the native conformation but increase in fluorescence when the original hairpin loop is hybridized to its extension product. (F) Dark quencher probes are initially quenched by a minor groove binder and the dark quencher. Hybridization to the target releases the fluorescence. (G) The short strand of partially double-stranded probes is displaced in the presence of target, releasing fluorescence from quenching.

Digital PCR

The sensitivity of real-time PCR, defined as a 95% detection rate, cannot be better than three copies per reaction because of variable partitioning of templates into any particular reaction (18). Digital PCR, however, uses partitioning to its advantage by running many PCRs with an average copy number typically between 0 and 1 (40). Each reaction is either positive or negative. Digital PCR can precisely determine the number of copies of a template (or variant) present at less than one copy per reaction if enough reactions are performed. Instruments that divide microliter PCR volumes into hundreds or millions of nanoliter to picoliter partitions on microfluidic chips or droplets are now available, promising highly sensitive and precise quantification. Digital MIQE guidelines defining the minimal information for publication of quantitative digital PCR experiments emphasize the unique requirements of digital PCR (41). The main uses of digital PCR in microbiology are (i) absolute quantification of reference materials, (ii) quantification of rare variants, for example, the emergence of a drug-resistant variant, and (iii) viral load testing.

Because digital PCR does not depend on a standard curve for absolute quantification, it is an ideal method to establish quantitative reference materials. For example, the U.S. National Institute of Standards and Technology produced a standard reference material for cytomegalovirus quantification by digital PCR (42), and many more are

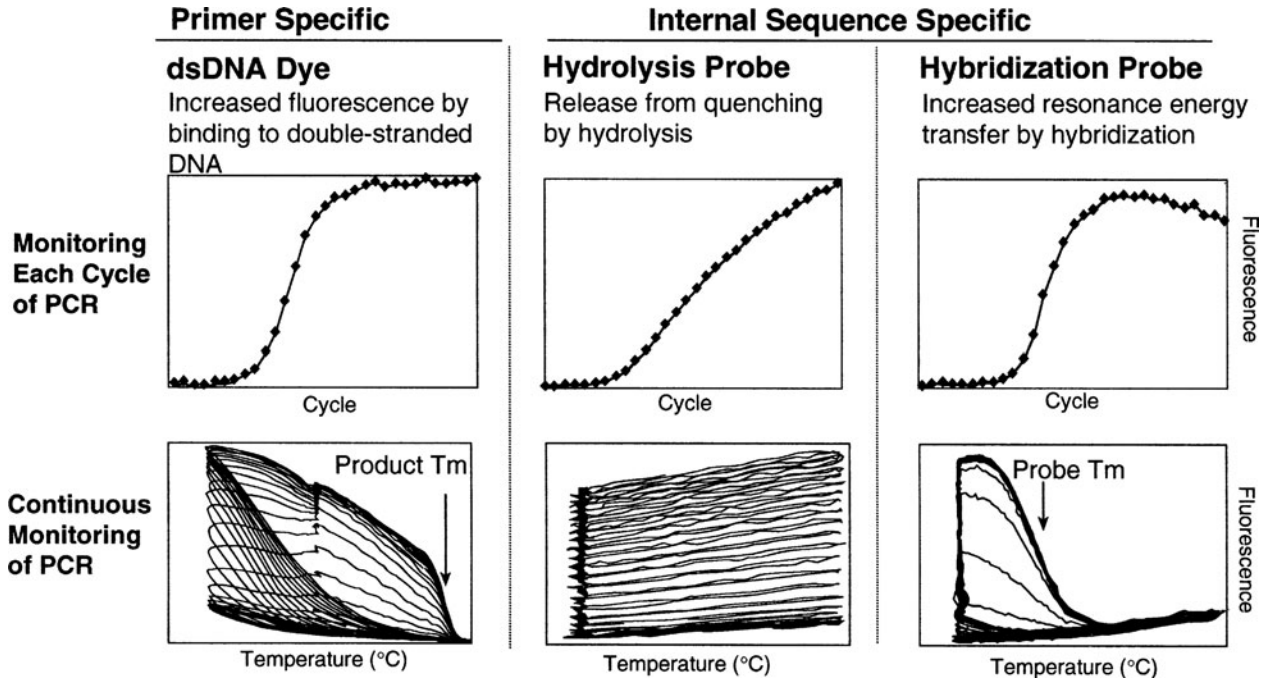

FIGURE 9 Typical real-time PCR amplifications monitored with SYBR Green I, hydrolysis probes, and hybridization probes. Both once-per-cycle and continuously monitored displays are shown. Note the hybridization information inherent in reactions monitored with SYBR Green I and hybridization probes.

likely to follow. Please see the chapter on digital PCR in this book for more details on the methods and clinical applications.

Detecting a small percentage of drug-resistant microbes in a population, or heteroresistance, is challenging by conventional methods. Digital PCR was successfully applied to heteroresistance in M. *tuberculosis*, targeting variants in four genes associated with isoniazid, rifampin, fluoroquinolone, and aminoglycoside resistance (43). Variants were detected at 0.01%, much more sensitive than real-time PCR or sequencing. Similar studies in HIV-1, HCV, and other viruses and bacteria are sure to follow.

Digital PCR for viral load testing has been compared to real-time PCR in several studies. In addition to the more common chip and droplet systems, novel rotational systems provide greater dynamic range, as demonstrated for HIV-1 and HCV (44). The proportion of chromosomally integrated human herpesvirus type 6 (HHV-6) to genomic DNA was precisely determined by digital PCR to prevent misdiagnosis and unnecessary treatment of active HHV-6 (45). Two studies comparing digital to real-time PCR for viral load testing of cytomegalovirus concluded that although there are theoretical advantages to digital PCR, practically clinical results are similar (46, 47).

Transcription-Based Amplification Methods

Nucleic acid sequence-based amplification (NASBA) and transcription-mediated amplification (TMA) are both isothermal RNA amplification methods modeled after retroviral replication (48–50). These methods are similar in that the RNA target is reverse transcribed into cDNA and then RNA copies are synthesized with an RNA polymerase. NASBA uses avian myeloblastosis virus RT, RNase H, and T7 bacteriophage RNA polymerase, whereas TMA uses an RT enzyme with endogenous RNase H activity and T7 RNA polymerase.

Amplification involves the synthesis of cDNA from the RNA target with a primer containing the T7 RNA polymerase promoter sequence (Fig. 11). The RNase H then degrades the initial strand of target RNA in the RNA-cDNA hybrid. The second primer then binds to the cDNA and is extended by the DNA polymerase activity of the RT, resulting in the formation of double-stranded DNA containing the T7 RNA polymerase promoter. The RNA polymerase then generates multiple copies of single-stranded, antisense RNA. These RNA product molecules reenter the cycle, with subsequent formation of more double-stranded cDNA molecules that can serve as templates for more RNA synthesis. A 10^9-fold amplification of the target RNA can be achieved in less than 2 h by this method.

The single-stranded RNA products of TMA in the Hologic/Gen-Probe tests are detected by the hybridization protection assay. Oligonucleotide probes are labeled with modified acridinium esters with either fast or slow chemiluminescence kinetics so that signals from two hybridization reactions can be analyzed simultaneously in the same tube. The probes are added after amplification and hybridize to the amplicons. A selection reagent is then added which differentiates between hydridized and unhybridized probes by inactivating the label on the unhybridized probes. The NASBA products in the bioMérieux tests are detected by hybridization with probes that are added after amplification, labeled with tris (2,2′-bispyridine)ruthenium and detected by electrochemiluminescence. NASBA has also been used with molecular beacons to create a homogeneous, kinetic amplification system similar to real-time PCR (51).

Transcription-based amplification systems have several strengths, including no requirement for a thermal cycler,

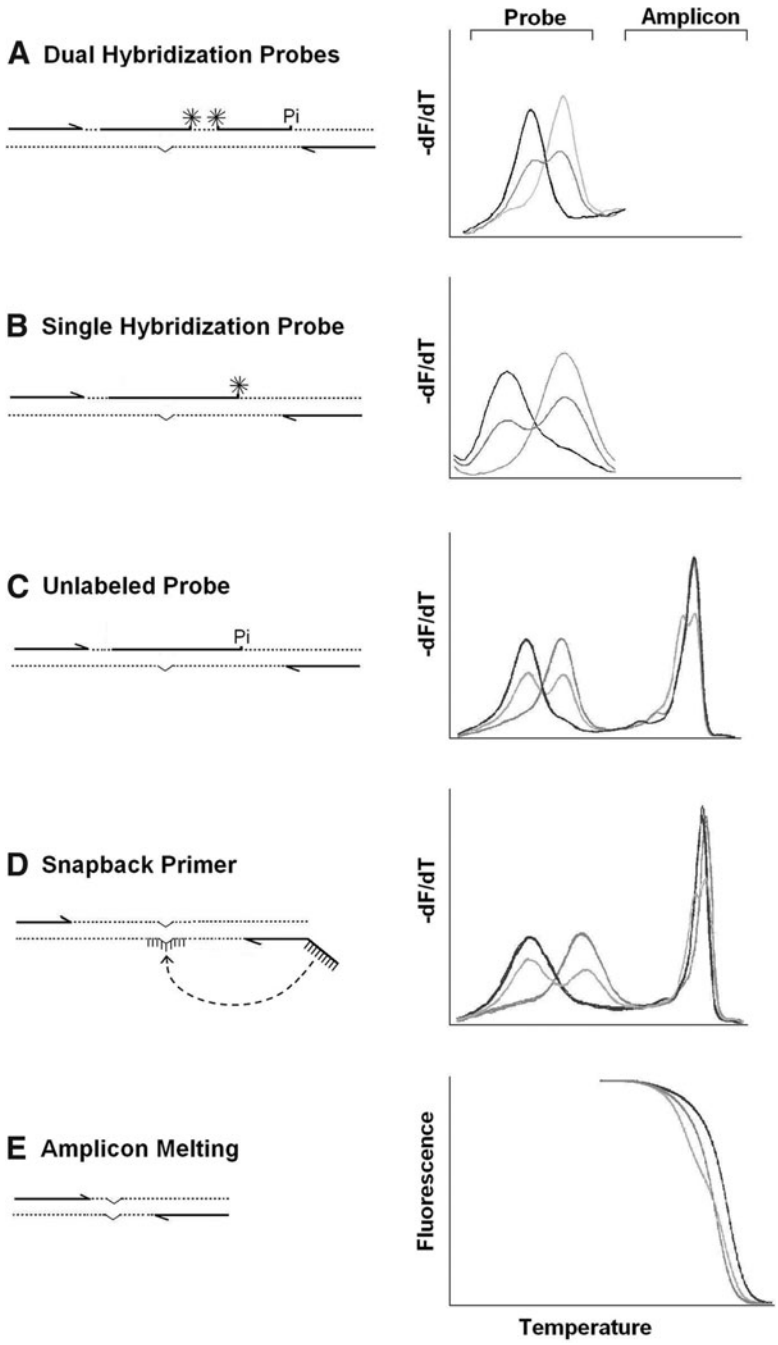

FIGURE 10 Variant typing by melting analysis. Primer and probe designs are shown on the left with typical data on the right. Dual (A) and single (B) hybridization probes use covalent fluorescent labels (asterisks), and typing is solely derived from the probe signal. Single hybridization probes discussed here include molecular beacons, scorpion primers, dark quencher probes, and partially double-stranded probes. Unlabeled probes (C) and snapback primers (D) require no covalent labels because fluorescence is provided by a dye that binds to dsDNA. With unlabeled probes and snapback primers, both probe and PCR product melting transitions are observed and can contribute to typing. Any free 3′ ends on the probes are terminated with a phosphate (Pi) or other blocker to prevent probe extension by the polymerase. The snapback primer (D) incorporates an unlabeled probe into the 5′ end of one primer, generating a self-probing amplicon that forms a hairpin. In panel E, no probe is present, but typing of the PCR product is still possible by high-resolution melting. High-resolution melting identifies heterozygotes by a change in curve shape and distinguishes homozygotes by T_m.

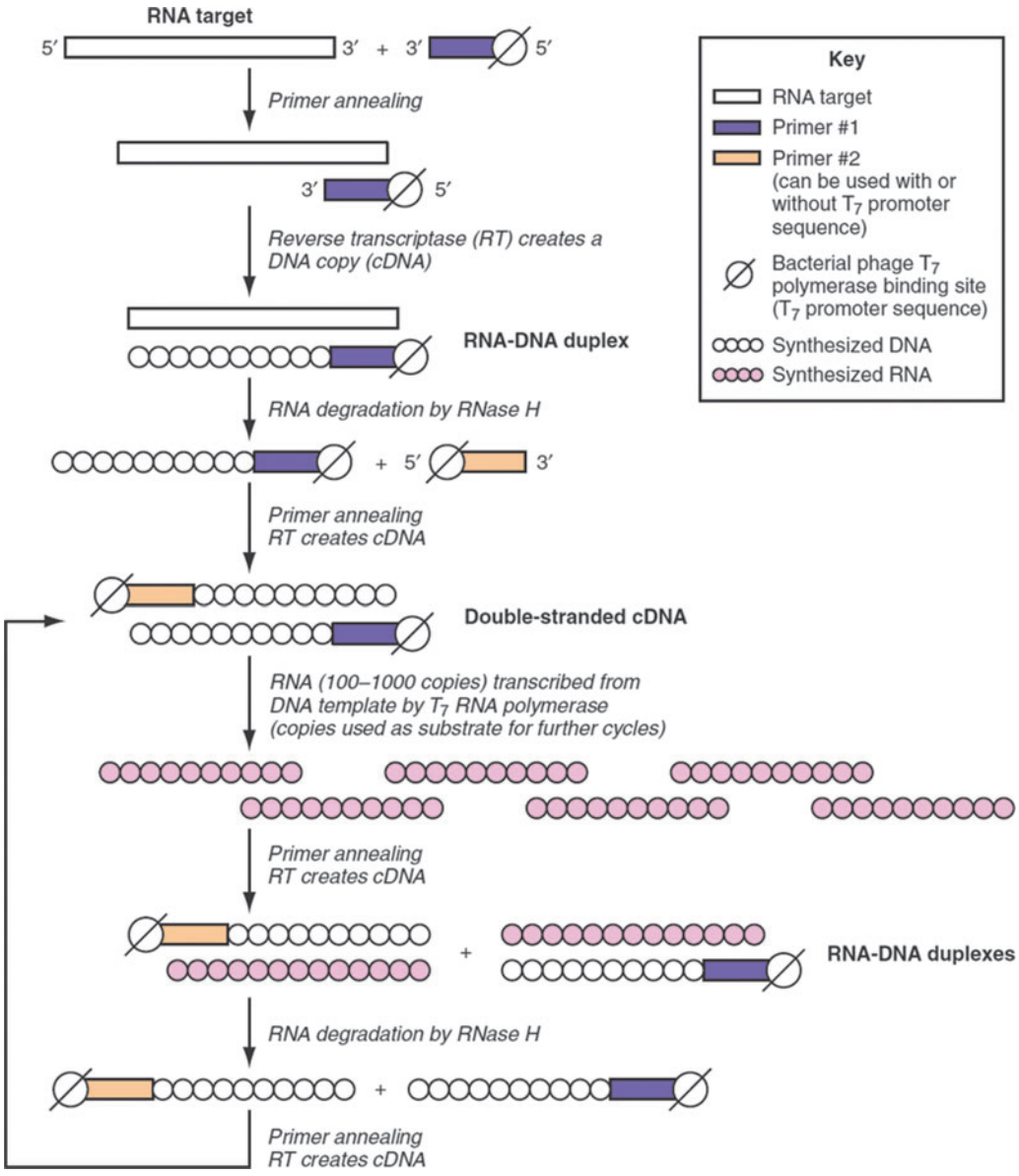

FIGURE 11 Transcription-based target amplification. NASBA and TMA are examples of transcription-based amplification systems. Reprinted with permission from reference 70.

rapid kinetics, and a single-stranded RNA product that does not require denaturation prior to detection. Also, single-tube clinical assays and a labile RNA product may help minimize contamination risks. Limitations include the poor performance with DNA targets and concerns about the stability of complex multienzyme systems. Hologic/Gen-Probe has developed FDA-cleared, TMA-based assays for detection of *M. tuberculosis*, *C. trachomatis*, *N. gonorrhoeae*, human papillomavirus, and *Trichomonas vaginalis*. NASBA-based kits (bioMérieux) for the detection and quantification of HIV-1 RNA and detection of enterovirus and MRSA were developed but are no longer commercially available. A basic NASBA kit is also available for the development of other applications defined by the user. In its latest iteration, NucliSens EasyQ, NASBA is coupled with molecular beacons for real-time amplification and detection of target nucleic acids (52).

Strand Displacement Amplification

Strand displacement amplification (SDA) is an isothermal template amplification technique that can be used to detect trace amounts of DNA or RNA of a particular sequence. SDA, as it was first described, was a conceptually straightforward amplification process with some technical limitations (53). Since its initial description, however, it has evolved into a highly versatile tool that is technically simple to perform but conceptually complex. SDA is the intellectual property of BD Diagnostics.

In its current iteration, SDA occurs in two discrete phases: target generation and exponential target amplification (54). Both are illustrated in Fig. 12. In the target generation phase, a double-stranded DNA target is denatured and hybridized to two different primer pairs, designated as bumper and amplification primers. The amplification primers include the single-stranded restriction endonuclease

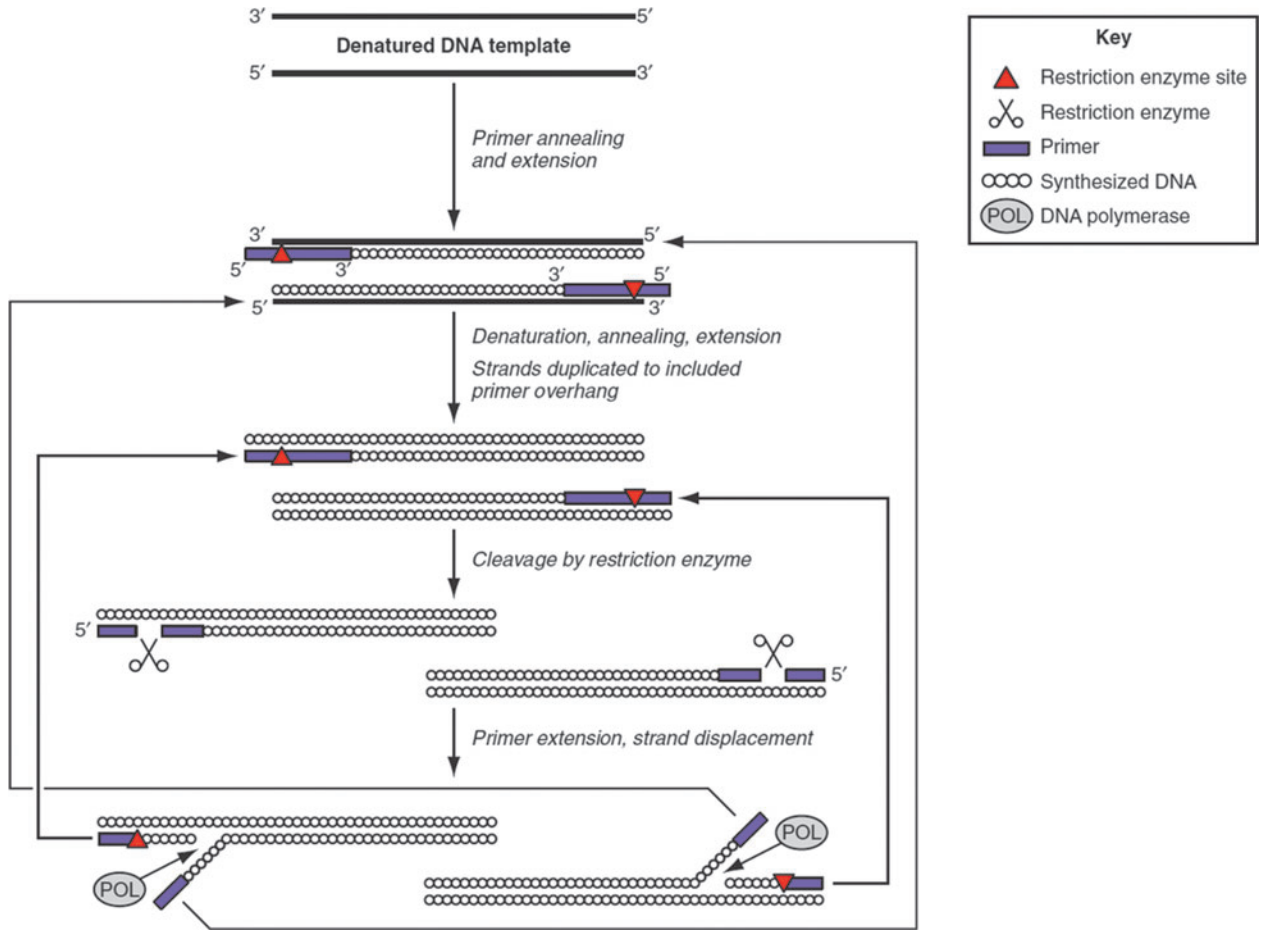

FIGURE 12 Strand displacement target amplification. The process is shown for only one strand of a double-stranded DNA target, but amplification occurs on both strands simultaneously. Reprinted with permission from reference 70.

enzyme sequence for BsoB1 located at the 5′ end of the target binding sequence. The bumper primers are shorter and anneal to the target DNA just upstream of the region to be amplified. In the presence of BsoB1, an exonuclease-free DNA polymerase, and a dNTP mixture consisting of dUTP, dATP, dGTP, and thiolated dCTP (C_s), simultaneous extension products of both the bumper and amplification primers are generated. This process displaces the amplification primer products, which are available for hybridization with the opposite-strand products with the opposite-strand bumper and amplification primers.

The simultaneous extension of opposite-strand primers produces strands complementary to the product formed by extension of the first amplification primer with C_s incorporated into the BsoB1 cleavage site. This product enters the exponential target amplification phase of the reaction. The BsoB1 enzyme recognizes the double-stranded site, but because one strand contains C_s, it is nicked rather than cleaved by the enzyme. The DNA polymerase then binds to the nicked site and begins synthesis of a new strand while simultaneously displacing the downstream strand. This step re-creates the double-stranded species with the hemimodified restriction endonuclease recognition sequence, and the iterative nicking and displacement process repeats. The displaced strands are capable of binding to opposite-strand primers, which produces exponential amplification of the target sequences.

These single-stranded products also bind to detector probes for real-time detection. The detector probes are single-stranded DNA molecules with fluorescein and rhodamine labels. The region between the labels includes a stem-loop structure. The loop contains the recognition site for the BsoB1 enzyme. The target-specific sequences are located 3′ of the rhodamine label. In the absence of a specific target, the stem-loop structure is maintained with the fluorescein and rhodamine labels in close proximity. The net effect is that very little emission for the fluorescein is detected after excitation. After SDA, the probe is converted to a double-stranded species, which is cleaved by BsoB1. The cleavage causes physical separation of the fluorescein and rhodamine labels, which results in an increase in emission from the fluorescein label.

SDA has a reported sensitivity high enough to detect as few as 10 to 50 copies of a target molecule (53). By using a primer set designed to amplify a repetitive sequence with 10 copies in the *M. tuberculosis* genome, the assay is sensitive enough to detect 1 to 5 genome copies from the bacterium. SDA has also been adapted to quantify RNA by adding an RT step (RT-SDA). In this case, a primer hybridizes to the target RNA and an RT synthesizes a cDNA molecule. This cDNA can then serve as a template for

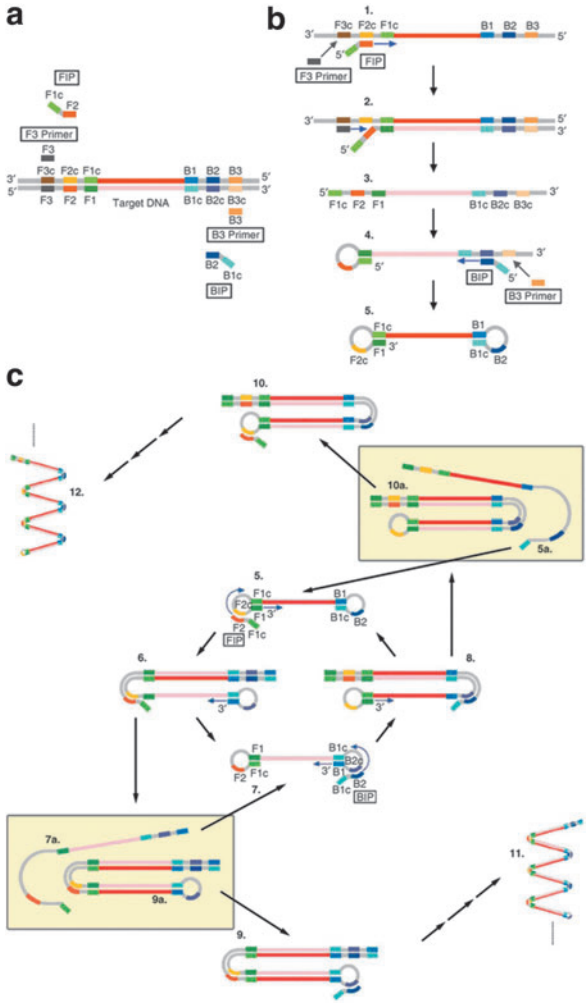

FIGURE 13 (a) Primer design of the LAMP reaction. For ease of explanation, six distinct regions are designated on the target DNA, labeled F3, F2, F1, B1c, B2c, and B3 from the 5′ end. Because c represents a complementary sequence, the F1c sequence is complementary to the F1 sequence. Two inner primers (FIP and BIP) and outer primers (F3 and B3) are used in the LAMP method. FIP (BIP) is a hybrid primer consisting of the F1c (B1c) sequence and the F2 (B2) sequence. (b) Starting structure producing step. DNA synthesis initiated from FIP proceeds as follows. The F2 region anneals to the F2c region on the target DNA and initiates the elongation. DNA amplification proceeds with BIP in a similar manner. The F3 primer anneals to the F3c region on the target DNA, and strand displacement DNA synthesis takes place. The DNA strand elongated from FIP is replaced and released. The released single strand forms a loop structure at its 3′ end (structure 3). DNA synthesis proceeds with the single-strand DNA as the template, and BIP and B3 primer, in the same manner as described earlier, to generate structure 5, which possesses the loop structure at both ends (dumbbell-like structure). (c) Cycling amplification step. Using self-structure as the template, self-primed DNA synthesis is initiated from the 3′ end F1 region, and the elongation starts from FIP annealing to the single strand of the F2c region in the loop structure. Passing through several steps, structure 7 is generated, which is complementary to structure 5, and structure 5 is produced from structure 8 in a reaction similar to that which led from structures 5 to 7. Structures 9 and 10 are produced from structures 6 and 8, respectively, and more elongated structures (11, 12) are also produced. Reprinted with permission from reference 57.

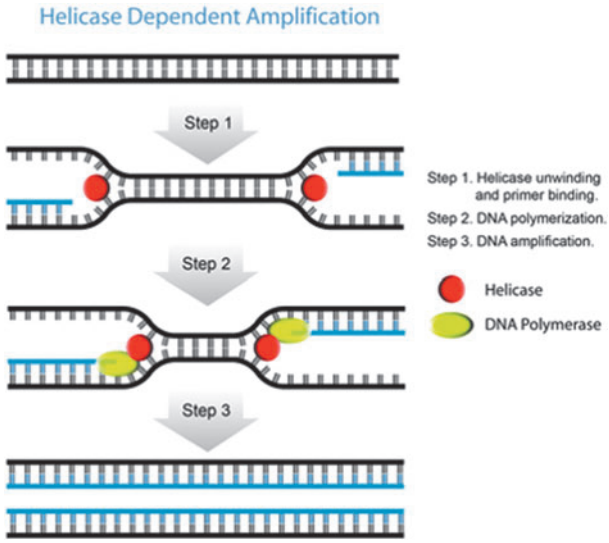

FIGURE 14 HDA amplifies target sequences using two sequence-specific primers flanking the fragment to be amplified and a mixture of enzymes for DNA strand separation and polymerization. In the first step of the HDA reaction, the helicase enzyme loads on to the template and traverses along the target DNA, disrupting the hydrogen bonds linking the two strands. Exposure of the single-stranded target region by helicase allows primers to anneal. The DNA polymerase then extends the 3′ ends of each primer using free deoxynucleotides (dNTPs) to produce two DNA replicates. The two replicated DNAs independently enter the next cycle of HDA, resulting in exponential amplification of the target sequence. Reprinted from http://www.biohelix.com/HDA_mechanism.asp.

primer incorporation and strand displacement. The products of this strand displacement then feed into the amplification scheme described above. RT-SDA has been used for the determination of HIV-1 viral load (55). FDA-cleared tests using SDA for the direct detection of C. *trachomatis*, N. *gonorrhoeae*, and HSV types 1 and 2 in urogenital specimens are available from BD Diagnostics. These assays can be run on either a semiautomated (ProbeTec) or fully automated (Viper) system.

The main advantage of SDA is that it is an isothermal process that, unlike PCR, can be performed at a single temperature after initial target denaturation. This eliminates the need for expensive thermal cyclers. Furthermore, samples can be subjected to SDA in a single tube, with amplification times varying from 30 min to 2 h. The main disadvantage of SDA lies in the fact that, unlike temperatures at which PCR is performed, the relatively low temperature at which SDA is carried out (52.5°C) can result in nonspecific primer hybridization to sequences found in complex mixtures such as genomic DNA. Hence, when the target is in low abundance compared to background DNA, nonspecific amplification products can swamp the system, decreasing the sensitivity of the technique. However, the use of organic solvents to increase stringency at low temperatures and the recent introduction of more thermostable polymerases capable of strand displacement have alleviated much of this problem.

Loop-Mediated Amplification

Loop-mediated amplification (LAMP) is an isothermal method that relies on autocycling strand displacement

DNA synthesis by *Bst* DNA polymerase and a set of four to six primers (56). Two inner and two outer primers define the target sequence, and an additional set of loop primers is added to increase the sensitivity of the reaction. The final products of the LAMP reaction are DNA molecules with a cauliflower-like structure of multiple loops consisting of repeats of the target sequence (Fig. 13) (57). The products can be analyzed in real time by monitoring of the turbidity in the reaction tube resulting from production of magnesium pyrophosphate precipitate during the DNA amplification. Amplification products can also be visualized in agarose gels after electrophoresis and staining with ethidium bromide or SYBR Green I.

LAMP has been used successfully in a number of laboratory-developed assays to detect DNA and RNA viruses (58–61) and diagnose mycobacterial infections (62). Since LAMP is an isothermal process and positive reactions can be detected by simple turbidity measurements or visualized directly with the naked eye, it requires no expensive equipment. These attributes make it an attractive technology for resource-poor settings and field use (63). However, primer design for LAMP is more complex than for PCR, with specialized training and software required for its design. Meridian Bioscience, Inc., Cincinnati, OH, has licensed LAMP technology from Eiken Chemical Company, Ltd., Tokyo, Japan, for the development of infectious disease diagnostics in the United States. Meridian currently has FDA-cleared tests for detection of *C. difficile*, *Mycoplasma pneumoniae*, group A and B beta-hemolytic streptococci, and *Bordetella pertussis* (64).

Helicase-Dependent Amplification

Helicase-dependent amplification (HDA) is an isothermal process developed by BioHelix, Beverly, MA, that uses helicase to separate dsDNA and generate single-stranded templates for primer hybridization and subsequent extension by a DNA polymerase (65). As the helicase unwinds dsDNA enzymatically, the initial heat denaturation and subsequent thermocycling steps required by PCR can all be omitted. In HDA, strands of dsDNA are separated by the DNA helicase and the ssDNA is coated with ssDNA-binding proteins. Two sequence-specific primers hybridize to each border of the target sequence, and a DNA polymerase extends the primers annealed to the target sequence to produce dsDNA. The two newly synthesized products are used as substrates by the helicase in the next round of amplification. Thus, a simultaneous chain reaction proceeds, resulting in exponential amplification of the selected target sequence (Fig. 14).

HDA is compatible with multiple detection technologies including qualitative and quantitative fluorescent technologies and with instruments designed for real-time PCR (66). Furthermore, HDA has shown potential for the development of simple, portable DNA diagnostic devices to be used in the field or at the point of care (67–69). FDA-cleared tests for detection of HSV type 1 and type 2, *C. difficile*, and group A and B beta-hemolytic streptococci based on HDA are available from Quidel (San Diego, CA).

FUTURE DIRECTIONS

Amplification methods and the probes that allow detection and quantification of nucleic acids are becoming faster, easier, and less expensive. Multiplexing and nesting extract more information, and better assay design provides greater clinical relevance. These trends will continue in the future. Entirely new amplification methods and probes will be created that may displace existing methods in some applications. Digital PCR and high-resolution melting will find greater use in clinical assays. Targeted amplification as presented here is currently being challenged by broad-spectrum mass spectroscopy and massively parallel sequencing. The needs of central clinical labs (cost efficiency and batching) will continue to clash with the ideals of rapid turnaround near the patient. No one knows the future, but it is exciting to be part of the process.

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Application of Identification of Bacteria by DNA Target Sequencing in a Clinical Microbiology Laboratory

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2

The identification of bacteria has traditionally been based upon the phenotypic properties of microorganisms grown in pure culture under optimal conditions. While useful in most circumstances, the physiological characteristics of bacteria are mutable and not always consistent within a given species. Phenotypic identification can, moreover, be tedious, subjective, and inconclusive when conflicting results are obtained. Even with the aid of semiautomated or automated instruments, these methods are still limited in that they cannot fully characterize all bacterial isolates, and the phenotype of an isolate may not be predictable (1, 2). We are beginning to appreciate the growing diversity of bacteria and the complexities in the evolution of a bacterial species. Similarly, we now more fully realize that the physiological properties of bacteria vary from the dynamic interplay between their environmental and ecological niches and their human hosts. With growing numbers of immunocompromised hosts who are susceptible to unusual infections, the distinction between environmental, colonizing, and clinically relevant bacteria is not always clear. Hence, commonly encountered bacteria with unusual physiological properties and the emergence of novel bacterial pathogens with unknown or poorly defined phenotypes pose significant challenges to clinical microbiologists. These challenges underscore the importance of characterizing bacteria by methods that are independent of a microorganism's biochemical characteristics.

Nucleic acid sequencing of various bacterial genes and other DNA targets has been used for determining the phylogeny of bacteria and for their identification (3) and aid in the description of novel organisms. With advances in technology, this approach has moved from research to the clinical laboratory. Even with newer technologies such as matrix-assisted laser deadsorption time-of-flight (MALDI-TOF) mass spectrometry gaining more widespread use in the clinical laboratory, DNA target sequencing remains the "gold standard" in bacterial identification. Compared to conventional methods, DNA target sequencing holds the advantage of speed, accuracy, and growth-independent identification (4–8). Once performed by using more laborious methods, nucleic acid sequencing can now be accomplished using high-throughput automated instrumentation. A brief overview of nucleic acid sequencing is shown in Fig. 1.

The rRNA genes (sometimes referred to as rDNA) and their intergenic regions found in bacteria are commonly used for prokaryotic phylogenetic studies (9). The small-subunit rRNA molecule is a fragment with a sedimentation coefficient of 16S and is encoded by an ~1,500-bp gene. The large-subunit rRNA contains 23S and 5S molecules. Partial sequencing of the 16S rRNA gene, with amplification of the first 500 bp, is usually used for bacterial identification in the clinical laboratory, including anaerobes and mycobacteria (1, 5, 10–15). Because it is commonplace to include a 16S rRNA sequence with the description of a new species and it is the most frequently used target for clinical and environmental metagenomic studies, the 16S rRNA databases cover more species than other targets. Most sequences that have been deposited in publicly available databases correspond to this region of the 16S rRNA gene. Using this method, researchers have discovered pathogenic bacteria such as *Tropheryma whipplei* and *Bartonella bacilliformis* (16, 17).

The 16S rRNA molecule contains alternating regions of sequence conservation and heterogeneity (Fig. 2), making it well suited as a target for sequence analysis (18). The conserved regions are ideal primer targets for amplification of this gene from all bacterial species. Regions of DNA sequence diversity between these conserved regions provide sequence polymorphisms that serve as "signatures" unique to a genus or species. The sequence obtained is compared to a database containing sequences of known microorganisms. The number of similar nucleotide bases between sequences is used to calculate the percent identity and ascertain the identification of the microorganism. While this strategy is adequate for the identification of many bacterial species, the degree of divergence observed within the 16S rRNA molecule may not be sufficient to distinguish some closely related species (19). Criteria for identification of bacteria to the genus or species level were initially determined empirically and differed from laboratory to laboratory. Only recently have standardized criteria been developed for use by clinical laboratories (20).

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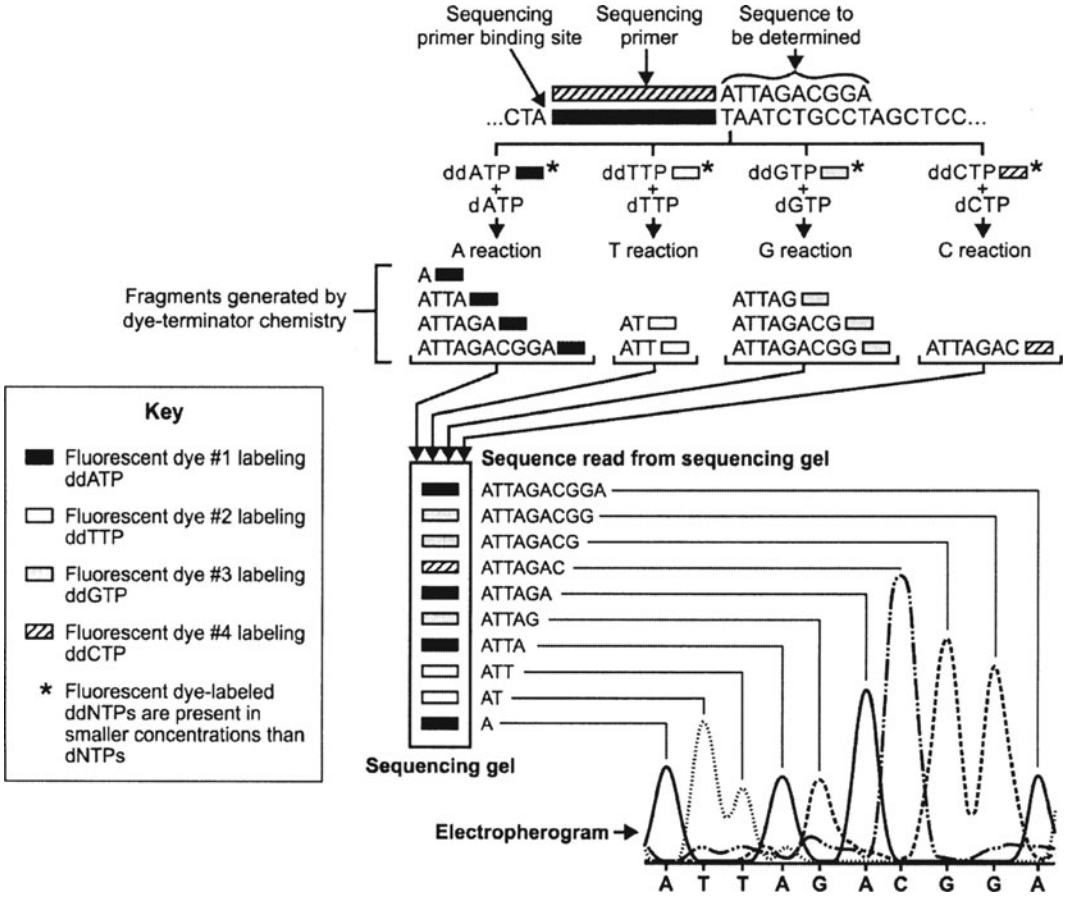

FIGURE 1 Dye-terminator cycle sequencing of amplified 16S rRNA gene. Purified PCR amplicon, sequencing primer, and limited concentrations of dideoxynucleotide triphosphates (ddNTPs) into which four fluorescent dyes have been incorporated are mixed with unlabeled deoxynucleotides (dNTPs). Synthesis terminates whenever a ddNTP instead of a dNTP is incorporated into a new strand. Strands of various lengths are synthesized and labeled as the terminal ddNTP is incorporated into the strand. Accumulated fragments are separated according to size by electrophoresis. During electrophoresis, labeled products are visualized by fluorescence, with each of the four fluorescent dyes indicating which of the terminal ddNTPs have been incorporated. Combining the terminal ddNTP information with the fragment size allows the determination of sequence information. Reprinted from reference 58 with permission from the publisher.

Other DNA targets have been used to better separate closely related species. These include *rpoB* (beta subunit of RNA polymerase), *sodA* (manganese-dependent superoxide dismutase), *gyrA* or *gyrB* (gyrase A or B), *tuf* (elongation factor Tu), *recA*, *secA*, and heat shock proteins (21–27). The utility of each target varies depending on the microorganism. Similar to the 16S rRNA gene, these alternative DNA targets have conserved regions flanking variable regions that can be used to differentiate closely related bacterial species. It should be noted that primers to the conserved regions are not universal to all bacteria, and targets should be selected based on the microorganism of

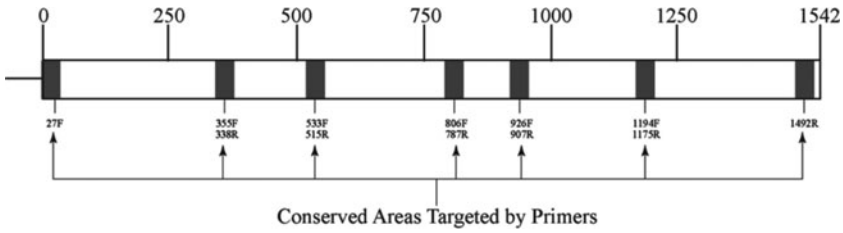

FIGURE 2 Schematic for 16S rRNA located on the small ribosomal subunit (30S). Arrows indicate the conserved regions that serve as primer targets for PCR amplification and DNA sequencing of bacteria.

interest. Databases for these alternative DNA targets are not as well populated as for the 16S rRNA gene, but the number of reference sequences is increasing rapidly. Use of DNA sequence information from more than one locus may be useful to distinguish some closely related species.

The routine use of sequencing can greatly enhance the ability of the clinical microbiology laboratory to identify bacteria on many levels. Once bacteria from a pure culture are isolated, the turnaround time for obtaining a sequence can be less than 24 h. When applied to fastidious, slow-growing, or biochemically inert microorganisms, such as anaerobes and *Nocardia* spp., the time required for microorganism identification can be decreased from weeks to within one day. In some cases, sequencing may be performed directly from a clinical specimen or from instrument flagged bottles, reducing the need for growth of individual colonies. Because sequence-based identification can replace the performance of many time-consuming and labor-intensive biochemical reactions, the average time spent per specimen is also dramatically reduced, allowing laboratory technologists more time to accomplish other necessary tasks. This is especially important in the current environment, in which there is a growing shortage of well-trained medical technologists. In our experience, sequence-based identification has decreased the personnel needed by at least one full-time-equivalent certified medical technologist.

Even in cases in which sequence-based identification is unable to provide a definitive answer, sequencing results can provide information on the isolate's phylogenetic relationship to more commonly known bacteria. Relatedness trees provide the clinician with more information about the microorganism's ecological and taxonomical niches than with conventional methods alone. One challenge of conventional biochemical identification and MALDI-TOF is the need for viable organisms under specific culture conditions, cultivation media, and sample preparation to achieve optimal identification. Sequencing overcomes this challenge in that it does not require viable or culturable organisms for identification. As our understanding of the role of the microbiome is increased, the role of non-cultivable organisms in specific medical conditions is becomingly increasingly important. Unlike MALDI-TOF or biochemical-based methods, sequencing data have less sample-to-sample and lab-to-lab variability, providing the opportunity for information to be exchanged between researchers and laboratories. Portability of unambiguous sequence data is important for furthering our understanding of the genetic relationship of microorganisms from a regional, national, and global perspective and defining their biological relevance.

Importantly, DNA target sequencing can serve as an adjunctive tool to conventional and MALDI-TOF-based identification methods. When MALDI-TOF libraries are insufficient to identify unusual or rarely encountered organisms, sequencing can act as a confirmatory or reference method for identifying such pathogens. We recommend that laboratories develop algorithm screening for microorganisms that can be identified by conventional methods with only a subset of isolates referred for 16S rRNA gene sequencing. For many bacteria, conventional testing, including MALDI-TOF, is less expensive, quicker, and more convenient than sequence-based methods. Conventional testing remains a cost-efficient and relatively accurate method to identify most microorganisms associated with clinical disease. Indeed, for microorganisms that share a high percent identity with 16S rRNA sequencing, simple and rapid biochemical tests can help differentiate between species and provide the definitive identification (Table 1). Of consideration in the routine use of DNA target sequencing is the need for technical expertise and its cost. Microbiologists who are less familiar with molecular techniques may find the transition to a molecular platform difficult. However, this technology is well received in the laboratory because it possesses high-throughput capabilities and many options for user-friendly software.

In addition to the use of sequencing in the identification of bacteria from pure colonies, direct amplification and sequencing from clinical specimens have become important diagnostic tools. Viable microorganisms may not

TABLE 1 Select microorganisms with indistinguishable 16S rRNA gene sequences and suggested supplemental phenotypic tests

Microorganism(s)	Phenotypic tests for species resolution
Bacillus anthracis and *Bacillus cereus*	Motility, hemolysis on blood agar, penicillin sensitivity Refer suspected *B. anthracis* isolates to Laboratory Response Network
Bordetella pertussis, *Bordetella parapertussis*, and *Bordetella bronchiseptica*	Urease, catalase, oxidase, motility, citrate
Campylobacter coli and *Campylobacter jejuni*	Hippurate hydrolysis, MALDI-TOF
Clostridium botulinum and *Clostridium sporogenes*	Refer to Laboratory Response Network for botulinum toxin testing in suspected cases
Escherichia coli and *Shigella* spp.	Spot indole, motility, lactose fermentation MALDI-TOF does not provide good species-level resolution
Streptococcus pneumoniae and *Streptococcus mitis*	Bile solubility
Pseudomonas spp.	For *Pseudomonas fluorescens* group, rule out *Pseudomonas aeruginosa* by phenotypic tests if necessary
Yersinia pestis and *Yersinia pseudotuberculosis*	Motility, refer suspected *Y. pestis* isolates to Laboratory Response Network

be available from a specimen due to low organism burden, previous antibiotic treatment, or the presence of highly fastidious organisms that do not grow well in routine culture conditions, such as *Coxiella burnetii*, *T. whipplei*, and *Bartonella quintana*. Additionally, sequencing can be performed from formalin-fixed paraffin embedded (FFPE) specimens—a performance characteristic with high value when the entire specimen is placed in formalin and not sent to the microbiology laboratory for culture.

Next-generation sequencing (NGS) has expanded our understanding of the microbial community in various tissue and body sites. The role of the microbiome has informed our understanding of disease pathogenesis and health outcomes. DNA target sequencing has become a helpful companion to NGS through characterization of the individual organisms that are identified in microbial communities. This is especially important because NGS identifies organisms that are difficult to culture or are noncultivable. Additionally, DNA target sequencing is not limited to monomicrobial infections. Although not as robust as NGS, in combination with various software algorithms, multiple microorganisms can be differentiated from mixed microbial populations identified using DNA target sequencing.

Caution with result interpretation is extremely important when performing amplification and 16S rRNA sequencing directly from clinical specimens. While the increased sensitivity of sequencing for detection of a pathogen is of value in the setting of previous antibiotic treatment or low organism burden, there is also a risk for increased detection of potential contaminants such as *Propionibacterium acnes* or coagulase-negative *Staphylococcus* species. Distinguishing between true infection and contamination is a challenge, and laboratories should make efforts to correlate sequencing results with Gram-stain or other clinical information prior to reporting results. Another challenge of sequencing directly from specimens is that multiple organisms may be detected from sources that historically were associated with monomicrobial infections. However, recent studies have demonstrated the important role of microbial communities in the pathogenesis of infection, and hence, identification of multiple pathogens from clinical specimens through DNA target sequencing will enhance our understanding of human disease and health.

METHODS: GENERAL CONSIDERATIONS

DNA Preparation

In this chapter, we address the preparation of DNA from pure culture, clinical specimens, and FFPE tissues. When starting from culture, the starting material can be either a broth culture (including positive blood culture or liquid AFB culture broth) or colonies on solid media. Centrifugation and washing the cells with sterile water or phosphate-buffered saline are recommended to dilute media because the composition of the media can affect the fidelity of the PCR. Preparation methods range from simple cell lysis to a robust DNA purification. The method of choice is usually influenced by the laboratory workflow and the spectrum of microorganisms that are being analyzed. For example, Gram-negative bacilli such as *Escherichia coli* may require no prelysis step, and cells may be directly added to the PCR, where the elevated 94°C denaturation step is sufficient to lyse the bacteria.

For methods that do not purify the DNA, the concentration of inocula is an important consideration to prevent PCR inhibition. For tissue, body fluid, or FFPE specimens, additional DNA purification steps should be performed to remove cellular debris. In fresh samples, body fluid specimens may go directly to DNA extraction, but tissue requires grinding of the sample prior to DNA extraction. For FFPE tissues, deparaffinization must be performed prior to DNA extraction. It should be taken into consideration that the process of formalin fixing and subsequent deparaffinization damages DNA and may inhibit the amplification of the target for sequencing. An internal control such as β-actin may be used to asses the PCR efficiency in these tissues.

Amplification and Sequencing

The selection of PCR reagents and enzymes should be influenced by laboratory workflow, the anticipated size of the generated amplicon, convenience, and considerations of contamination control. When amplification is performed directly from pure culture, contamination control via uracil *N*-glycosylase is not critical. For laboratories that cannot adequately separate the sample preparation and amplification areas, use of uracil *N*-glycosylase is strongly recommended. In clinical laboratories, the first 500 bp of the 16S rRNA gene is the most common portion of the gene used for identification. The 16S rRNA 500-bp fragment will identify most microorganisms and can be bidirectionally sequenced with a single forward and reverse primer. To reduce costs, some laboratories have favored the use of only the forward or reverse sequence. While more expensive, use of both forward and reverse strands allows evaluation of the impact of copy variants that can be present within a single 16S rRNA genome for many bacterial pathogens (28, 29). Table 2 provides information about several versions of primers targeting similar regions of the 500-bp region. PCR conditions and cycling times are influenced by amplification reagents and available instrumentation. For example, Applied Biosystems offers two versions of the MicroSeq 500-bp kit. One version controls for contamination with use of dUTP instead of dTTP and requires ~2 h to complete the PCR step. Their second version amplifies the gene in ~45 min by use of a "fast

TABLE 2 Frequently used primer sequences for gene sequence-based identification of bacteria

Gene target	Primer name	Orientation	Sequence (5′ to 3′)
16S rRNA	4F	Sense	TTGGAGAGTTTGATCCTGGCTC
16S rRNA	27F	Sense	AGAGTTTGATCMTGGCTCAG
16S rRNA	534R	Antisense	TACCGCGGCTGCTGGCAC
16S rRNA	801R	Antisense	GGCGTGGACTTCCAGGGTATCT

kit." Preparation of the PCR for sequencing can be performed by using shrimp alkaline phosphatase and exonuclease I or by affinity matrixes such as magnetic beads or column purification. (Note that when uracil *N*-glycosylase is included in the PCR, enzymatic purification will not be adequate.)

The alternative sequencing targets, *sodA*, *hsp*65, and *rpoB*, are used when the 16S rRNA gene does not provide sufficient discrimination. The *rpoB* gene is often used as an alternative target to 16S for routine sequencing of bacterial isolates. Of particular value is that it is generally a monocopy gene, reducing the challenges that arise with multiple copies of 16S rRNA in a bacterial genome. Additionally, the *rpoB* gene reflects more accurately DNA-DNA hybridization than has been described in the 16S rRNA gene. The *rpoB* gene and other alternative targets have been demonstrated to be effective in providing resolution within groups of closely related bacteria and may refine phylogenetic identification of bacterial genera. However, the use of targets other than the 16S rRNA gene is limited due to the lack of standardization in the development of kits and databases specific to those targets.

Several methods exist for inferring DNA sequence data such as pyrosequencing, mass spectrometry, a massively parallel sequence often referred to as next-generation sequencing or NGS, and the most common method, capillary electrophoresis. Data from capillary electrophoresis are viewed as electropherograms, which contain the sequence and quality information. The Phred score or quality value (QV) is a score for each base call that estimates the probability that the base was correctly called. For example, a QV of 10 indicates a 10% probability that the error was called incorrectly, and a QV of 20 indicates a 1% probability of error. It is important to realize that multiple copies of the 16S rRNA gene often exist in a bacterial genome, and sequence differences between copies can affect the ability to analyze the sequence.

Controls

Controls are useful for monitoring DNA preparation (extraction), amplification, and sequencing steps. A negative control and a positive control should be incorporated at the DNA preparation step. The DNA preparation negative control should be the same solution that serves as the starting material for the isolates to be analyzed. For the DNA preparation positive control, an uncommon isolate that is not a human pathogen is recommended. A second set of positive and negative controls should be added at the amplification step to monitor the components of the PCR. The positive control at this step should be purified DNA devoid of inhibitors and also an uncommon microorganism that is different from the DNA preparation control. Sterile water is recommended for the negative control. Sequencing controls are not critical to the process but can be helpful in monitoring sequencing reagents in cases of a complete sequencing run failure. A plasmid, pGEM, is often provided in sequencing kits and is a suitable control to monitor this step.

Interpretation of Results

Definitions

The "percent identity" for a sequence is defined as the percentage of nucleotide bases between the query and reference sequence that are identical in the aligned region. "Percent separation" indicates the distance between the query and subject sequence and is simply 100% minus the percent identity. These two values are usually used when establishing criteria for microorganism identification. It is also important to consider the query coverage when using programs like BLAST. The query coverage represents the percentage of the query sequence used to generate the alignment, which is the query length aligned / query length.

Interpretation

Because of the inherent issues with any database, it is advisable for laboratory personnel to review more than just the first few references from a BLAST search to analyze for possible aberrant references. At least the first 20 matches should be reviewed to detect any outlying or erroneous references. Whenever there is a question, the origin of a reference sequence should be examined to assess its validity, which can be based on several parameters such as derivation of the sequence (type strain, peer-reviewed publication, and year) and whether the species is formally recognized by DSMZ or other reputable collections. As a general practice, for viable, cultivable microorganisms, sequencing results should always be correlated with colony morphology prior to reporting a final result to avert laboratory errors.

SEQUENCING SOFTWARE

Sequence analysis and sequence alignment are two core tasks addressed by software. Many platforms are available for sequence analysis, which consist of manual and automated editing of the electropherograms, and consensus generation when more than one overlapping sequence fragment is generated from a sample. Sequence alignment is used to compare the edit query sequences to a reference database. Sequence alignment may be incorporated into products that allow sequence editing, but these two tasks are often split among software packages. In this chapter we provide software resources that mainly target Sanger-based sequencing, since this is the most common form of sequencing used on pure culture. Other chapters in this book specifically address NGS applications. In some cases the software is agnostic in regard to the method by which the sequence was generated.

Software for sequence analyses includes MicroSeq (Life Technologies, Grand Island, NY) SmartGene (SmartGene, Lausanne, Switzerland), RipSeq (Isentio, Palo Alto, CA), DNA Baser Assembler (Heracle Software, Germany), SeqMan (DNASTAR, Inc., Madison, WI), Geneious (Biomatters, Auckland, New Zealand), and CLC Workbench (CLC Bio, Aarhus, Denmark). In general, these applications provide a graphical view of the electropherogram with the text sequence underneath. The interface can be used to edit base calls with ambiguity or bases with low quality scores. Settings based on sequence quality values can also be used to automatically trim the sequence or resolve base conflicts between two sequencing fragments. The speed at which a sequence can be identified is influenced by a number of parameters, including whether the alignment is performed locally or on a server, the number of reference sequences in the database, the settings that are used to seed an alignment, and the number of processors used to run the alignment. Ultimately, the choice of software will be influenced by the number of expected sequences that will be analyzed in any given run and the

cost of the software. There are many commercial and free options available to analyze sequence data.

Sequence comparison or alignment to a reference database is performed after generating a high-quality consensus sequence. Common sequence alignment algorithms for sequence alignment include BLAST and Needleman-Wunsch. BLAST is one of the most popular sequence alignment programs. It allows the query sequence or subject sequence to align to its partner. BLAST is provided as an aligner in SmartGene, RipSeq, Geneious, and CLC Workbench. BLAST can also be accessed directly on NCBI using NCBI references as the reference database. In Geneious and the CLC Workbench, the NCBI BLAST web server is accessed when performing searches to NCBI databases. Alternatively, a custom database can be set up to run BLAST locally. SmartGene and Isentio are web-based software, and BLAST is performed on their servers with maintained databases. BLAST can also be set up locally and used with custom local databases. This is performed via the command line interface, so it may not be suitable for labs without a sufficient IT or bioinformatics infrastructure.

MicroSeq performs an alignment using the Needleman-Wunsch algorithm. Needleman-Wunsch differs from BLAST in that it is a global rather than local alignment and is typically slower than BLAST. The query sequence is aligned end-to-end, so both the reference and query sequence should contain the same region or the alignment values will be compromised.

An additional tool for sequence comparison is the RDP classifier (http://rdp.cme.msu.edu/) (30). The RDP classifier uses sequences (commonly referred to as kmers; e.g., 8-mers) of a length of 8 bp to determine the relationship to reference sequences. Unlike an alignment program, where a sequence is compared directly to a subject sequence, the RDP classifier requires that a database have an overlying taxonomic structure that describes how the references are related. The classifier is then trained on the database. The taxonomic lineage of the query sequence is determined from its kmer content. In this approach, the taxonomic lineage is reported rather than the top reference hit, a feature that may be helpful for placing novel organisms into the appropriate taxonomic location. Routine use of the RDP classifier for the identification of microorganisms in the clinical microbiology laboratory may be limited by the fact that the default database reports sequences only to the genus level. SmartGene, MicroSeq, and Isentio provide complete packages for microorganism identification that include tools for sequence analysis and sequence alignment with reference databases suited for microorganism identification to the species level. Such packages are sufficient for clinical laboratories to perform organism identification.

Database Selection

Both proprietary, commercially available, and free access, public reference databases exist for sequence comparison. A summary of the strengths and limitations of these databases is provided in Table 3. Databases that are available to the public include GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), European Molecular Biology Laboratory (EMBL) Nucleotide Sequence Database (http://www.ebi.ac.uk/embl//), DNA Data Bank of Japan (DDBJ) (http://www.ddbj.nig.ac.jp/), Ribosomal Database Project (RDP) (31) (http://rdp.cme.msu.edu/), Greengenes (32) (http://greengenes.secondgenome.com/), and Silva (33) (http://www.arb-silva.de/). GenBank, EMBL, and DDBJ form the International Nucleotide Sequence Collaboration (INSC), in which data are exchanged on a daily basis. In general, public databases contain many more sequences (e.g., RDP-II, Release 10, contains >700,000 aligned 16S rRNA sequences). However, the user should be aware that public reference sequences might contain errors with potentially poor quality sequences, inaccurate terminology, and outdated nomenclature.

The DNA target of interest will influence the choice of database. Some of the databases mentioned above only collect and curate data from ribosomal sequences. For example, the RDP database consists of small-ribosomal subunit (SSU) sequences for bacteria and large-ribosomal subunit (LSU) sequences for fungi; the Silva database contains LSU and SSU sequences for eukaryotic, bacterial, and archaeal sequences; and Greengenes contains bacterial/archaeal SSU sequences. These databases all draw from publicly available data from the International Nucleotide Sequence Collaboration and have different levels of curation, quality filtering, and database size. Silva and RDP offer a set of tools to query sequences on their servers. Greengenes references are downloaded by the user and must be used locally. Greengenes 16S rRNA sequences are clustered at different identity cutoffs, which provide a non-redundant dataset with each sequence representing an "operational taxonomic unit." Ribosomal sequences, or more specifically SSU sequences, offer the most species coverage in public databases and have been used extensively as phylogenetic markers. For alternative genetic markers, curated datasets are less common, and institutions may need to sequence the references internally to create a suitable clinical reference set.

The quantity and quality of reference sequences in a database are important to determine when making a selec-

TABLE 3 Comparison of features of various nucleotide sequence databases[a]

Database	Fee for use?	Database size (no. of sequences)	Comprehensive phylogenetic analysis	Creation of private sequence database	Automatic creation of searchable clinical isolate database	Use of flagging of questionable reference sequences	Quality control	Updates
SmartGene (v.3.2.3)	Yes	~130,000	Yes	Yes	Yes	Yes	Partial	Weekly
MicroSeq (v1.4.3)	Yes	~2,000	Yes	Yes	No	No	All type strains	Periodically
RIDOM	No	~250	Yes	No	No	No	All type strains	Periodically
RDP-II	No	>700,000	Yes	Yes	Yes	No	Partial	Monthly
GenBank	No	>700,000	No	No	No	No	No	Daily

[a]Modified from reference 48 with permission.

tion. Proprietary databases include SmartGene, Isentio, and MicroSeq. These databases contain 16S rRNA sequences for bacteria. Unlike other databases that contain mostly type strains, MicroSeq consists entirely of culture collection strains that may reflect the wider genetic variability within a species. MicroSeq has been evaluated by a number of investigators for identification of a wide breadth of bacteria (34). However, there are fewer sequences in the database, and it has been shown to lack some clinically important organisms (34).

SmartGene and Isentio build reference databases from sequence data in the public domain, using profile-based filters to exclude non-useful sequences and annotations. The SmartGene 16S rDNA database contains approximately 500,000 sequences, as of September 2014, and is updated by SmartGene on a daily basis. This approach accommodates species variability, including recently described organisms. All sequences included in the SmartGene databases have a link to their original source. SmartGene also has several datasets that have been created through collaborations with academic institutions, and SmartGene allows users to report suspected errors in sequence annotation, which can then be flagged for correction or removal. As targets other than 16S rRNA are being more widely used, databases have begun to incorporate these additional sequences. For example, SmartGene has additional databases for the *rpoB* sequence for bacteria and provides services to add user-specified targets. Isentio also provides alternative DNA targets in their database. Few studies have been undertaken to systematically compare the strengths and limitations of these proprietary databases (35). The choice of reference database ultimately depends on numerous factors, such as laboratory budget, computer skill level of the technologists, and personal preference for the various features unique to each product.

Criteria for Microorganism Identification

General Considerations

Sequences should be reviewed for read length and length of alignment. For sequencing of the first 500 bp of the 16S rRNA gene, at least 300 bp should be aligned with the database reference sequences to ensure that areas of sequence variability are captured. When the number of aligned base pairs is <300, the quality of the sequence should be questioned, and proper alignment with the reference sequence should be reviewed. Improper alignment may occur due to the presence of ambiguous base pairs, in which case manual alignment may rectify the issue. Ideally, the forward and reverse strands will form a consensus sequence. The Clinical and Laboratory Standards Institute (CLSI) has published standards for result analysis and interpretation (20). This document addresses criteria for identification of a bacteria, mycobacteria, and fungi to the genus and species levels for clinical microbiology laboratories. For most bacteria, except for aerobic actinomycetes and mycobacteria, ≥99.0% identity with an acceptable reference sequence is required for species identification, and ≥97.0% identity is required for genus identification. When the identity score is <97.0% but ≥95.0%, the microorganism cannot be identified to the genus or species level but can be reported as most closely related to a genus. For isolates that share less than 95% identity with a reference sequence, the reference database may be insufficient and/or the isolate may represent a novel species. The cutoff for species identification may vary if alternative targets are used for sequencing depending on the level of shared sequence identity in the target. These general guidelines should certainly be considered in the context of the clinical scenario and microorganism phenotype. The guideline is a pragmatic approach for implementing DNA target sequencing in clinical laboratories and attempts to develop a uniform standard for laboratory specialists to identify microorganisms. The guideline does not reflect strict taxonomical classifications since microbial taxonomy remains an evolving field and the concept of species is largely unresolved.

Staphylococcus spp. and Related Gram-Positive Cocci

Although identification of *Staphylococcus aureus* is usually straightforward with conventional testing, difficult to identify *S. aureus* isolates are occasionally encountered. The 16S rRNA target can identify *S. aureus*, *Staphylococcus warneri*, and *Staphylococcus lugdunensis*. For many other coagulase-negative *Staphylococcus* spp. such as *Staphylococcus epidermidis*, *Staphylococcus capitis*, *Staphylococcus caprae*, *Staphylococcus schleiferi*, and *Staphylococcus intermedius*, the use of alternative targets such as sodA provides better resolution to the species level (36). *Staphylococcus saprophyticus* and *Staphylococcus xylosus*, both of which are novobiocin resistant, may not be differentiated using 16S rRNA, and use of *tuf* or *rpoB* genes would be better for this purpose (24, 37–39). The importance of identifying these species of coagulase-negative staphylococci to the species level lies in their different antibiotic susceptibility profiles and potential differences in virulence. Increased resistance to glycopeptides has emerged in species such as *S. epidermidis*, *Staphylococcus haemolyticus*, and *S. lugdunensis* associated with native valve endocarditis (40). While beyond the scope of this chapter, it is noted that *rpoB* gene mutation analysis can also provide information on rifampin resistance in *Staphylococcus* spp. (24, 41). For other staphylococcus-like microorganisms, such as *Micrococcus* spp., *Kocuria* spp., and *Kytococcus* spp., 16S rRNA provides adequate resolution to the species level.

Streptococcus spp. and *Streptococcus*-Like Microorganisms

Identification of streptoccoci to the species level can be challenging, even using conventional methods, given the prominent role of horizontal gene transfer in this genus. 16S *Streptococcus pyogenes* and *Streptococcus agalactiae* as well as the former "nutritionally variant streptococci," *Abiotrophia* spp. and *Granulicatella* spp., are easily identified by using 16S rRNA as the gene target. However, there is no single gene target to reliably identify beta-hemolytic streptococci of Lancefield group C and G (42). For viridans group streptococci, resolution to the genus level with the 16S rRNA target is usually possible, but there is limited resolution to the species level; *rpoA* has been demonstrated to provide better species resolution for the viridans group streptococcus (25, 43, 44). *Streptococcus cristatus*, *Streptococcus oralis*, and members of the *Streptococcus anginosus* group are exceptions, with 16S rRNA providing sufficient resolution to the species level (45, 46).

Enterococci are usually resolvable to the species level, but a high level (>99.0%) of shared identity may be seen between the *Enterococcus* species *Enterococcus faecium* and *Enterococcus durans*, *Enterococcus hirae*, or *Enterococcus azikeevi* (47). *Enterococcus casseliflavus* and *Enterococcus flavescens* share a similarly high percent identity, and it is

now believed that they represent a single species, *E. casseliflavus* (48).

Anaerobes

DNA target sequencing is still the most accurate method of anaerobic identification, especially since databases from conventional methods often are not current and do not reflect the tremendous genetic diversity within anaerobic taxa. More accurate identification is highly relevant clinically because antimicrobial resistance is increasing. Determining the optimal percent identity to identify an anaerobic isolate is difficult due in large part to evolving taxonomical classifications and uncertainty about the taxonomical significance of genetic heterogeneity. 16S rRNA gene sequence provides resolution to the species level for many anaerobic Gram-negative bacilli, such as *Bacteroides* spp., *Parabacteroides* spp., *Porphyromonas* spp., *Prevotella* spp., *Veillonella* spp., *Anaerobiospirillum* spp., *Bilophila* spp., *Desulfovibrio* spp., *Tissierella* spp., and *Tannerella* spp. (49–51). Speciation of *Fusobacterium* spp. may be limited using the 16S gene, and *rpoB* may be the preferred target for this genus (52). The 16S rRNA gene sequence remains the preferred target for many Gram-positive anaerobes as well, including *Bifidobacterium* spp., *Eubacterium* spp., *Eggerthella* spp., *P. acnes*, *Finegoldia magna*, *Parvimonas micra*, *Peptostreptococcus anaerobius*, *Peptoniphilus asaccharolyticus*, and *Anaerococcus* spp. (53, 54). For *Clostridium* spp., resolution to the species level is typically good, with the notable exceptions of *Clostridium botulinum* and *Clostridium sporogenes*, which often cluster together. Testing for botulinum toxin is necessary for definitive identification.

Gram-Negative Bacilli: Enterics, Nonfermenters, and *Campylobacterales*

Many species in the family *Enterobacteriaceae* can be identified by use of the 16S rRNA gene target. However, use of automated identification methods is in many cases quicker and more convenient without compromising accuracy. When 16S rRNA fails to provide adequate identification, *rpoB*, *gyrB*, *tuf*, or F-ATPase beta-subunit (*atpD*) genes can provide better resolution (55, 56). The utility of 16S rRNA sequencing depends on the genera of the organisms in question. For example, the genera *Proteus*, *Providencia*, *Morganella*, *Xenorhabdus*, *Cedecea*, *Edwardsiella*, and *Hafnia* demonstrate good separation. On the other hand, *E. coli* and *Shigella* spp. cannot be distinguished based on 16S rRNA sequencing because genetically, they can be considered the same species. *Aeromonas* species cannot be reliably identified using the 16S gene, and resolution to the species level can be achieved by sequencing the *gyrB* or *rpoD* gene (57, 58).

Classification of microorganisms belonging to the genera *Klebsiella*, *Enterobacter*, *Citrobacter*, and *Pantoea* based on phenotypic characteristics does not correlate well with phylogenetic classification using the 16S rRNA region. Our understanding of their taxonomic relationship is still evolving, and conventional methods of identification may be more helpful at this time. *Citrobacter freundii*, *Enterobacter aerogenes*, and *Pantoea agglomerans* may cluster closely together, as can *Citrobacter koseri* and some *Salmonella* spp. Identification of *Klebsiella* spp. and *Raoultella* spp. to the species level is usually limited, but *Klebsiella oxytoca* is well separated from the *Klebsiella pneumoniae* group (*K. pneumoniae*, *Klebsiella milletis*, and *Klebsiella granulomatis*) (59). Slightly different criteria should be applied to interpreting sequencing results for these microorganisms. The cutoff for species identification should be raised to 99.5% with separation of >0.5% between species. For sequence identities of ≥97.0% but with poor separation between genera, it may be necessary to report all closely related genera. However, species of different genera from the *Enterobacteriaceae* family typically share between 97 and 99% 16S rRNA gene sequence identity. Alternative targets such as *rpoB* or *gyrB* may be more useful for providing greater resolution to the species level for *Klebsiella* spp., but shared sequence identity may still be observed with the *rpoB* target between newly classified groups of *Enterobacter* species (shared identity ranges from 88 to 100%) (60).

For the glucose-nonfermenting Gram-negative bacilli, 16S rRNA gene target sequencing is a valuable tool because many such microorganisms are difficult to separate phenotypically. Resolution to the species level is well attainable for species of *Stenotrophomonas*, *Pasteurella*, *Moraxella*, *Achromobacter*, *Alcaligenes*, *Actinobacillus*, *Aggregatibacter*, *Oligella*, *Ralstonia*, *Pandoraea*, *Eikenella*, and *Kingella*. Identification to the species level using 16S rRNA is attainable for most *Burkholderia* spp., and greater resolution of the *Burkholderia cepacia* complex can be achieved by sequencing the *recA* gene (61). For *Haemophilus* spp., the 16S rRNA sequence does not allow for adequate speciation because *Haemophilus influenzae* can cluster with *Haemophilus haemolyticus* or *Haemophilus aegyptius*. While phylogeneic relationships of *Haemophilus* and *Aggregatibacter* are continuing to be evaluated, multitarget sequencing may be required for the most accurate identification (62).

For *Acinetobacter* spp., resolution may be to only the genus level with the 16S rRNA gene and to groups using the *rpoB* gene (63). If *Acinetobacter baumannii* needs to be excluded, MALDI-TOF is not able to distinguish *A. baumanii* from other related *Acinetobacter* species; phenotypic tests (growth at 42°C and acid production from lactose) are helpful. For *Pseudomonas* spp., *Pseudomonas aeruginosa* can usually be discerned, but other members may only be classifiable to groups, e.g., *Pseudomonas fluorescens*, *Pseudomonas stutzeri*, and the *Pseudomonas alcaligenes* group. The *Bordetella* species *Bordetella pertussis*, *Bordetella parapertussis*, and *Bordetella bronchiseptica* share >99% sequence identity, and therefore resolution is usually limited to the genus level only (64). Phenotypic tests or use of *recA* sequence analysis can provide resolution to the species level (65). *Legionella* spp. are adequately identified to the genus level by 16S rRNA sequencing, but resolution to the species level requires additional targets such as *mip* or *rpoB* (66).

Neisseria meningitidis is not well demarcated from *Neisseria lactamica*, *Neisseria cinerea*, or *Neisseria gonorrhoeae* using the 16S rRNA target. Laboratories should be aware that there are a great number of *N. meningitidis* reference sequences in databases, such that they can obscure the presence of other *Neisseria* spp. that appear much lower on the list of matches but that are closely related as well. Misidentification of commensal *Neisseria* species as *N. meningitidis* has also occurred with MALDI-TOF (67). A fragment of the 50S ribosomal protein L6 (*rplF*) gene has been demonstrated to distinguish between *Neisseria* species (68). *Bartonella* spp. can be identified to the genus level and, in some cases, to the species level. For *Rhizobium*, *Agrobacterium*, *Brucella*, and *Ochrobactrum* spp., 16S rRNA analysis can provide resolution to the genus level, but *recA* or *gyrB* can better provide resolution to the species level (69).

Campylobacter coli and *Campylobacter jejuni* cannot be differentiated by 16S rRNA sequencing. We have found

that phenotypic methods such as hippurate hydrolysis or MALDI-TOF are valuable for differentiating these two species. Other *Campylobacter* spp. can frequently be identified to the species level with 16S rRNA sequence analysis. *Helicobacter* spp. and *Arcobacter* spp. can usually be resolved to the species level with the 16S rRNA target. Other targets, such as *hsp60* and *atpA*, have been shown to give better resolution of *Campylobacter*, *Helicobacter*, and *Arcobacter* to the species level (70).

Coryneform Gram-Positive Bacilli

Once believed to largely represent a skin contaminant when isolated in culture, coryneform bacteria are now recognized as important pathogens, especially in immunocompromised patients. Identification of a clinically significant coryneform organism is important, because some have characteristic antimicrobial susceptibility patterns, with some well known for multidrug resistance (71). As mentioned previously, copy variants of the 16S rRNA may be seen more frequently in coryneform bacteria, creating the appearance of mixed bases in the sequencing data. With proper manual editing, a consensus sequence can usually be reached. Identification to the species level can be achieved for most coryneform bacteria, including *Actinobaculum* spp., *Arcanobacterium* spp., *Brevibacterium* spp., *Gardnerella vaginalis*, *Rothia* spp., and *Dermabacter hominis* (1). For the genus *Corynebacterium*, resolution to the species level is good for some members, but there is poor separation between *Corynebacterium ulcerans* and *Corynebacterium pseudotuberculosis*, between *Corynebacterium pseudodiphtheriticum* and *Corynebacterium propinquum*, and among *Corynebacterium amycolatum*, *Corynebacterium xerosis*, and *Corynebacterium freneyi* (72). Due to the lack of resolution between species in this genus, the *rpoB* gene may be the preferred sequence to resolve to the species level (73). For *Actinomyces* spp., there is good resolution to the species level except for *Actinomyces meyeri*, *Actinomyces odontolyticus*, and *Actinomyces lingae*, which cluster as a group (12). Alternative targets such as *rpoB*, *atpA*, or *metG* may be necessary to resolve the *Actinomyces* spp. to the species level (74).

Nocardia spp. and Other Aerobic Actinomycetes

In general, ≥99.6% sequence identity in the 16S rRNA is sufficient to identify an organism to the genus and species level for *Nocardia* spp. and other aerobic actinomycetes, provided that there is at least 0.4% separation between different species. Between 99.0 and 99.5% identity, an organism can be identified to the genus level. A sequence identity of ≥95% but <99.0% is insufficient to definitively identify an aerobic actinomycete to the genus level, but the organism may be reported as "most closely related" to the genus in question.

Many *Nocardia* spp. can be identified to the species level using the 16S rRNA target, including *Nocardia beijingensis*, *Nocardia brasiliensis*, *Nocardia farcinica*, *Nocardia otitidiscaviarum*, and *Nocardia kruczakiae*. For better resolution to the species level, *secA*, *rpoB*, and *gryB* have been used successfully (21, 75, 76). In laboratories where the technology and expertise are available, multilocus sequence analysis using a combination of these genes may be the optimal approach (77). For other aerobic actinomycetes, including *Actinomadura* spp., *Gordonia* spp., *Rhodococcus* spp., and *Tsukamurella* spp., 16S rRNA is an appropriate target to resolve an organism to the species level. *Streptomyces* spp. can frequently only be resolved to the genus level, which is usually sufficient for clinical purposes (15).

Bioterrorism Agents and Other Microorganisms

For agents of bioterrorism, i.e., *Bacillus anthracis*, *Brucella* spp., *Clostridium botulinum*, *Francisella tularensis*, and *Yersinia pestis*, 16S rRNA sequencing has varying utility. There is poor separation between *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis*; between *C. botulinum* and *C. sporogenes*; and between *Y. pestis* and *Yersinia pseudotuberculosis*. *F. tularensis* and *Brucella* spp. are conveniently identified with the 16S rRNA target. In any suspected cases, the isolate should immediately be referred to the nearest Laboratory Response Network laboratory (http://www.bt.cdc.gov/lrn).

We have found 16S rRNA sequencing to be valuable in identifying other bacteria worth mentioning. *Streptobacillus moniliformis* is easily identified, and in nearly all cases we have been able to extract a patient history of rat exposure. *Mycoplasma hominis* has been identified from various body sites, including soft tissue, abdominal fluid, and cerebrospinal fluid. In many cases growth of a faint haze of microorganism is seen on sheep blood agar during routine culture. Sequencing can confirm the organism identity more quickly than most phenotypic assays for identifying *M. hominis*.

Detection of Organisms from Primary Specimens

The utility of 16S sequencing has expanded beyond sequencing from isolated colonies to sequencing from the primary clinical specimen. In cases where organisms are slow growing, require specific cultivation environments, or are noncultivable, direct sequencing provides the only means of identification of the pathogen. Sequencing can also be applied from direct specimen for more timely results for slowly growing microorganisms. Direct sequencing of whole blood to identify *Borrelia* spp. is a tool to aide in the diagnosis of tick-borne relapsing fever, particularly in cases of low spirochetemia (78). Causes of culture-negative endocarditis can be ascertained through sequencing of infected valves, particularly for *B. quintana* and *C. burnetii* (79). In cases of direct sequencing of clinical specimens, the 16S gene is likely the best target to sequence since there are more well-characterized databases to query for organism identification.

Novel Organism Identification

One of the valuable applications of DNA target sequencing is in the identification of novel organisms. Currently, the 16S rRNA gene is the benchmark for novel organism identification; however, because sequencing technologies are evolving, interrogation of additional targets has expanded the opportunity to identify novel organisms. The *International Journal of Systematic and Evolutionary Microbiology* (*IJSEM*) is the journal responsible for the taxonomy, nomenclature, identification, and characterization of all microorganisms. The journal requires that upon proposing a new organism, authors provide evidence that type strain sequences are deposited in recognized culture collections in two different countries. Additional guidelines for the description of a new taxon are available at http://ijs.sgmjournals.org. Sequencing a single locus such as the 16S gene to identify a novel species has limitations, and

sequencing of the entire 16S gene, use of multilocus sequencing, and whole-genome sequencing will likely become the gold standards for novel organism identification and taxonomy assignment (80).

REPORTING RESULTS

In most cases, a result can be reported as genus and species provided that the sequencing results have met established criteria. For microorganisms with more than one top-scoring match, it is possible to report all top matches, using a slash mark in between, for example, "*Neisseria meningitidis/Neisseria lactamica*." When the top references do not separate well enough, it may also be necessary to report that the second most closely related species could not be ruled out, or adjunctive phenotypic testing may be able to provide resolution to species. Occasionally isolates will be identified as a bacterial species that is completely novel to the laboratory and clinician. If the laboratory information system permits, it would be highly informative to clinicians for the laboratory to provide a phylogenetic representation of the microorganism. This way, the most closely related bacteria are demonstrated, facilitating clinical decision making (Fig. 3).

CONCLUSION

Molecular studies have enhanced our knowledge about the taxonomical diversity among bacteria and have improved our ability to conduct surveillance of emerging pathogens. Numerous pathogens once believed to be rare clinical isolates or even contaminants are now being identified more precisely by nucleic acid sequencing and recognized as being clinically relevant. Our taxonomical understanding continues to evolve along with our understanding of their clinical correlates.

Although sandwiched between the widely attainable and inexpensive option of MALDI-TOF and the broadly applicable option of NGS, DNA target sequencing remains an important part of the clinical microbiology toolbox. As conventional identification methods, including MALDI-TOF, increase the numbers of microorganisms in their databases, the role of 16S rRNA sequencing has shifted from routine to use as a reference method or as a method to identify novel or uncultivable pathogens. Application of 16S rRNA sequencing has defined the etiologic agent for various culture-negative infections, such as meningitis, brain abscess, septic arthritis, endocarditis, and sepsis. Although MALDI-TOF has emerged as a routine tool for identification of microorganisms from culture-dependent methods, it continues to rely on sequencing to confirm identification of newly identified organisms.

Sequencing remains a powerful tool in clinical diagnostic laboratories, and its data is portable and transferable. The technology is culture independent, enabling direct detection of microorganisms from clinical specimens—a valuable tool particularly for patients on antecedent antimicrobial therapy. Software has been developed to tease apart mixed sequences and perform multiplex sequencing reactions in the same tube. Evolution of routine Sanger sequencing to newer sequencing applications will allow us to gain insight into the contribution of previously unidentified organisms including complex microbial communities contributing to human health and disease. These sequencing-based innovations combined with improvements in cost, automation, and ease of use will accelerate the ap-

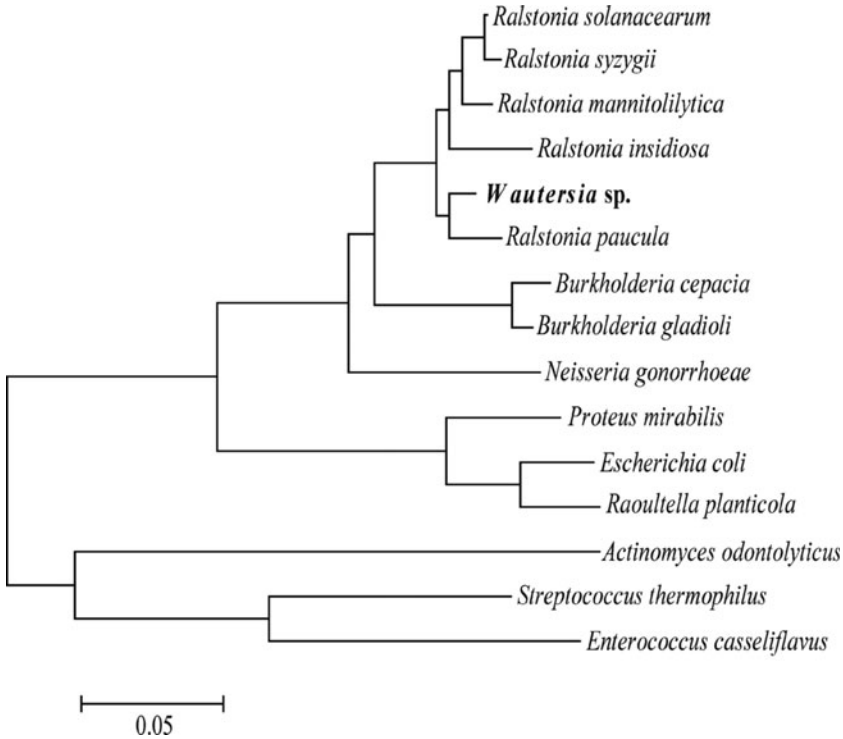

FIGURE 3 Phylogenetic representation of an unusual clinical isolate, such as this *Wautersia* sp., can provide useful information to the clinician if included with the patient results.

plication of sequence analysis for clinical microbiology laboratories.

This chapter contains information presented in the second edition of this text in the chapter by Rosemary She.

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Microbial Whole-Genome Sequencing: Applications in Clinical Microbiology and Public Health

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3

The genomic era of bacteriology began in 1995 when the first bacterial genome (*Haemophilus influenzae*) was sequenced using the Sanger sequencing method (1). A decade later, the introduction of high-throughput or next-generation sequencing (NGS) technologies allowed sequencing of bacterial genomes to be performed in days rather than months or years (2). These newer technologies use different processes but rely on a combination of template preparation, sequencing and imaging, and genome alignment and assembly methods (3). The major advantages of NGS over Sanger sequencing are speed of sequencing combined with lower costs (4). The ability to readily sequence the whole genome of microorganisms has enabled the performance of large-scale comparative and evolutionary studies that were unimaginable even a few years ago (5–9). Furthermore, the development of rapid benchtop sequencing platforms (10) that are able to sequence a microbial genome in a day makes them increasingly appropriate for introduction into the diagnostic microbiology laboratory environment (11). This chapter will briefly review the currently available NGS technologies and platforms, followed by an in-depth review of the potential clinical applications of whole-genome sequencing (WGS) in the microbiology laboratory. We will also present the challenges for implementation of WGS in the clinical setting and consider some future directions.

THE CURRENT DIAGNOSTIC MICROBIOLOGY PARADIGM

Clinical microbiology is a laboratory discipline that primarily focuses on the detection and characterization of pathogens that cause disease in humans (diagnostic microbiology) (12, 13). The laboratory also has a secondary function: the surveillance and monitoring of infectious diseases (public health microbiology). This is particularly important for detecting outbreaks of infection and the emergence and spread of new pathogens or highly drug-resistant or virulent organisms. Bacterial pathogens account for a large proportion of the global burden of infectious diseases, and the majority of samples submitted to the microbiology laboratory for investigation fall into this category.

Culture, Identification, and Antimicrobial Susceptibility Testing of Bacterial Pathogens

Clinical specimens are submitted to the laboratory and cultured to detect pathogens, which then undergo identification using a variety of methods. These include colonial appearance and morphology, Gram staining and microscopy, and biochemical tests which may be performed at the bench or using an automated platform such as the Vitek 2 system (Biomérieux, Marcy l'Etoile, France) or BD Phoenix (Becton Dickinson, Oxford, England). Antimicrobial susceptibility testing is performed in parallel to identify drugs to which the isolate is susceptible and resistant and to enable appropriate treatment. Recently, matrix-assisted laser desorption ionization–time of flight mass spectroscopy, a method that can rapidly identify many bacterial pathogens within a few minutes at low cost (14), has been introduced into many diagnostic microbiology laboratories. However, although it has many advantages over more traditional identification methods, it cannot reliably distinguish certain species (e.g., *Escherichia coli* and *Shigella sonnei*), and does not provide antimicrobial susceptibility data.

Epidemiological Typing and Detection of Virulence Determinants

In the context of suspected outbreaks of infection, bacterial isolates collected from infected patients undergo epidemiological typing to determine their degree of relatedness. For certain organisms such as carbapenem-resistant *Enterobacteriaceae* or multidrug-resistant or extensively drug-resistant tuberculosis, additional antimicrobial susceptibility testing is required, usually at a reference laboratory. For other species such *Staphylococcus aureus* and group A streptococci, testing for virulence determinants such as toxins may also be performed. The time taken to provide a final result (turnaround time) increases with the number of tests performed on the bacterial isolate. This can sometimes be

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too long to guide initial patient management, particularly for samples that require reference laboratory testing. The complexity of the current diagnostic microbiology paradigm is illustrated in Fig. 1.

Identification of Other Pathogens

Similar principles apply to the identification of other pathogens including fungi, viruses, and parasites, although the tests that are performed may differ. Fungi are cultured on specialist media and undergo identification and antifungal susceptibility testing in a diagnostic or reference laboratory. The diagnosis of viral infections is predominantly based on molecular diagnostics directed at detection of virus antigens or nucleic acids using amplification methods. Parasites are usually diagnosed by microscopy or molecular methods. This review will focus primarily on the clinical applications of NGS in the diagnosis and management of bacterial and viral pathogens, to reflect applications that are likely to be adopted first into day-to-day clinical practice. However, because the technology is pathogen agnostic, it could potentially be applied to other pathogens.

NGS TECHNOLOGIES AND PLATFORMS

Sequencing technologies include a number of methods that involve template preparation, sequencing, and imaging and data analysis (3). The type of data produced by each platform (e.g., length of DNA sequence read and depth of coverage) depends on the unique combination of methods for these steps and can present challenges when comparing data quality and costs between platforms.

Template Preparation

There are two methods for preparing templates for NGS reactions: clonally amplified templates arising from single DNA molecules and single DNA molecule templates (3). Current methods are based on randomly shearing genomic DNA from which templates are created. The template is then attached or immobilized to a solid surface or support, which enables thousands or millions of sequencing reactions to be performed simultaneously. The two most common methods for clonal amplification are emulsion PCR (15) and solid-phase amplification (16) (Fig. 2). Although clonally amplified methods offer some advantages over

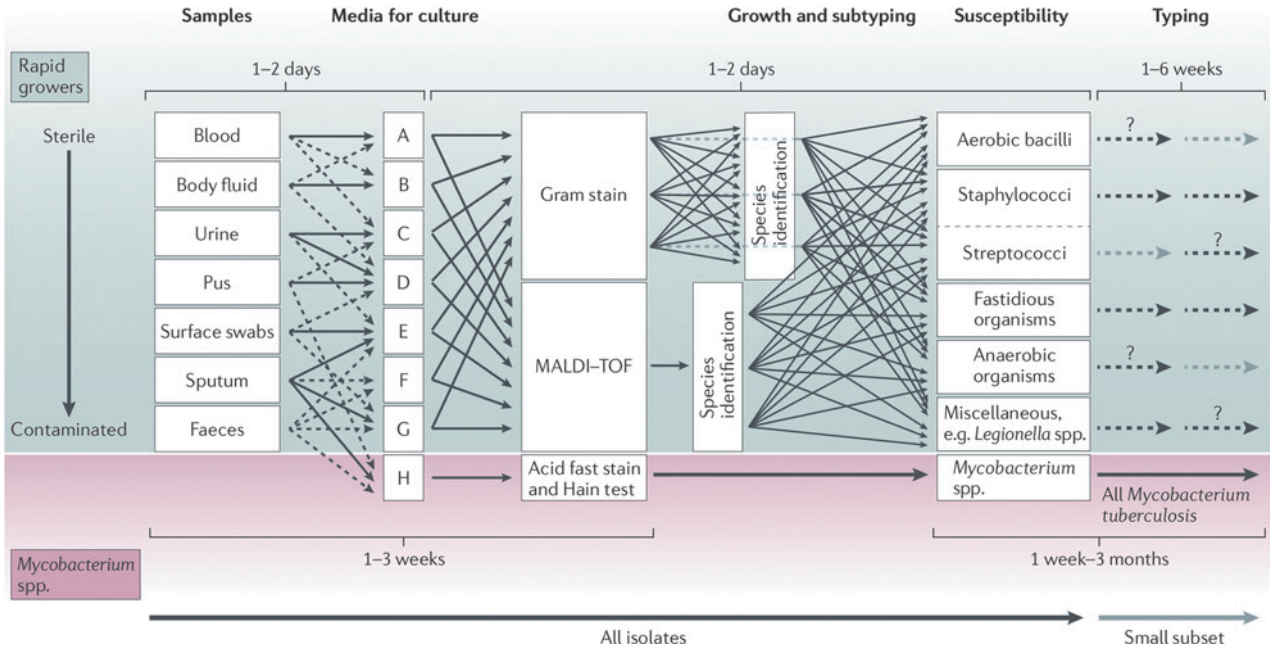

FIGURE 1 The current diagnostic microbiology processes for bacterial pathogens. A schematic representation of the current workflow for processing samples for bacterial pathogens is presented, showing high complexity and a typical timescale of a few weeks to a few months. The schematic is an approximation that highlights the principal steps in the workflow; it is not intended to be a comprehensive or precise description. Samples that are likely to be normally sterile are often cultured on a rich medium that will support the growth of any culturable organism. Samples that are contaminated with colonizing flora present a challenge for growing the infecting pathogen. Many types of culture media (referred to as selective media) are used to favor the growth of the suspected pathogen; this approach is particularly important for culturing pathogens from feces. Boxes A to H arbitrarily represent the many different media for culture. Medium H represents a medium designed for growing mycobacteria that have specific growth requirements. When an organism is growing, the morphological appearance and density of growth are properties that need specialist knowledge for deciding whether it is likely to be pathogenic. The likely pathogens are then processed through a complex pathway that has many contingencies to determine species and antimicrobial susceptibility. Broadly, there are two approaches. One approach uses matrix-assisted laser desorption ionization–time of flight mass spectrometry for species identification before setting up susceptibility testing. The other uses Gram staining followed by biochemical testing to determine species; susceptibility testing is often set up simultaneously with biochemical tests. Categorization of pathogens into groups of species is needed to choose the appropriate susceptibility-testing panel. Finally, depending on the species and perceived likelihood of an outbreak, a small subset of isolates may be chosen for further investigation using a wide range of typing tests that are often only provided by reference laboratories. The dashed lines and question marks are positioned arbitrarily to indicate that the further investigation is varied and happens in only a small number of cases. (Reprinted from reference 12 with permission.)

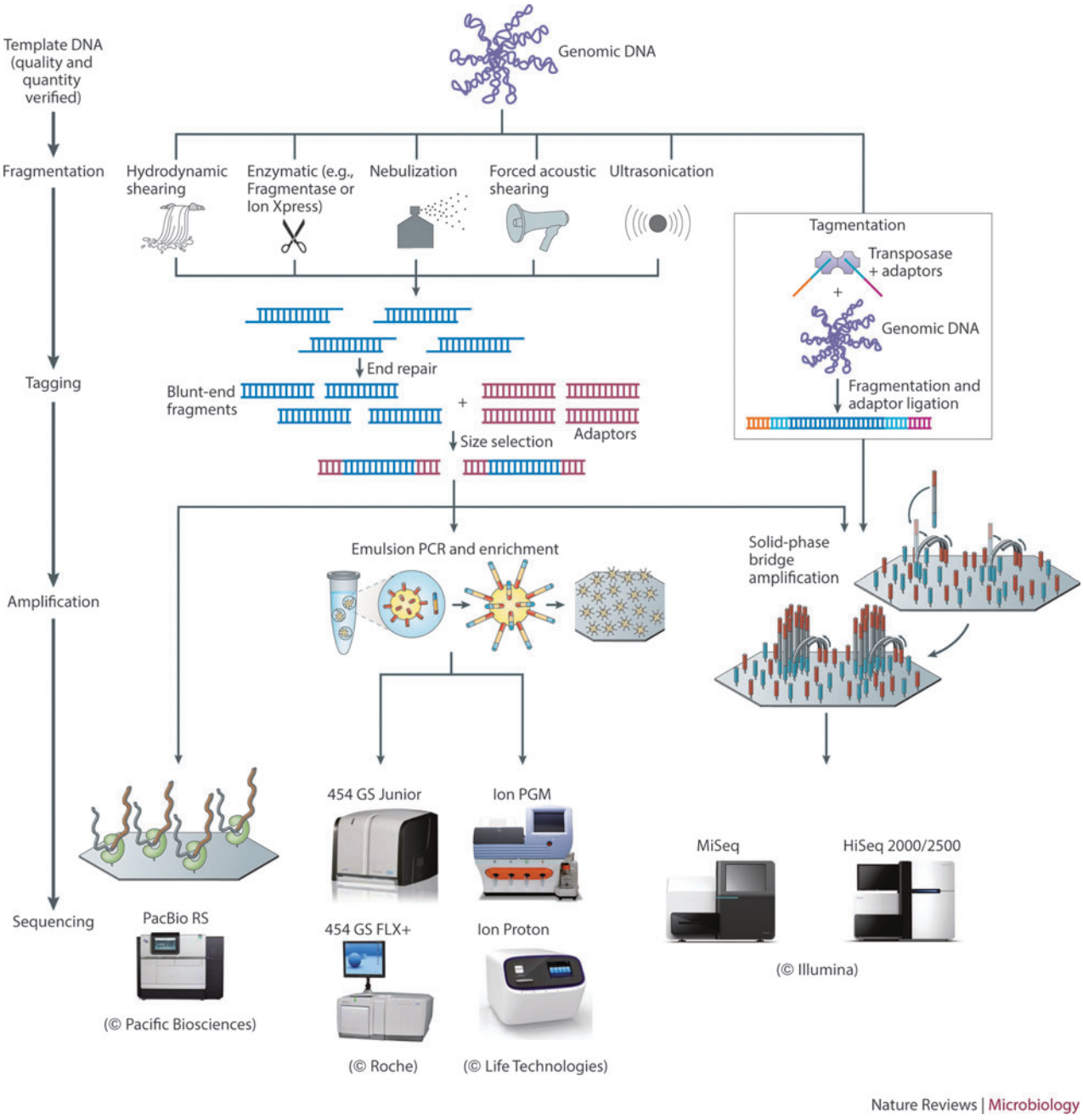

FIGURE 2 NGS platforms. The schematic shows the main high-throughput sequencing platforms available to microbiologists today and the associated sample preparation and template amplification procedures. PGM, personal genome machine. (Reprinted from reference 10 with permission.)

bacterial cloning, some of the protocols are cumbersome and require large amounts of starting genomic DNA (3 to 20 μg). Furthermore, AT-rich and GC-rich target sequences may show amplification bias in product yield, which can result in their under-representation in genome alignments and assemblies. The preparation of single DNA molecule templates is more straightforward by comparison because it requires less starting material (1 μg DNA) and does not require PCR (which can introduce mutations that may be falsely interpreted as sequence variants). Single-molecule templates are immobilized on solid supports using one of three approaches: immobilization by a primer, a template, or a polymerase.

Sequencing and Imaging

There are fundamental differences in sequencing of clonally amplified and single-molecule templates (3). Clonal amplification results in a population of identical templates, each of which has undergone the sequencing reaction. Upon imaging, the observed signal is a consensus of the nucleotides or probes added to the identical templates for a given cycle. This places a greater emphasis on the

efficiency of the addition process, and incomplete extension of the template ensemble results in dephasing (i.e., the growing primers move out of synchronicity for any given cycle). Signal dephasing increases fluorescence noise, causing base-calling errors and shorter reads. In contrast, dephasing is not an issue with single-molecule templates, so the requirement for cycle efficiency is relaxed. Single molecules, however, are susceptible to multiple nucleotide or probe additions in any given cycle. Here, deletion errors will occur owing to quenching effects between adjacent dye molecules or no signal will be detected because of the incorporation of dark nucleotides or probes (i.e., those that do not contain a fluorescent signal).

A number of sequencing and imaging methods are available; these include cyclic reversible termination, sequencing by ligation, single nucleotide addition, and single-molecule real-time sequencing. A brief description of these methods is given below, but they are comprehensively reviewed elsewhere (3).

Cyclic reversible termination uses reversible terminators in a cyclic method that comprises nucleotide incorporation, fluorescent imaging, and cleavage (2). In the first step, a DNA polymerase, bound to the primed template, adds or incorporates just one fluorescently modified nucleotide, which represents the complement of the template base. The termination of DNA synthesis after the addition of a single nucleotide is an important feature of cyclic reversible termination. Following incorporation, the remaining unincorporated nucleotides are washed away. Imaging is then performed to determine the identity of the incorporated nucleotide. This is followed by a cleavage step, which removes the terminating/inhibiting group and the fluorescent dye. Additional washing is performed before starting the next incorporation step. This technology is used by the Illumina sequencing platforms, which currently dominate the NGS market.

Sequencing by ligation is another cyclic method that differs from cyclic reversible termination in its use of DNA ligase and either one-base-encoded probes or two-base-encoded probes (17, 18). In its simplest form, a fluorescently labeled probe hybridizes to its complementary sequence adjacent to the primed template. DNA ligase is then added to join the dye-labeled probe to the primer. Nonligated probes are washed away, followed by fluorescence imaging to determine the identity of the ligated probe. The cycle can be repeated either by using cleavable probes to remove the fluorescent dye and regenerate a 5′-PO4 group for subsequent ligation cycles, or by removing and hybridizing a new primer to the template. This technology has been commercialized by Life/APG and is called the support oligonucleotide ligation detection, or SOLiD, system.

Single nucleotide addition, also known as pyrosequencing, is a nonelectrophoretic, bioluminescence method that measures the release of inorganic pyrophosphate by proportionally converting it into visible light using a series of enzymatic reactions (19, 20). Unlike other sequencing approaches that use modified nucleotides to terminate DNA synthesis, the pyrosequencing method manipulates DNA polymerase by the single addition of a deoxynucleoside triphosphate (dNTP) in limiting amounts. Upon incorporation of the complementary dNTP, DNA polymerase extends the primer and pauses. DNA synthesis is reinitiated following the addition of the next complementary dNTP in the dispensing cycle. The order and intensity of the light peaks are recorded as flowgrams, which reveal the underlying DNA sequence. This technology was commercialized by 454 Life Sciences (Branford, Connecticut, USA) and is now less popular than the Illumina platforms. The last addition to the market of second-generation sequencing instruments is the Ion Torrent, manufactured by Life Technologies (Paisley, United Kingdom). This device is based on a technique similar to 454 Life Sciences, but it senses the incorporation of a nucleotide electrically by using a small, sensitive complementary metal-oxide semiconductor–compatible pH meter (21). Instead of detecting the incorporation of a nucleotide through a fluorescent dye, the generation of a hydrogen atom, which is released each time a nucleotide is added into the growing strand, is probed. The pH meter chip replaces the camera and fluorescent molecules in the 454 device and permits cheaper sequencing through lower reagent and device costs.

Single-molecule real-time sequencing involves imaging the continuous incorporation of dye-labeled nucleotides during DNA synthesis (22). The first NGS instrument, developed by Pacific Biosciences (Menlo Park, California, USA), is referred to as single-molecule real-time sequencing and has been available since 2011. Single-molecule real-time sequencing is based on a chip pioneered by Levene et al. (23) that contains an array of zero-mode waveguides. A single DNA polymerase is attached to the bottom of the well, and the zero-mode waveguides create an illuminated volume that is small enough to allow observation of the incorporation of a single nucleotide. Each time a nucleotide is added to the DNA at the bottom of the well, the dye is detected before being cleaved off and diffusing away. Although the devices are very expensive, they are ideal for *de novo* sequencing of unknown genomes due to their long read lengths of 8 kbp. They even permit the quantification of methylation, DNA damage, and other epigenetic information.

Another NGS technique that has been gaining much attention recently is based on nanopores (24, 25). The first detection of DNA using the biological nanopore α-hemolysin was accomplished in 1996 by John Kasianowicz et al. (26). The nanopore was incorporated into a phospholipid bilayer, which separated two reservoirs filled with a KCl solution. Using two electrodes placed on opposite sides of the bilayer, an electrical potential can be applied, causing an ionic current. DNA, which is negatively charged, can therefore be forced to translocate through the nanopore by applying a positive potential to the electrode on the opposite side of the membrane. The translocation velocity varies, depending on parameters such as the electrical potential, the type of nanopore, and whether the DNA is single-stranded or double-stranded (27). Oxford Nanopore Technologies (Oxford, United Kingdom) is developing a device based on an array of biological nanopores (28, 29). A commercial launch has not yet been disclosed, but if the technology is coupled with a device that enables reliable decoding of long sequences with an acceptable error rate, it could change the current landscape of DNA sequencing.

Genome Alignment and Assembly

After NGS reads have been generated they are aligned to a known reference sequence or assembled *de novo*. The decision to use either strategy depends on the biological application as well as cost, effort, and time considerations. For example, identifying and cataloguing genetic variation in multiple strains of highly related genomes are best

achieved by mapping to a previously sequenced reference genome. This enables phylogenetic and epidemiological analysis (5–9) and is of clinical utility when investigating outbreaks of infection in hospital (30–35) and community settings (33, 36–41). Single-nucleotide variants can be easily identified, although validation of these findings may be required, for example, by visually examining the raw sequence data and checking its alignment to the reference genome.

There are, however, some limitations to the alignment approach. First, there needs to be a previously sequenced reference genome to which others are mapped. Second, there may be difficulties in mapping within repetitive regions of the reference genome or if there are no corresponding areas in the reference genome, e.g., as a result of gaps in the reference genome or the presence of structural variants. The latter problem may be resolved to some extent with mate pair reads (i.e., reads that are generated from sequencing from both ends of a DNA fragment). *De novo* assemblies are usually performed for genomes that are relatively small compared with the human genome such as bacteria, viruses, or fungi, either when the organism identity is unknown (e.g., a new or emerging pathogen) or when no previously sequenced reference genome is available. One strategy to improve the quality of alignment or assembly is to increase the coverage (i.e., the number of times a nucleotide is read during the sequencing process). An alternative strategy is to combine sequence read data generated by different NGS platforms, e.g., Roche 454 and Illumina, which has been shown to improve *de novo* assemblies of microbial genomes (42).

An overview of NGS technologies, instrument performance and costs, pros and cons, and recommendations for biological applications is presented in Table 1. The rapid pace of technological advances in the field means that this information is likely to become outdated.

CLINICAL AND RESEARCH APPLICATIONS OF NGS

The main driver for platform development has been the sequencing of human genomes to catalogue single-nucleotide variants and their association with phenotypic differences, with the eventual goal of personalized genomics for medical purposes. To this end, the United Kingdom government has committed to sequence 100,000 human genomes (43), and similar initiatives are ongoing in other countries, e.g., the Cancer Genome Atlas (44) and the 1000 Genomes project (45). Although not the primary intended audience, diagnostic and public health microbiology can derive major benefit from these technical developments. This includes large-scale projects such as the 100K Foodborne Pathogen Genome Project, an international collaboration to sequence 100,000 bacterial genomes with a view to addressing issues relating to food-borne infectious diseases (46).

The clinical applications of genomics in microbiology can be divided into two categories: those that require a bacterial isolate (e.g., bacterial typing, outbreak investigation, and surveillance and determination of resistance and virulence determinants) and those that can be applied directly to clinical samples (e.g., metagenomics and population profiling). In the following sections we will discuss the background literature that will inform current and future applications of NGS in clinical microbiology and public health.

Global Evolutionary Studies

In recent years WGS has been used to examine the evolution and spread of medically important bacterial pathogens at local, national, and international levels. Although many of these were conducted as research studies, they have been crucial in preparing the ground for clinical sequencing and generating databases of reference genomes. The first paper to demonstrate this was a study by Harris and colleagues (5), who sequenced 63 methicillin-resistant *S. aureus* (MRSA) ST239 isolates: a global collection of 43 MRSA isolates collected between 1982 and 2003, and a local collection of 20 MRSA isolates from a hospital in Thailand. Using this approach they were able to demonstrate intercontinental spread of MRSA isolates as well as evidence of local, intra-hospital transmission of MRSA. A second study, by McAdam and colleagues, examined 87 hospital-associated MRSA isolates (including 60 epidemic MRSA 16 [EMRSA-16] and 27 additional clonal complex 30 isolates collected from three continents over a 53-year period (47). They found that the three major pandemic clones to originate from the clonal complex 30 lineage had a common ancestor about 100 years ago and that EMRSA had emerged about 35 years ago. Genome-wide analysis revealed molecular correlates of hospital- or community-associated pandemics represented by mobile genetic elements and nonsynonymous mutations affecting antibiotic resistance and virulence. Phylogeographic analysis indicated that EMRSA-16 spread within the United Kingdom by transmission from hospitals in large population centers in London and Glasgow to regional health care settings.

A third study, by Holden and colleagues, examined the emergence and spread of another epidemic clone, EMRSA-15, which was first detected in the United Kingdom in the early 1990s (48). They sequenced 193 MRSA ST22 isolates from 15 countries collected between 1990 and 2009 and found that the current pandemic population of EMRSA-15 descended from a health care–associated MRSA epidemic that spread throughout England in the 1980s. Furthermore, the emergence of fluoroquinolone resistance in this EMRSA-15 subclone in the English Midlands during the mid-1980s may have played a role in triggering the pandemic spread. Using comparative genomic analysis, they identified the genetic basis of 99.8% of the antimicrobial resistance phenotypes of the isolate collection, highlighting the potential of pathogen genome sequencing as a diagnostic tool. They also documented the genetic changes associated with adaptation to the hospital environment and with increasing drug resistance over time, and how MRSA evolution may have been influenced by differences in antibiotic use between countries. Collectively, these studies have provided new insights into the emergence and spread of epidemic MRSA clones and the factors that may have contributed to their epidemiological success.

WGS has also been applied to the epidemiological analysis of important enteric pathogens such as *Vibrio cholerae*, *Salmonella enterica* serovar Typhi (*S.* Typhi), *S. sonnei*, and *Clostridium difficile*. *V. cholerae* is a globally important pathogen that causes 3 to 5 million reported cases of cholera every year. Historically, seven cholera pandemics have been recognized, with recent outbreaks in Zimbabwe and Haiti being included in the seventh pandemic. Only isolates in serogroup O1 (consisting of the classical and El Tor biotypes) and serogroup O139 can cause epidemic cholera. The first six cholera pandemics were caused by

TABLE 1 Comparison of NGS platforms[a]

Machine	Manufacturer	Chemistry	Avg read length	Run time	Output range	Advantages	Disadvantages
GS FLX Titanium XL+	Roche	Pyrosequencing	700 bp	23 hours	0.7	Long read lengths	Appreciable hands-on time High reagent costs High error rate in homopolymers
GS FLX Titanium XLR70	Roche	Pyrosequencing	450 bp	10 hours	0.45	Long read lengths	Appreciable hands-on time High reagent costs High error rate in homopolymers
HiSeq X Five HiSeq X10	Illumina	Reversible terminator	2 × 150 bp	<3 days	900–1,800	Minimal hands-on time Massive throughput Cost-effective	Long run time Short read lengths
HiSeq 2500 HiSeq 3000 HiSeq 4000	Illumina	Reversible terminator	2 × 125 bp 2 × 150 bp 2 × 150 bp 2 × 150 bp	Rapid run 7–60 hours High-output 1–6 days <1–3.5 days <1–3.5 days	10–300 Gb 50–1,000 Gb 125–750 Gb 125–1,500 Gb	Minimal hands-on time High throughput Cost-effective	Long run time Short read lengths
NextSeq 500	Illumina	Reversible terminator	2 × 150 bp	Mid-output 15–26 hours High-output 12–30 hours ×	20–39 Gb 30–120 Gb	Minimal hands-on time High throughput Cost-effective	Long run time Short read lengths
MiSeq	Illumina	Reversible terminator	2 × 300 bp (maximum)	5–55 hours	0.5–15 Gb	Minimal hands-on time Short run time	Short read lengths
SOLID 5500 W system 550 xI system	Life Technologies	Ligation	1 × 75 bp or 2 × 50 bp	8 days	80–320 Gb	Massive throughput Low error rate	Short read lengths Long run times
PacBio RS II	Pacific Biosciences	Single-molecule real-time sequencing	1,400 bp (maximum 400 kb)	30–240 min	0.5–1 Gb	Simple sample preparation Low reagent costs Very long read lengths	Expensive High error rate
Ion Proton system	Life Technologies	Proton detection	200 bp	2–4 hours	10 Gb	Flexible chip reagents Short run times	Appreciable hands-on time High error rate for homopolymers
Ion PGM™	Life Technologies	Proton detection	200–400 bp	2.3–7.3 hours	30 Mb–2 Gb	Suitable for small numbers of samples Flexible chip reagents Short run times	Appreciable hands-on time High error rate for homopolymers

[a]Adapted from reference 10.

the classical biotype, but the El Tor biotype has subsequently spread globally. Standard epidemiological typing methods have relied on subgenomic regions, which are less discriminatory than WGS data. A recent study examined the phylogeny of 154 geographically and temporally diverse *V. cholerae* isolates and found that the seventh pandemic had spread from the Bay of Bengal in three separate but overlapping waves, with a common ancestor in the 1950s as shown in Fig. 3 (6). Furthermore, the acquisition of the SXT element (an integrating and conjugating

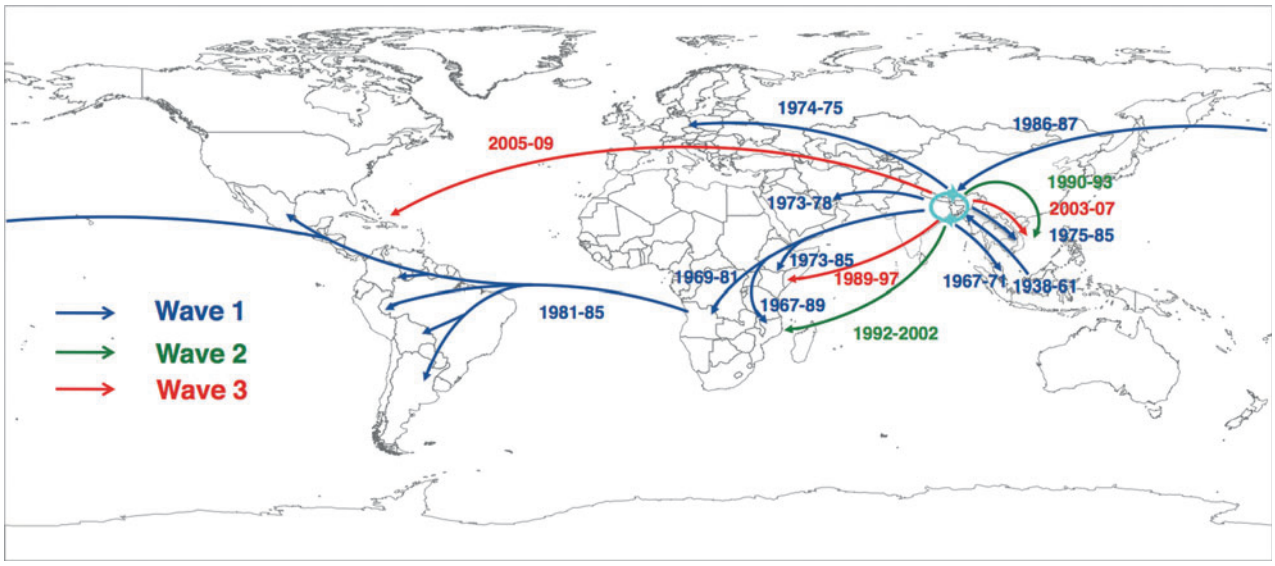

FIGURE 3 Global spread of the seventh cholera pandemic. Transmission events inferred for the seventh-pandemic phylogenetic tree, drawn on a global map. The date ranges shown for transmission events are taken from the BEAST analysis and represent the median values for the most recent common ancestor of the transmitted strains (later bound) and the most recent common ancestor of the transmitted strains and their closest relative from the source location (earlier bound). (Reprinted from reference 6 with permission.)

element that encodes resistance to sulfamethoxazole, trimethoprim, chloramphenicol, and streptomycin) appears to have shaped the pandemic spread.

S. Typhi is the major cause of enteric fever, which is estimated to affect 16 million people per year, and accounts for 500,000 to 600,000 deaths per year. Chloramphenicol-resistant *S.* Typhi was first reported in 1950 (49), and by the 1970s multidrug-resistant strains (resistant to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole) had been reported (50). Multidrug resistance in *S.* Typhi is almost exclusively conferred by IncHI1 plasmids carrying antimicrobial resistance genes (51). The first study to examine genome variation and evolution in *S.* Typhi using WGS sequenced 19 *S.* Typhi isolates from 11 countries collected between 1916 and 2000 (52). Comparative analysis showed little evidence of selection, antigenic variation, or recombination between isolates, which was notable when compared to patterns observed among other human bacterial pathogens. Only 3 *S.* Typhi genes contained more than 6 single-nucleotide polymorphisms (SNPs), and 16 genes contained independent nonsynonymous SNPs in the same or neighboring amino acids. The scarcity of evidence for antigenic variation in *S.* Typhi suggested that this pathogen was not under strong selective pressure from the human immune system. If human carriers provide the main reservoir for *S.* Typhi, this could account for the patterns of genetic drift and lack of recombination or gene acquisition observed, because the human reservoir is likely to be small and physiologically isolated. Furthermore, adaptive mutations arising during symptomatic typhoid infections may have no fitness advantage in the carrier state and may therefore not persist in the long-term *S.* Typhi population.

S. sonnei is another globally important enteric pathogen, which causes outbreaks of bacterial dysentery. Historically, this has been associated with outbreaks in developed countries but is emerging as a problem in the developing world, where it appears to be replacing *Shigella flexneri* as a cause of dysentery. Holt and colleagues sequenced 132 globally distributed *S. sonnei* isolates collected between 1943 and 2008 (7). Phylogenetic analysis revealed that the current *S. sonnei* population descended from a common ancestor that existed less than 500 years ago and diversified into several lineages with unique characteristics. The majority of this diversification appears to have taken place in Europe and then disseminated to other continents through pandemic spread of multidrug-resistant clones.

In contrast to the above, *C. difficile* is an enteric pathogen that is the most common cause of antibiotic-associated diarrhea (53). The emergence of an epidemic strain, designated *C. difficile* 027/BI/NAP1, was associated with nosocomial outbreaks and high morbidity and mortality (54). This fluoroquinoline-resistant strain was previously uncommon, and the reasons underlying its evolutionary success were unknown. To investigate this, He and colleagues sequenced a global collection of 151 *C. difficile* 027/BI/NAP1 isolates from hospital patients between 1985 and 2010 (8). They found not one, but two distinct epidemic lineages (designated FQR1 and FQR2) that had both emerged in North America but showed different patterns of global spread. The FQR2 lineage, in particular, had spread more widely and caused outbreaks in the United Kingdom, continental Europe, and Australia.

Mycobacterium tuberculosis is a globally important pathogen causing 8.6 million cases of tuberculosis (TB) and 1.3 million deaths in 2012 (55). In common with other crowd diseases, *M. tuberculosis* is believed to have emerged about 10,000 years ago during the Neolithic demographic transition. However, recent studies suggest an earlier origin. Comas and colleagues explored the evolutionary history of *M. tuberculosis* (9) by sequencing a global collection of 259 isolates of *M. tuberculosis* complex representing all seven lineages. They found that *M. tuberculosis* complex emerged about 70,000 years ago, accompanied migrations of anatomically modern humans out of Africa, and expanded as a consequence of increases in human population density. A second study examined the evolution of drug resistance in

M. tuberculosis (56). Using 116 new and 7 previously sequenced genomes, Farhat and colleagues reconstructed a phylogeny and identified targets of convergent evolution using a simple statistical test. They identified all of a set of 11 known drug resistance determinants, as well as 39 genes and intergenic regions newly associated with resistance. These regions encoded pathways of cell wall biosynthesis, transcriptional regulation, and DNA repair—mutations that could directly confer resistance or compensate for fitness costs associated with resistance.

Collectively, these studies demonstrate the superiority of WGS over current typing methods for investigating the global spread of bacterial pathogens, particularly those that are highly clonal in nature. In addition, WGS can provide information about antibiotic resistance mutations/genes and virulence determinants that may help to explain the evolutionary success of particular epidemic or pandemic lineages.

Outbreak Investigation

The major application of WGS in clinical microbiology and public health is likely to be the investigation and management of suspected outbreaks of infection, in both hospital and community settings. Early recognition and confirmation of outbreaks can result in rapid implementation of appropriate infection control and/or public health measures and thus potentially bring the outbreak to a close more quickly. Conversely, being able to refute an outbreak (57) may be reassuring to clinicians and allow de-escalation of infection control measures, which can be time-consuming and expensive. Over the past few years a number of studies have demonstrated the potential utility of this approach for pathogens such as *E. coli* (41), MRSA (30, 34), *Klebsiella pneumoniae* (31), *Acinetobacter baumannii* (58), *Enterococcus faecium* (35), *Enterobacter cloacae* (35), *Pseudomonas aeruginosa* (59), *C. difficile* (33, 60), *M. tuberculosis* (37, 61), *Mycobacterium abscessus* (32), viruses (62–64), and fungi (65). Most of these studies were conducted retrospectively and did not influence immediate clinical management, although a few were conducted in real time (34, 41). As clinical microbiology laboratories become more adept at sequencing and bioinformatic analysis becomes automated, it is likely that WGS will replace conventional typing methods and provide clinically actionable data for outbreak investigation and management.

One of the earliest and most high-profile examples of the use of WGS was during the investigation of an outbreak of *E. coli* 0104:H4, which affected more than 3,000 people in Germany in 2011 (41). This outbreak was characterized by a number of unusual features, namely a high incidence in adults (particularly in females) and the hemolytic-uremic syndrome. Rohde and colleagues sequenced a sample obtained from a patient involved in the outbreak using a rapid benchtop sequencing platform, the Ion Torrent PGM. Sequence data were released into the public domain on day 3 and rapidly analyzed by bioinformaticians on four continents. By 24 hours after the release of the sequence data the genome had been assembled, and 2 days after its dissemination it had been assigned to an existing sequence type. Five days after the release of the sequence data, strain-specific diagnostic primer sequences had been designed and released. In less than a week, this study revealed that the outbreak strain belonged to an enteroaggregative *E. coli* lineage that had acquired genes for Shiga toxin 2 and for antibiotic resistance, illustrating the potential power of open source genomic analysis to investigate outbreaks of emerging infectious diseases.

A second study published simultaneously used third-generation single-molecule real-time DNA sequencing (using the PacBio platform) to determine the complete genome sequence of the German strain, as well as the genome sequences of seven diarrhea-associated enteroaggregative *E. coli* serotype O104:H4 strains from Africa and four enteroaggregative *E. coli* reference strains belonging to other serotypes (66). Genome-wide comparisons were performed using these enteroaggregative *E. coli* genomes, as well as those of 40 previously sequenced *E. coli* isolates. The investigators found that the enteroaggregative *E. coli* O104:H4 strains were closely related and formed a distinct clade among *E. coli* and enteroaggregative *E. coli* strains. The German outbreak strain contained a prophage encoding Shiga toxin 2 and a distinct set of additional antibiotic resistance and virulence factors, suggesting that horizontal genetic exchange preceded the emergence of this strain.

One of the first studies to demonstrate the potential utility of WGS in nosocomial outbreak investigations was conducted by Köser and colleagues in the United Kingdom (30). The investigators retrospectively investigated an MRSA outbreak in a neonatal intensive care unit by sequencing 14 MRSA isolates: 7 from infants on the unit and 7 from control patients located elsewhere in the hospital. Phylogenetic analysis revealed a cluster of outbreak strains that were clearly separated from the control isolates and detected a previously unsuspected transmission event between two of the control patients. The investigators also created an artificial "resistome" of antibiotic resistance genes and demonstrated concordance between genotypic and phenotypic antimicrobial susceptibility results, thus highlighting another potential use for WGS. A second study, by Harris and colleagues, applied WGS during the course of a suspected ongoing MRSA outbreak in neonates (34). In this study, the investigators sequenced MRSA isolates from 12 infants who were admitted to the special care baby unit over a 6-month period. These were found to be a novel MRSA strain (ST2371) that carried the gene for Panton-Valentine leukocidin. Sequencing of additional MRSA isolates with the same antibiotic susceptibility profile identified 24 cases that were epidemiologically linked, indicating spread between infants on the special care baby unit, mothers on the postnatal ward, and family members in the community. A new case, which occurred 64 days after the previous case, prompted screening of health care workers on the unit. One person was found to be carrying the outbreak strain, and no further cases were detected after they were successfully decolonized. The epidemiology and phylogeny of this outbreak are illustrated in Fig. 4. Of note, this was the first study to demonstrate the feasibility of using WGS in real time to investigate and manage a nosocomial infection outbreak.

K. pneumoniae is an important nosocomial pathogen in intensive care units, particularly among immunocompromised patients. In recent years *K. pneumoniae* strains resistant to carbapenems have emerged and spread worldwide. In 2011, the U.S. National Institutes of Health experienced an outbreak of carbapenem-resistant *K. pneumoniae* that affected 18 patients, 11 of whom died (31). Snitkin and colleagues sequenced *K. pneumoniae* isolates from the affected patients and found that the outbreak was monoclonal despite a 3-week gap between the index case and subsequent cases. They identified at least three independent transmission events from the index case, resulting in

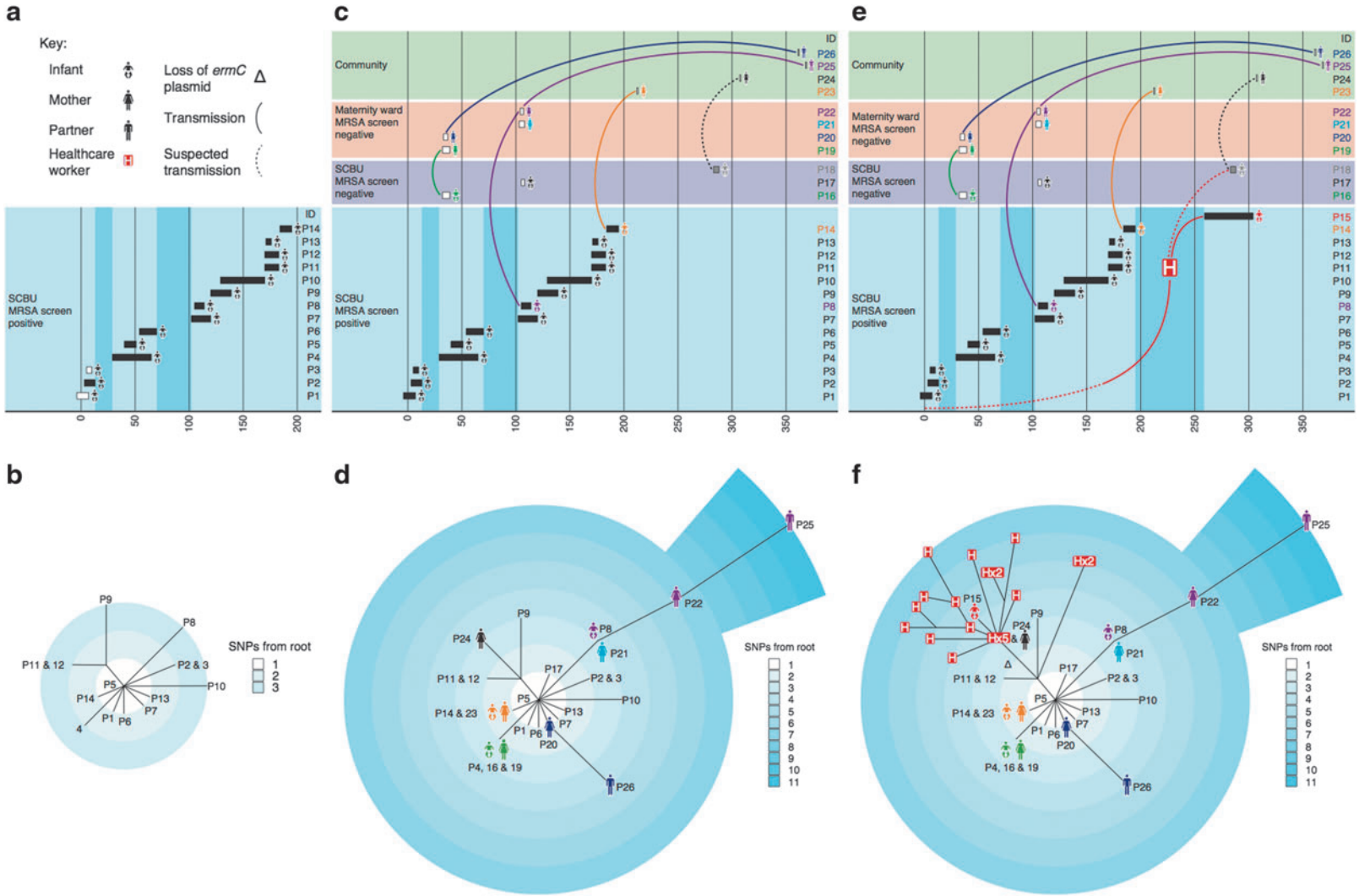

FIGURE 4 Epidemiology and phylogeny of a neonatal MRSA ST2371 outbreak. (a) Epidemiological map of 14 infants (patients 1 to 14) on the special care baby unit (SCBU). Boxes shown for infants in the SCBU in panel a represent duration of hospital stay (black boxes show infants included by the infection-control investigation and white boxes show infants excluded by the infection-control team). Gray vertical blocks in panels a, c, and e show the time periods on the SCBU when there were no known carriers of MRSA. (b) Phylogenetic tree based on WGS of MRSA isolates from patients 1 to 14. (c) Epidemiological map of patients 1 to 14 and 10 other patients (patients 16, 17, and 19 to 26) with linked MRSA infection detected in the community. The colored lines link members of the same family. (d) Phylogenetic tree based on WGS of MRSA isolates from patients 1 to14 and patients 16, 17, and 19 to 26. (e) Epidemiological map of all cases of MRSA identified by WGS and one patient (patient 18) suspected of being linked to the outbreak but for whom no MRSA colonization was detected. (f) Phylogenetic tree of all cases of MRSA in the outbreak. Twenty individual MRSA colonies from a staff member are shown in red boxes, with multiple colonies from the staff member shown in parentheses. MRSA, methicillin-resistant *Staphylococcus aureus*; SCBU, special care baby unit; SNP, single-nucleotide polymorphism; P, patient. Note: Out-group was the sequence type 22 reference genome. (Reprinted from reference 34 with permission.)

two major clusters of colonized patients. One patient could be linked to a contaminated ventilator. They also identified putative resistance mutations in isolates that had become resistant to colistin.

A number of other studies have used WGS to investigate nosocomial outbreaks caused by a variety of pathogens including multidrug-resistant *A. baumannii* (58), vancomycin-resistant *E. faecium* (35, 67), carbapenem-resistant *E. cloacae* (35), and *P. aeruginosa* (59). WGS has also been used to investigate *Legionella pneumophila* outbreaks both retrospectively (39) and in real time (68). More recently, WGS has been applied to the investigation of a milk-borne outbreak of *Campylobacter jejuni* (69) and a food-borne outbreak of *Listeria monocytogenes* (70). In all of these studies the investigators were able to distinguish outbreak from nonoutbreak strains and to delineate probable transmission pathways.

A similar approach has been applied to the investigation of *C. difficile* cases in health care and community settings. A study by Eyre and colleagues sequenced 1,223 isolates from symptomatic patients with *C. difficile* infection between 2007 and 2011 (33). Thirty-five percent of isolates had no more than two single-nucleotide variants between them, which was considered to be consistent with transmission. Of these, 38% had ward contact with another patient (admitted to the same ward at the same time), 2% may have acquired the organism from the ward environment (ward contamination), 9% had hospital contact (admitted on different hospital wards at the same time), and 6% had both ward contamination and hospital contact. In terms of community contact opportunities, 10% of patients were registered at the same general practice and 11% lived in the same postal code district. The remaining 36% had no evidence of hospital or community contact. Forty-five percent of isolates had more than 10 single-nucleotide variants from previous cases, representing transmission from sources other than symptomatic cases included in the study. These findings suggest that *C. difficile* transmission arises from genetically diverse sources, which may include asymptomatic patients and/or the environment. The use of WGS for *C. difficile* could, therefore, be targeted to investigate cases that are epidemiologically linked with a view to preventing further spread, and also to monitor performance of institutional infection control programs. A second study by the same group considered the role of asymptomatic carriage in the transmission of *C. difficile* (60). Of the 227 patients recruited, 132 provided one or more stool samples; 18 were culture positive for *C. difficile*, and 13 carried toxigenic strains. Several plausible cases of transmission to asymptomatic carriers were identified, but there was no clear evidence of onward transmission from an asymptomatic case in this small study.

In developed countries, tuberculosis control programs use genotyping (e.g., IS6110 RFLP or MIRU-VNTR [Mycobacterial Interspersed Repetitive Units-Variable Number of Tandem Repeats] typing) to identify clusters of patients affected with the same strain, to target contact tracing of associated cases with active disease or latent infection. The first study to compare WGS with traditional genotyping methods for TB outbreak investigation was conducted by Schürch and colleagues in the Netherlands (36). They sequenced *M. tuberculosis* isolates from 3 of 104 patients involved in the Harlingen outbreak between 2001 and 2008 and compared these to the results obtained by IS6110 RFLP typing. WGS proved highly discriminatory and enabled them to identify transmission chains within the Harlingen outbreak that were not apparent by conventional typing.

A second, larger study by Gardy and colleagues investigated a 3-year TB outbreak in Canada (37). Sequencing of 32 outbreak isolates and four historical controls revealed two genetically distinct lineages with identical MIRU-VNTR genotypes. Integration of phylogenetic and social network analysis revealed several transmission events, including some involving "super-spreaders." Both lineages descended from a common ancestor that had been present in the community prior to the outbreak, suggesting a social rather than a genetic reason for spread. Further epidemiological investigation indicated that the outbreak coincided with increased crack cocaine use in the community.

A third study, conducted by Walker and colleagues in the United Kingdom, aimed to estimate the genetic diversity of *M. tuberculosis* strains in the Midlands and to determine how this might be used to investigate outbreaks (61). They sequenced 390 *M. tuberculosis* isolates from 254 patients and characterized them into four groups: cross sectional, longitudinal, household, and community. They measured pair-wise nucleotide differences within hosts and between hosts in household outbreaks. The estimated rate of change in DNA sequence was 0.5 SNPs per genome per year, and divergence was rarely higher than five SNPs in 3 years. Ninety-six percent of paired isolates from individuals and households differed by five or fewer SNPs. More than five SNPs separated none of the epidemiologically linked patients, 15% of the possibly linked patients, and 17% of the unlinked patients. Phylogenetic analysis suggested that super-spreaders were present in two community clusters. Other studies have confirmed the utility of WGS in TB outbreak investigation in different settings (38, 71, 72). Collectively, the findings of these studies indicate that WGS can be used to distinguish patients who are in a recent TB transmission chain from those who are not and have substantial implications for contact tracing, particularly in complex transmission settings or where epidemiological data are incomplete.

M. abscessus is a major respiratory pathogen, particularly in patients with cystic fibrosis, in whom it can lead to rapid decline in lung function and may preclude transplantation. Three subspecies have been recognized—*M. abscessus* subsp. *abscessus*, *M. abscessus* subsp. *massiliense*, and *M. abscessus* subsp. *bolletti*—but these are not usually distinguished in the clinical microbiology laboratory. Previous studies have suggested that *M. abscessus* was acquired from the environment and that there was limited evidence for person-to-person transmission. A recent study by Bryant and colleagues has challenged this view (32). The investigators sequenced 168 *M. abscessus* isolates from 31 adults attending a cystic fibrosis unit in the United Kingdom between 2007 and 2011. Phylogenetic analysis revealed two clustered outbreaks of near-identical isolates of *M. abscessus* subsp. *massiliense* that differed by less than 10 SNPs, suggesting patient-to-patient transmission. Furthermore, there was evidence of transmission of acquired drug resistance mutations between individuals, some of whom had not been exposed to the antibiotics to which their isolate was resistant. An epidemiological investigation identified numerous opportunities for cross-infection between patients within the hospital setting, but environmental sampling for *M. abscessus* was negative. Thus, WGS revealed transmission of multidrug-resistant *M. abscessus* strains between patients with cystic fibrosis despite standard infection control measures. This study has led to changes in

infection prevention and control guidance for *M. abscessus* in the United Kingdom.

WGS has been applied to the epidemiological analysis of the influenza AH1N1/09 pandemic in the United States and the United Kingdom. Nelson and colleagues undertook a large-scale phylogeographic analysis of viruses sampled in three locations in the United States to determine if there were different epidemiological patterns between the spring and autumn waves of the pandemic (73). Between March and July 2009 no specific viral lineage dominated in Texas, whereas outbreaks in Milwaukee and New York State were each dominated by a different viral lineage. By August 2009 a single viral lineage (the dominant lineage in New York during the spring wave) had also become dominant in all three locations. Baillie and colleagues sequenced 153 pandemic influenza H1N1/09 strains—127 from the first wave and 26 from the second wave (74). The investigators found that the United Kingdom epidemic was composed of many cocirculating lineages, at least 13 of which were exclusively or predominantly United Kingdom clusters. The estimated divergence times of two of the clusters predated the detection of influenza H1N1/09 in the United Kingdom, suggesting that the virus was already circulating before the first clinical case was reported. Two United Kingdom clusters contained similar sequences in the first and second waves of infection, suggesting persistent transmission in the community. These studies shed light on the changing epidemiology of influenza strains and highlight a potential role for WGS influenza surveillance, which could be used to inform vaccine design.

WGS has also been applied to the investigation of outbreaks of viral infections in hospital and community settings, including norovirus (62), hepatitis A virus (63), West Nile virus (WNV) (64), and Middle Eastern respiratory syndrome (MERS) coronavirus. Norovirus is a leading cause of viral gastroenteritis worldwide and is associated with outbreaks of infection in health care and community settings. Kundu and colleagues investigated a cluster of norovirus infections that occurred in children undergoing bone marrow transplantation at a United Kingdom hospital in 2010 (62). Thirteen complementary DNA samples from five patients were amplified and sequenced using the Roche 454 platform and assembled *de novo*. Phylogenetic analysis indicated that two of the three patients on the same ward had closely related viruses, indicating the possibility of cross-infection despite protective isolation. Analysis of deep sequencing data established the likely direction of nosocomial transmission.

Hepatitis A virus is a common cause of acute viral hepatitis and usually causes a self-limited infection. Infection in children is usually asymptomatic but can, rarely, result in fulminant liver failure. Vaughan and colleagues investigated a hepatitis A virus outbreak in five children, two of whom developed acute liver failure and died (63). Samples were obtained and sequenced from the two fatal cases and two asymptomatic cases in one household and from a symptomatic case in another household. Both households were infected with hepatitis A virus subtype 1A, but the infecting strains in the two households were different. Interestingly, the strain recovered from the asymptomatic household contact was nearly identical to the strain causing acute liver failure.

WNV is an arbovirus infection transmitted by mosquitoes, and may be asymptomatic or cause a febrile illness, meningitis, or encephalitis. Since 2010, WNV has caused a number of large epidemic outbreaks in Europe. Barzon and colleagues used WGS to investigate cases of WNV infection associated with an ongoing outbreak in northern Italy (64). Analysis of six WNV sequences obtained from clinical samples in 2013 indicated a new strain of WNV lineage 2, which was similar to strains circulating in Greece and Hungary. They also sequenced a WNV strain from a blood donor collected during the 2012 outbreak, which was found to be WNV lineage 1. Thus, WGS identified two strains of WNV that were cocirculating in northern Italy in 2012 to 2013.

In September 2012 the WHO reported the first cases of MERS (75). Whole-genome sequencing identified a novel human coronavirus, which was isolated from the sputum of a patient who died of pneumonia and renal failure in a hospital in Saudi Arabia (76). Since then MERS coronavirus has been reported from several countries and associated with person-to-person transmission in a hospital setting (77). As of June 2014, 699 laboratory-confirmed cases of MERS coronavirus and 209 deaths had been reported to WHO (78).

Finally, WGS has also been used to investigate an outbreak of fungal infections caused by *Exserohilum rostratum* (65) and *Apophysomyces trapeziformis* (79). In the first study, *E. rostratum* caused an outbreak of fungal meningitis and other infections that were associated with contaminated methylprednisolone injections. The investigators sequenced 22 isolates of *E. rostratum* from 19 patients, 6 isolates from contaminated vials, and 7 isolates unrelated to the outbreak. All 28 isolates associated with the outbreak were closely related, with a total of eight SNPs between them and no more than two SNPs between any of the two outbreak genomes. In contrast, the control isolates showed considerable genetic variability, and the closest isolate was >100,000 SNPs distant from the outbreak isolates. In the second study, 13 patients in 6 hospitals developed mucormycosis after sustaining tornado-related injuries (79). Sequencing of 11 isolates showed that 4 strains infected the patients. Clonally related isolates did not cluster geographically but were scattered throughout the tornado path, the two predominant clones causing infections in patients living closest to the touch-down site of the tornado.

Collectively, these studies indicate the clinical utility of using rapid microbial WGS for the investigation of suspected outbreaks in hospital and community settings. It is likely that this technology will supplant conventional epidemiological typing methods in the future, but significant challenges remain. As WGS becomes faster and cheaper, the key barrier to implementation into routine clinical care is the lack of automated sequence data analysis tools for the common human pathogens. In addition, although large genomic databases that provide a genomic context exist for some common pathogens, these are not always readily accessible for comparison of genome sequences generated during outbreak investigations.

Detection and Surveillance of Antimicrobial Resistance

WGS can be used to detect drug resistance mutations and acquired genes and to elucidate mechanisms of resistance. Several studies have compared phenotypic antimicrobial susceptibility testing with genotypic predictions by WGS for a variety of pathogens (80–82). These have shown good concordance between phenotypic and genotypic susceptibility results that are well within the limits set by the U.S. FDA for marketing approval (<1.5% for a very major

discrepancy and <3% for a major discrepancy). For most common bacterial pathogens, however, phenotypic antimicrobial susceptibility testing is rapid and inexpensive and is likely to remain the first-line testing method for some time to come.

There are specific clinical scenarios where rapid WGS could play a role in the detection of antibiotic resistance. The first is when phenotypic drug susceptibility testing is too slow to guide initial patient management. A prime example is *M. tuberculosis*, for which drug susceptibility testing can takes weeks to months, particularly for multidrug-resistant or extensively drug-resistant isolates for which a wider panel of drugs are tested. Molecular tests exist but only target the most common mutations for a limited number of drugs. A study by Köser and colleagues used WGS to investigate a case of extensively drug-resistant tuberculosis (83). They detected evidence of mixed infection with two Beijing strains and were able to demonstrate concordance between genotypic and phenotypic susceptibility testing. Furthermore, they were able to detect resistance mutations for additional agents that were not routinely tested for in the reference laboratory. WGS therefore shows great promise not just for epidemiological typing of *M. tuberculosis* isolates, but also for clinical management of infection caused by highly drug-resistant isolates. Caveats include the fact that genotype-phenotype associations have not been determined for all drugs and that current technology may be not be able to detect mutations that are present at 1% or less in the total population unless deep sequencing is performed (that is, the genome is sequenced many times over).

Another potential application is where elucidation of the resistance mechanism is important for infection control purposes. One example is linezolid-resistant *E. faecium*, when determination of the basis of resistance (mutation versus plasmid-mediated resistance) may influence the decision to implement enhanced infection control measures (84). A second example is carbapenem-resistant *Enterobacteriaceae*, which may be caused by carbapenemase production (which requires enhanced infection control) or by a combination of resistance mechanisms (e.g., extended-spectrum β-lactamase production plus porin loss) (35, 85). It is important to note that these resistance mechanisms are currently detected using multiplex PCR or array technologies, so WGS is unlikely to be cost-effective if only used for the detection of known resistance mechanisms.

WGS can also be used to study the emergence of antibiotic resistance in real time under a variety of conditions (86). Toprak and colleagues developed a microbial cultivation system capable of maintaining a constant antibiotic pressure and then monitored the evolution of resistance in *E. coli* to trimethoprim, chloramphenicol, and doxycycline over 20 days (87). Resistance levels increased dramatically, with parallel populations showing similar phenotypic trajectories. WGS of the evolved strains identified mutations both specific to individual drugs and shared in resistance to multiple drugs.

Safi and colleagues used WGS to study the evolution of ethambutol resistance in *M. tuberculosis* (88). Both epidemiological and laboratory evidence suggests that mutations in the *embCAB* operon are responsible for resistance to ethambutol. *M. tuberculosis* isolates with a range of ethambutol MICs were selected and cultured. Ethambutol MICs were determined using the 7H10 agar proportion method and by the BACTEC 460TB method. Isolation of genomic DNA and PCR were performed, and *Rv3806c*, *Rv3792*, *embC*, and *embB* mRNA expression levels were measured by quantitative real-time PCR. Direct Sanger sequencing of PCR products was performed on an ABI 3100 Genetic Analyzer. Mutations in decaprenylphosphoryl-β-D-arabinose biosynthetic and utilization pathway genes *Rv3806c*, *Rv3792*, *embB*, and *embC* accumulated to produce a wide range of ethambutol that depended on mutation type and number.

In addition to elucidating the mechanisms of resistance, WGS also has a role in determining the rate at which resistance emerges. For example, Ford and colleagues reported that lineage 2 *M. tuberculosis* strains acquired rifampicin resistance more rapidly than lineage 4 strains (89). WGS has also established some of the genetic factors that have facilitated the spread of drug-resistant *M. tuberculosis*. For example, WGS has identified mutations in the α- and β-subunits of the RNA polymerase that alleviate fitness costs of resistance to rifampicin (90). These compensatory mutations are widespread in multidrug-resistant *M. tuberculosis* complex strains in high-incidence countries and are associated with ongoing transmission (91). WGS has also highlighted the importance of antimicrobial resistance in the evolution and spread of microbial pathogens, as discussed previously. For example, the emergence of fluoroquinolone resistance preceded the global spread of the MRSA ST22 strain (48) and the *C. difficile* 027/BI/NAP1 strain (8).

Development of Novel Antibiotics

WGS has been used to develop a novel antibiotic for the treatment of *M. tuberculosis*. In 2005, 454 pyrosequencing was used to identify the F0 subunit of a mycobacterial ATP synthase (92), which resulted in the development of the novel TB drug bedaquiline (93). This has enabled researchers to sequence this gene in a genetically diverse collection of reference isolates to ensure that it is conserved among all *M. tuberculosis* complex lineages (94). This represents an important step in drug development and will allow pharmaceutical companies to confirm the presence of a drug in a large bacterial population prior to investing in clinical trials of new agents. The early elucidation of drug resistance mechanisms also has implications for clinical trial design because it may enable the detection of resistance mechanisms that raise the MIC of a drug, but it may be overcome by using higher doses. It may also detect cross-resistance between different antibiotic classes, e.g., bedaquiline and clofazimine (95), which would then not be used in combination.

Microbiome Analysis

One of the most powerful applications of NGS has been its role in understanding the complex diversity of the human microbiome. We are only just beginning to understand what constitutes normal and abnormal flora in the gut, respiratory tract, urinary tract, vagina, skin, and wounds (96, 97). Gut microbiome analyses are also providing new insights into the pathogenesis of inflammatory conditions such as Crohn's disease (98). A further possibility is the ability to monitor changes in microbial populations during treatment, which might provide useful prognostic information. For example, a study of the human gut microbiome in bone marrow transplant recipients found that postantibiotic overgrowth with vancomycin-resistant enterococci was associated with a higher risk of bacterial sepsis from this organism (99). For further information on metagenomics please see chapters 10 to 14.

Viral WGS for Genotyping and Resistance Monitoring

WGS has the potential to improve the clinical management of viral infections such as human immunodeficiency virus (HIV). Since its introduction in the 1990s, highly active antiretroviral therapy has transformed HIV infection from a rapidly fatal disease into a treatable condition with near-normal life span and quality of life (100, 101). One of the key principles of antiretroviral therapy is to select a combination drug regimen to which the virus is susceptible and which suppresses HIV viral replication below the limit of detection for as long as possible. Current clinical practice involves examining regions of the viral genome for mutations that confer resistance to antiretroviral drugs and using this information to guide treatment decisions, both at initiation of therapy and when treatment failure/resistance occurs (102, 103). WGS provides detailed information on the entire viral genome, rather than just the parts of the genome coding for drug resistance. It can also simultaneously provide information on regions that are used to genotype viruses, thus providing genotyping and drug resistance data in one step, in a rapid and potentially cost-effective manner. As directly acting antivirals are introduced for other infections such as hepatitis C and hepatitis B, it seems likely that WGS will be used to guide therapy in these conditions.

CONSIDERATIONS FOR IMPLEMENTATION OF WGS INTO CLINICAL PRACTICE

A number of challenges remain before WGS can be implemented into routine clinical practice (11). Sequencing platforms are under constant development and refinement, and the choice of platform will depend upon their relative advantages and disadvantages for their intended use. Reassuringly, an evaluation of three sequencing platforms used in an investigation of an MRSA outbreak gave clinically comparable results (104). A second consideration is how best to design a WGS service. The current paradigm of clinical microbiology involves detection and antimicrobial susceptibility testing at a local diagnostic laboratory followed by referral of specimens to reference laboratories for further specialist testing. However, numerous studies have demonstrated the feasibility of performing WGS in clinical microbiology laboratories, reducing the delays incurred by transporting specimens to a centralized laboratory, and generating sequence data more rapidly. An additional consideration is whether to use the infrastructure and expertise that already exists, for example in clinical genetics laboratories in the United Kingdom. Standard operating procedures are required for DNA extraction, library preparation, sequencing and data handling, storage, and reporting. These procedures need to be validated locally and undergo internal control and external quality assurance. The laboratory should be accredited by the relevant authorities to perform WGS to ensure that the information produced is robust and fit for its purpose.

The production of billions of NGS reads has also challenged the infrastructure of existing information technology systems in terms of data transfer, storage and quality control, computational analysis to align or assemble read data, and laboratory information management systems for sample tracking and process management. Advances in bioinformatics are ongoing, and improvements are needed if these systems are to keep pace with the continuing developments in NGS technologies. A major limitation to the implementation of WGS into routine clinical practice is the lack of automated data interpretation tools to enable rapid analysis and reporting of clinically relevant findings from genome sequence data. Furthermore, there is a need for large open-access genomic databases against which to compare sequenced isolates, a problem that was highlighted by a study that sequenced all isolates processed in a diagnostic laboratory over the course of one day (105). An additional challenge is the integration of clinical and genomic data, which will need to be stored securely but also be accessible to relevant individuals, e.g., clinicians, infection control teams, and public health officials. This potentially raises ethical issues with respect to the collection and storage of such information with individual patient consent, as well as potential concerns about breaches in patient confidentiality if clinical data are not held securely.

THE FUTURE OF GENOMICS IN CLINICAL MICROBIOLOGY

There are several situations where sequencing directly from a clinical sample would simplify and/or improve diagnostic microbiology. This includes fastidious or slow-growing bacterial species, which can be difficult and labor-intensive to isolate. Culture-based approaches are also of limited value if the patient has received antibiotic therapy prior to sample collection or is infected by nonculturable organisms. New developments in sample preparation are required that facilitate direct sample sequencing, which is currently difficult because of a low copy number of the bacterial target of interest combined with contamination with other flora and human DNA.

The use of single-cell genomics is emerging as a strategy for the investigation of microbial communities and uncultured organisms (106). A study by Raghunathan and colleagues provided proof-of-principle that DNA from a single bacterium could be sequenced (107). Single-cell sequencing has already provided draft genome sequences of major bacterial taxa that were not previously available in databases. Single-cell genomics can also be combined with metagenomics to provide an inventory of the microbial community and genetic linkages of sequences in a single organism (108).

Our understanding of microbial diversity in health and disease is currently rudimentary, but human microbiome analysis may ultimately become a part of patient management. Furthermore, the influence of host genetics on the susceptibility and outcome of infectious diseases has been established for certain pathogens, e.g., *M. tuberculosis*, HIV, and hepatitis C virus. Parallel investigation of host and bacterial genomics may improve our understanding of host-pathogen interactions and patient management.

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Digital PCR and Its Potential Application to Microbiology

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4

A digital PCR (dPCR) takes a PCR and subdivides it across a large number of smaller reactions, termed partitions, so that a number of the partitions contain no template molecules. Many of the ideas that underpin dPCR were described in the late 1980s and early 1990s (1) and applied at that time using conventional or nested PCR. However, the procedure originally required partitioning of a single sample using individual tubes or a 96-well plate, followed by amplicon detection by agarose gel electrophoresis. This initial format represented a very unwieldy technique and an inefficient use of time and resources.

The term "digital PCR" was coined by Vogelstein and Kinzler (2). They used dPCR to improve the ability of PCR to detect rare mutations in a sample predominated by wild-type sequence. The partitioning in this study essentially reduced the wild type/mutant ratio, thus improving the assay sensitivity by reducing the effect of competition from the wild type sequence (this is described in more detail in reference 3). Partitioning was performed using the same 96-well plate approach that had been described almost a decade earlier. Consequently, at that time, dPCR offered the potential of a powerful experimental method to quantify and detect DNA molecules, but it was not practical for routine use in an applied diagnostic setting.

The advent of real-time quantitative PCR (qPCR) (4) offered a far simpler method for quantification that had the added benefit of being automated, requiring no subsequent handling postamplification to determine whether any target sequence had been detected. Uptake of qPCR rapidly rendered the complex, inefficient, and reagent-hungry original dPCR method obsolete. qPCR continued to advance in many areas of clinical and biological research, including microbiology.

The use of qPCR for clinical (both communicable and noncommunicable diseases) applications was, arguably, led by the virologists with monitoring of viral loads used to guide treatment in numerous viral diseases (5–7) (see chapters 1 and 30 through 32 of this volume). This was followed by applications in bacterial infectious disease (see chapter 1 and sections II, III, and V), hand in hand with the development of fully automated "black box" qPCR point-of-care instrumentation developed to reduce the requirement for expert scientists to perform the assays (8, 9) (see sections VIII and X).

While qPCR-based methods have been making considerable inroads into research and applied diagnostic microbiology, dPCR has been experiencing something of a renaissance. This has been made possible by technical advances in microfluidic engineering and emulsion chemistries that have enabled partitioning across thousands to millions of nanoliter and subnanoliter partitions (10). With these technological advances, dPCR is being increasingly applied in a variety of research areas including microbiology because it is able to offer more precise measurement (11) and, when sample volumes are matched, higher sensitivity (12, 13) than qPCR. The data generated are more reproducible than with qPCR, with a more predictable variance, thus making power calculations simpler (11, 14) and sources of random error easier to determine (15).

These characteristics make dPCR a potentially valuable tool for microbial research and offer an independent means of quantifying calibrators for other methods (16) such as qPCR and next-generation sequencing. It also provides opportunities for translation to clinical laboratory analysis, potentially offering more accurate and reproducible detection and quantification for the diagnosis and monitoring of infectious diseases. In this chapter we describe how dPCR is performed and discuss some of the most common formats and sources of error. In addition, we discuss how it has been applied in molecular microbiology and how it has the potential to advance pathogen measurement in the context of disease.

SPECIFIC EXPERIMENTAL CONSIDERATIONS FOR dPCR

For dPCR to be performed, a PCR must be distributed across a large number of partitions. The resurgence of dPCR over the last 5 or so years has largely been due to the development of instruments that have simplified this partitioning. The current instruments can be split into two categories depending on how they partition the reaction, either by using nanofluidic solid partitions or water-in-oil emulsions. Details of the current commercially available instruments can be found in Table 1. The choice of machine will inevitably depend on a range of factors. For those

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TABLE 1 Details of the currently available dPCR instruments

Instrument (company)	Analyzed reaction vol[a]	No. reactions per run	Partition no./reaction	Partition vol	Run duration[b]	
Biomark (Fluidigm)	4.6 µl (12-panel array)	12	765	~4 nl	Chip loading	<10 min
					PCR	~120 min
	0.65 µl (48-panel array)	48	770	~1 nl	Chip loading	<40 min
					PCR	~120 min
QX200 (Biorad)	Up to 18 out of 20 µl	96	Up to ~20,000	~0.85 nl	Droplet generation	<2 min (per eight samples)
					PCR	~120 min
					Droplet reading	<10 min (per eight samples)
RainDrop (Raindance)	Full recovery mode: 25–50 µl	8	4,500,000–9,000,000	~5 pl	Droplet generation	20–30 min
					PCR	~180 min
					Droplet reading	<240 min
	1M read mode: 5 µl	8	~1,000,000	~5 pl	Droplet generation	20–30 min
					PCR	~180 min
					Droplet reading	<120 min
QuantStudio 3D Digital PCR System (Thermofisher)	14.5 µl	24 chips	Up to 20,000	~0.865 nl	Chip loading	<4 min (per chip)
					PCR	~2.5 hours
					Chip reading	~1 min (per chip)
Constellation dPCR platform (Formulatrix)	2.2 µl	96-well plate	496	~4 nl	Plate priming	<12 min
					PCR	~60 min
					Plate reading	<12 min

[a]Total reaction excluding losses due to dead volume.
[b]This does not include reaction setup but does include instrument-specific pre- and postmanipulation.

who are familiar with qPCR, some technical considerations that set dPCR instruments apart are described in the following subsections.

Number of Partitions

Partition number is a key factor because it determines the dynamic range of the instrument. Those with fewer partitions will still enable precise quantification, but this may require dilution of the sample to ensure that the template concentration is not too high. Such dilution also requires prior knowledge of the sample concentration and may require initial analysis of the sample to determine whether, and how much, dilution is needed. When measuring rare mutations, the partition number also determines the sensitivity. Consequently, increased partitions enable greater dilution, thereby increasing the ratio of rare mutants to wild type in those partitions that contain the mutant sequence.

Reaction Volume

Reaction volume is an important consideration because, unlike qPCR, only a proportion of the reaction that is loaded into the instrument ends up being analyzed. This is because there is a dead volume associated with partition loading and/or selecting the partitions to be included in the analysis. Consequently, experiments need to be designed to account for this dead volume wastage. Additionally, unlike qPCR, most dPCR instruments are not readily scalable, which limits the volume of sample that can be analyzed in any one reaction. This can be compensated for somewhat by performing multiple reactions, but such an approach may require committing considerable amounts of sample to dead volume as well as additional costs associated with increased reagent use. Current instruments that offer >10,000 partitions per reaction all have variable reaction volumes because the number of partitions generated and analyzed varies from run to run. This is a unique concept for dPCR because other PCR methods usually perform replicate, control, and calibration reactions with the same reaction volume.

Protocol Steps

All of the current dPCR instruments require an initial step to partition the reaction. Depending on the partitioning format, this can comprise priming or loading a chip or generating water-in-oil emulsions. Whichever format is chosen, this step adds time. There is currently only one instrument that offers real-time analysis and an amplification plot of each reaction; this is clearly not crucial, but it is a useful tool to have when interpreting data, with the added benefit that data analysis is automated requiring no post-reaction manipulation. For the majority of dPCR instruments a subsequent end-point analysis step is required after the PCR. Compared to qPCR, this also increases the time needed to perform the reaction, and this varies between instruments (Table 1).

QUANTIFICATION BY dPCR

One of the key metrics for dPCR is the average number of template copies per partition (also termed lambda, λ). Where λ is low (<0.1 copies per partition) the majority of positive partitions contain a single molecule only, and their number is roughly equivalent to the estimated copies (Fig. 1a). This principle can be used to measure multiple

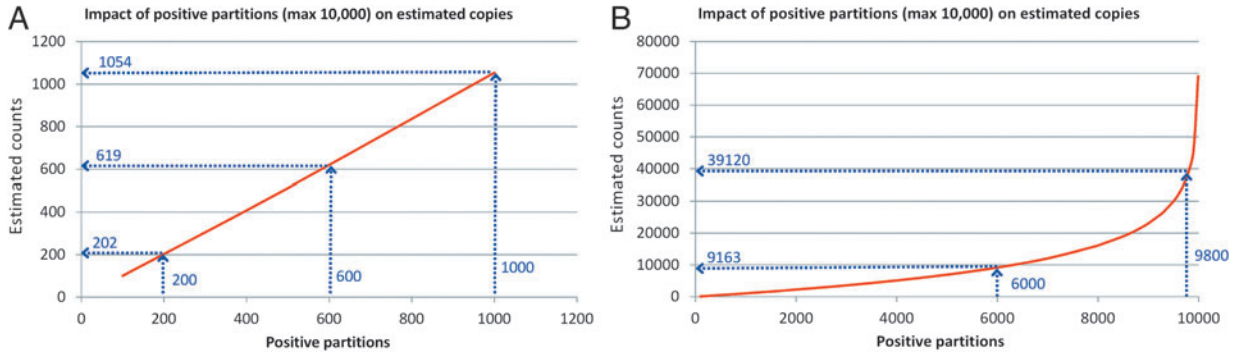

FIGURE 1 Influence of the number of positive partitions on the estimated copy calculations. Graph demonstrating the linear relationship between the number of positive partitions and estimated copies (red line) when a total of 10,000 partitions are measured. (A) When λ is low (<0.1), the number of positive partitions is almost equal to the number of molecules (blue dashed lines). (B) As λ increases, the total number of estimated copies exceeds the number of positive partitions (blue dashed lines), so the dynamic range is greater than the total number of partitions.

targets (multiplexing) using probes with different amounts of the same fluorophore (17), thus increasing the number of targets that can be measured in a single reaction (18). This unique aspect of dPCR is only practical if the instrument has very large numbers of partitions because the vast majority must contain no template to ensure that those that do only contain a single molecule.

As λ increases, the number of positive partitions containing more than one molecule also increases (Fig. 1b). This leads to a potential problem because dPCR only measures partitions as positive or negative. While negative partitions are assumed to contain no template (molecular dropout is discussed in more detail below), there is currently no physical means of precisely establishing whether a positive partition contains one or more template molecules, a fact that is crucial for accurate quantification. To compensate for this, the random distribution of the template DNA can be used and λ can be estimated using equation 1:

$$\lambda = -\ln\left(1 - \frac{k}{n}\right) \quad (1)$$

where k is the number of positive partitions and n is the total number of partitions. As well as providing a precise estimation of the template concentration, this signifies that the dynamic range of any dPCR instrument is greater than the number of partitions used to perform the reaction (Fig. 1b).

Because dPCR simply counts positive and negative reactions, the output is potentially more reproducible than with qPCR. Furthermore, dPCR does not require a calibration curve to provide a numeric value for the measurement. Together, these facts give dPCR a substantial advantage over qPCR that may facilitate more reproducible measurements between laboratories.

Without the application of a common calibration standard, qPCR results generated by different laboratories may vary greatly (19). This establishes the need for reference materials in the field of applied molecular measurement when performing qPCR. With the support of international WHO reference materials, quantification of viral load for pathogens such as HIV and the hepatitis viruses using qPCR revolutionized clinical analysis with increasing agreement between laboratories. Despite this there may be residual interlaboratory differences, and physicians should be cautious when comparing results from different laboratories. This is, in part, likely to be due to the challenges associated with the reproducibility of qPCR. Furthermore, WHO reference materials are not available for all pathogens.

While dPCR does not require a calibration curve to assign a quantitative value, we cannot agree with the assertion of some users that dPCR is "calibration-free." This is because instrument calibration is crucial to determine factors such as thermal profile and partition size, which may differ between instruments (or the same instrument over time) and must be monitored. Although relatively robust, dPCR remains susceptible to sources of error that may impact on measurement accuracy, and thus calibrators may still be needed to compensate for this.

SOURCES OF ERROR

When discussing quantification error, the following points should be considered. First, error comes in two guises: fixed and random. Random error is the component that varies with replicate measurements and determines variance, standard deviation, and precision. Fixed error is the component that leads to bias, which leads to deviation from the true value. Second, a given technique will be susceptible to intrinsic (linked to the technique in question) and extrinsic (linked to factors that affect the result but not due to the technique) error.

While the advantages of a novel approach frequently focus on the intrinsic benefits of the technique in question, the extrinsic factors must also be considered in any discussion about applications and potential translation to routine practice. This is particularly pertinent for molecular methods since extrinsic factors including sample storage and extraction are frequently ignored while paradoxically being the main source of error in the measurement.

Variance

As with any measurement method, dPCR provides a value that can be influenced by both variance and bias. When measuring the quantity of a given DNA molecule by qPCR, variance is dependent on both the addition of the template to the reaction as well as "noise" intrinsic to the

qPCR, which is influenced by factors such as optimization and PCR efficiency. Notably, the intrinsic variance of a given qPCR reaction can vary considerably depending on a variety of factors including primers, platform, level of optimization, etc. Where dPCR differs is that the intrinsic noise is more fixed because it follows a random distribution which is used by the Poisson calculation to estimate the numbers of copies (11, 14).

dPCR also differs from qPCR in how precision is influenced by the template concentration being measured. A considerable strength of a well-optimized qPCR is that it offers a similar variance as a proportion of the mean across a wide dynamic range: i.e., the error is homoscedastic in the logarithmic scale. The variance of dPCR differs because it is dependent upon the λ (Fig. 2), which means that for the most precise measurement, the sample needs to be at or diluted to a concentration that is approximately 1.5 to 1.6 copies per partition (20), and thus for the most precise measurements some idea of the concentration of the sample is needed prior to analysis.

As with qPCR, dPCR is susceptible to variance associated with the addition of template to the reaction as well as "upstream" extrinsic factors required to sample and prepare the template for analysis. These upstream factors are frequently the predominant source of variance for both qPCR (21) and dPCR (15). Consequently, experiments should be performed which reflect this by replicating steps including sampling and extraction to more accurately estimate the variance associated with the whole procedure required to analyze the sample in question.

Bias

One of the major problems associated with qPCR is that it is prone to bias. The output (the quantification cycle, Cq [22], formerly designated Ct or Cp) is an arbitrary metric dependent upon a host of factors including instrument, threshold level, plastic ware, choice of probe chemistry, and batch of reagents to name but a few. Most methods applying qPCR to quantify microbial abundance compare the unknown sample to a calibration curve to convert the Cq to DNA or RNA copies.

The problem with applying calibration curves is that they can give very precise but potentially highly biased results because accurate quantification of the calibrator is difficult and/or choosing a calibrator that is readily commutable to the sample being analyzed is very challenging. Consequently, laboratories can differ widely in the magnitude of the measurements (19). This may hamper research into aspects such as the dynamics of a given infection and can impair comparisons between data generated by different laboratories.

One of the major potential benefits of dPCR is that it does not require a calibration curve to provide a quantitative result. While this is a clear advantage, dPCR is not infallible and will be susceptible to bias that may in cases require calibration in some form. Because volume is crucial to dPCR, it is essential that instrument manufacturers accurately define both the volume and volume variance of their partitions. Other than such volume-related issues, there are few routes for a well-optimized and specific dPCR reaction to overestimate the quantity. As with any method, contamination could have such an impact, but the only other route to overestimation of dsDNA is if the strands become denatured and populate two different partitions. Consequently, there could potentially be a 2-fold overestimation bias (23).

The potential for dPCR to underestimate the abundance of a target is arguably a more prominent source of bias. dPCR may be less susceptible to certain inhibitors than qPCR (24, 25). However, molecular dropout, where a molecule is present but does not amplify, is known to occur (26), and inhibition remains a potential problem and must be considered and controlled for, especially where a sample is reported as negative for a given pathogen. Internal positive controls are therefore likely to be as important for dPCR when used diagnostically as they are for qPCR.

Underestimation may also occur if some of the assumptions applied when performing dPCR are not met. dPCR is dependent upon random distribution of template across partitions. If, for example, template molecules are linked *in cis*, they will not distribute randomly because they will colocalize in the same partition. Because there is no current way to quantify the number of molecules present in a

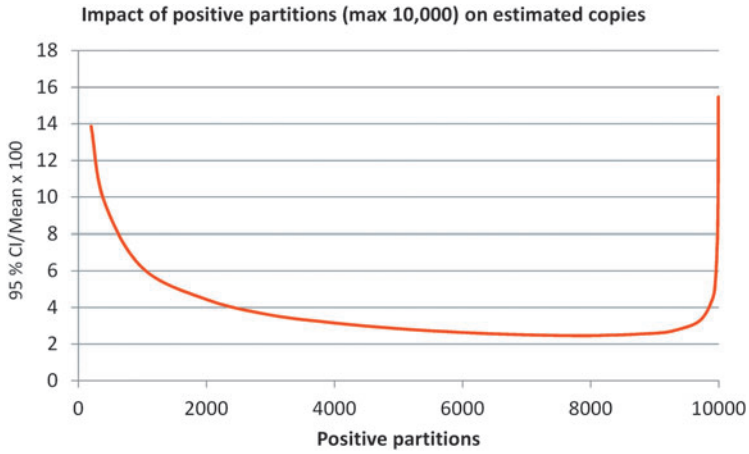

FIGURE 2 Influence of the number of positive partitions on precision. Graph demonstrating the correlation between the precision and the proportion of positive partitions observed. At very low or high λ, the precision is reduced. The highest precision is observed when $1.5 < \lambda < 1.6$, where the number of positive partitions is between 7,700 and 8,000. Graph calculated using Ucount (https://dna.utah.edu/ucount/uc.html).

given partition, they will be treated as a single molecule, and with equation 1 the true number will be underestimated. This could be rectified by separating the linked sequences using methods such restriction digestion or sonication.

Many other extrinsic sources of bias that must be considered when performing qPCR equally affect dPCR. For example, if an extraction only recovers a small proportion of the sample, or the sample is degraded, then dPCR will not be able to accurately measure the amount of nucleic acid originally present within the sample. Where microbial, or patient, gene expression is being measured by targeting RNA, or an RNA virus is of interest, then the target must be converted to complementary DNA (cDNA) using reverse transcription (RT) that can be both variable and biased (27). The implications of the effects of extrinsic factors such as extraction and RT on dPCR and the need for calibration are discussed in more detail below.

dPCR can be used to measure single nucleotide polymorphisms (SNPs), for example, in the detection of drug-resistant *Mycobacterium tuberculosis* mutations (28). A PCR-based SNP assay is usually performed with universal primers that amplify both the wild type and mutant sequences with the specificity being conferred by a probe for either the wild type or mutant variant. While dPCR partitioning can improve sensitivity to rare mutations, the presence of wild type sequences within the sample can lead to two signal populations being observed: one containing the amplification of the mutant sequence and a second containing the amplification of the wild type sequence due to probe cross-reactivity leading to a reduced signal amplitude (Fig. 3a). If these two populations are not clearly separated, an overestimation of the mutant sequence could occur depending on where the threshold is set; consequently, wild-type-only controls are crucial in the experimental design. In such situations it may be preferable to perform such analyses in duplex (measuring both wild type and mutant sequences concomitantly using different fluorophores) and visualize the results using a two-dimensional plot. This would allow the user to determine which partitions contain mutant, wild type, or both sequences (Fig. 3b).

The measurement of rare variants is also heavily dependent upon template concentration; for example, to detect a mutant SNP present at 0.1% of the population, at least 1,000 copies must be added to the reaction. This may seem obvious, but it is an important consideration where the pathogen in question may be at a low level and a potential resistance genotype may be missed due to insufficient sampling. A further consideration to compound this matter is the fidelity of the DNA polymerase. At some point, the frequency of the mutant sequence in the sample will be lower than the error rate at which the polymerase introduces the specific mutant SNP into the wild type sequence, thereby contributing to reduced sensitivity if it is defined or specificity if it is not.

When a partition contains both wild type and mutant SNP sequences, a phenomenon we call "partition-specific competitive PCR" occurs. When the primers are universal, they amplify both sequences within the partition, resulting in roughly half of each. This results in a reduced amount of template for each probe to bind to, leading to a reduced signal output (Fig. 3c). This may not be cause for concern for many samples if the thresholds can be set to separate the different populations. However, high-concentration samples may be more difficult to analyze because a number of partitions contain more than one copy of the two sequences. If, for example, a partition contains five copies of the wild type and one copy of the mutant sequences, then the wild type sequence may outcompete the mutant sequence entirely, resulting in almost no mutant signal. In such cases, the partitions containing both sequences may resemble an arc across the two-dimensional plot, causing difficulty in selecting the correct position for the thresholds (Fig. 3c). In such situations, it may be appropriate to

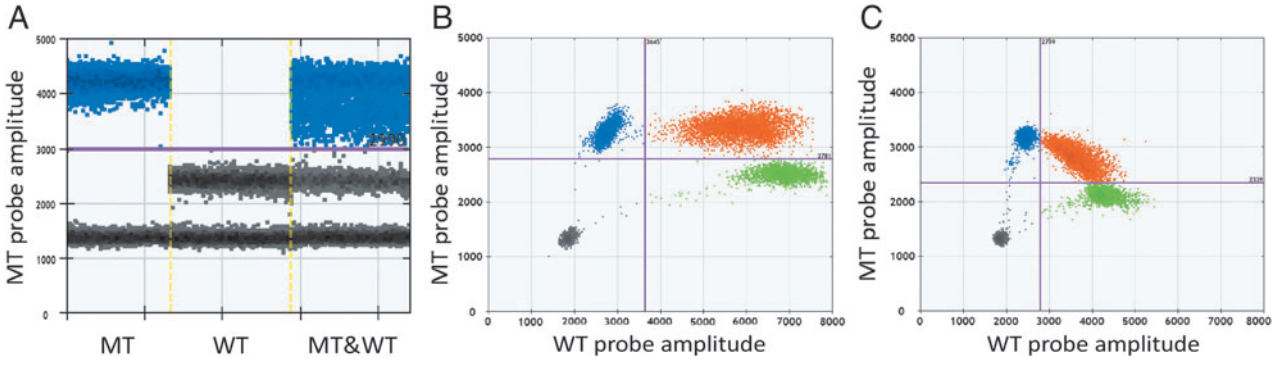

FIGURE 3 Detection of single nucleotide polymorphisms using droplet dPCR. Example of graphs produced with the QuantaSoft Software from the QX200 Droplet Digital PCR System (Bio-Rad). The horizontal and vertical pink lines represent the thresholds between negative and positive droplets. (A) One-dimensional plot illustrating positive and negative droplets using a uniplex reaction containing a mutant-specific probe only. The mutant-only sample (MT) gives two distinct droplet populations: positive (blue droplets) and negative (gray droplets). The presence of the wild type sequence in the wild type-only sample (WT) and mixed sample (MT & WT) generates a second population of droplets that falls between the negative and positive droplets due to the probe binding with lower affinity to the wild type sequence. (B) Two-dimensional plot of the mixed sample shown in (A) with a duplex reaction containing both the mutant and wild type probes. Each droplet has both a mutant and wild type signal, so four possible outcomes are observed: MT only (blue), WT only (green), MT & WT (orange), and negative (gray). (C) Two-dimensional plot of a mixed sample showing partition-specific competitive PCR. Droplets containing both MT and WT sequences (orange) merge with the MT only (blue) and WT only (green) droplets to form an "arc" across the plot with reduced amplitude of one or both signals, thus making confident positioning of the thresholds difficult.

perform dilutions to reduce the effect of partition-specific competitive PCR.

THE ABILITY OF dPCR TO PERFORM ACCURATE QUANTIFICATION

A comprehensive description of the sources of bias and variance will provide a better understanding of the accuracy of dPCR. However, the groundswell of interest that accompanies a novel method frequently comes with much hype and inflated expectations. The speculated ability of dPCR to provide an "absolute" measure of DNA quantity is of note because the vast majority of other molecular methods used in clinical and biological quantitative measurement (e.g., qPCR, enzyme-linked immunosorbent assay) are made relative to some kind of calibrator. Part of the problem with current molecular quantification is defining what that calibrator should be so that laboratories' performance can be readily compared in a manner that is appropriate (or commutable) to the sample being analyzed.

dPCR has caught the interest of the metrology community and national measurement institutes including the European Commission's Joint Research Center, the Korean Research Institute of Standards and Science, LGC in the UK, the National Institute of Biology in Slovenia, the National Institute of Metrology in China, the National Institute of Standards and Controls in the United States, the National Measurement Institute in Australia, and TÜBİTAK National Metrology Institute in Turkey. This is because dPCR offers measurement that counts individual molecules without being compared to a calibrator. It is the national measurement institutes that have made major contributions to some of the initial research performed on dPCR. To date, research into the technique has focused on defining the actual performance of the method and critically evaluating emerging dogmas.

This has included demonstrating with purified DNA that dPCR quantification can be precise and linear (12) and that primers targeting different regions of the same molecule can perform with very good agreement (12, 16, 29). When measuring purified DNA, dPCR can offer improved precision which facilitates smaller-fold change measurements and thus finer discriminations (26).

When compared to orthogonal methods such as spectrophotometry (the only other readily available molecular method that does not require comparison to a calibrator) or methods like qPCR, which are frequently calibrated using spectrophotometry, the agreement with dPCR is frequently reported to be within 2-fold (12, 29). dPCR has also been demonstrated to perform with good agreement to other orthogonal methods such as isotope dilution mass spectrometry (30). Taken together this suggests that when measuring a purified DNA molecule, the estimated quantity that is reported is likely to be close to the "real" value, and dPCR can therefore be regarded as accurate. More focused research has been performed to improve the accuracy of dPCR to better understand partition volume and error (31, 32), with further discussion on how to determine the impact of partition volume associated error (3, 33).

While dPCR may currently offer the most accurate method to measure a small quantity of a specific DNA sequence in a complex background, some key factors must be considered when it is applied to microbial analysis. First, dPCR, like any PCR reaction, measures an amplicon. This typically follows qPCR design parameters and thus is usually <100 bp long. Any link between this measurement and that of the microbial genome being measured must make the fairly substantial assumption that a 100-bp molecule is representative of a genome in the kilobase (viral), megabase (bacterial), or >10 Mb (fungal, parasite) range. This assumption has generally proved adequate for established applications using qPCR, and we have shown that dPCR can demonstrate good agreement with next-generation sequencing methods for metagenomics analysis (34, 35). However, because neither PCR nor next-generation sequencing requires intact large-molecular-weight genomic DNA, it can be difficult to know whether the measured DNA is from an intact genome or a degraded fragment, which may mean different things; it is important to appreciate that the detection of such small genomic fragments provides no direct evidence of viability or infectivity of a given microbe.

POTENTIAL APPLICATIONS OF dPCR TO MICROBIAL RESEARCH AND DIAGNOSTICS

In the short term, the biggest impact the technique of dPCR will have on microbial analysis is likely to be within research rather than clinical diagnostics. The growth in research applying dPCR has either capitalized on the advanced features of dPCR to investigate a specific question or compared dPCR to alternative methods. The latter is done either to specifically evaluate dPCR performance or to apply it as an additional method to back up findings generated by other techniques.

dPCR has been applied in a number of infectious disease settings. The improved precision of dPCR has been demonstrated in DNA viral quantification of human cytomegalovirus (hCMV) (36, 37), HIV LTR circles, and total HIV DNA (38, 39). dPCR has also been used to measure chromosomally integrated human herpesvirus 6 (40, 41).

Where clinical sensitivity has been compared in viral analysis, dPCR has been reported as worse than qPCR for hCMV (36) and hepatitis B virus (42). Digital technologies have also been applied to isothermal methods such as loop-mediated isothermal amplification for measuring hCMV (25) and recombinase polymerase chain reaction in the analysis of methicillin-resistant *Staphylococcus aureus* (43).

Identification of methicillin-resistant *S. aureus* using dPCR has also been directly compared with in-house qPCR and near-patient PCR-based instruments (44). Unlike the above viral comparisons, the authors concluded that dPCR performed with comparable sensitivity and specificity to the other methods, which are routinely applied clinically. Clinical application of dPCR to the analysis of other pathogenic bacteria has included detection of ocular *Chlamydia trachomatis* infections (45). *C. trachomatis* is one of the few bacterial pathogens for which molecular diagnosis has been the gold standard for detection in sexually transmitted infection for some time.

Roberts et al. compared dPCR with the Roche Amplicor CT/NG test as a diagnostic test for the cause of trachoma due to *C. trachomatis* infections, concluding that the former was an effective diagnostic technology suitable for both research and clinical use in the application (45). The same group used dPCR to demonstrate that there was no association with plasmid abundance and severity of ocular *C. trachomatis* infection, suggesting that dPCR did not concur with many of the qPCR reports (46).

The application of dPCR has also been assessed for the analysis of anthrax to enumerate chromosomal and virulence plasmid copies (47), and the authors reported that dPCR was in good agreement with direct sequencing. Pholwat et al. used dPCR to investigate mixed genotypes of *M. tuberculosis* to quantify heteroresistance and compared this to the agar proportion method, reporting good agreement between the methods (28). They reported that the dPCR approach was more sensitive than qPCR or Sanger sequencing; notably, they performed this dPCR with 384-well plates, demonstrating that a dPCR instrument is not essential to perform the method.

The measurement of RNA expression or RNA viral genomes is less well documented, but possible applications of RT-dPCR have been described in hand, foot, and mouth disease (48) and for measuring variant sequences in hepatitis C infections (49). A multivolume dPCR instrument has also been used to measure HIV and HCV viral load (50). Additionally, dPCR measurement of patient HLA-DR mRNA expression as a surrogate marker of sepsis has been reported as a more accurate alternative to RT-qPCR (51).

As previously mentioned, a further challenge to RNA measurement is the potential bias attributable to the RT step required to convert the RNA to cDNA. Historically, RT-qPCR has depended on the qPCR as a measurement of performance, and conversion efficiencies of RNA to cDNA were not extensively evaluated. Essentially, as long as the quantification was linear and maximal sensitivity was not essential, RT enzymes were generally assumed suitable and conversion efficiencies were not routinely considered, despite some evidence of wide differences (27).

dPCR will place the RT step under a higher level of scrutiny because different enzymes and primers convert RNA to cDNA with different efficiencies. This will lead to a different number of positive partitions when measuring the same template, and there is the potential for wide disagreement between different approaches (13). Consequently, early application of RT-dPCR needs to be approached with caution, and calibration to compensate for the associated bias may be a key consideration. The measurement of positive partitions associated with dPCR will enable RT-dPCR to evaluate conversion efficiencies of reverse transcriptase and most suitable priming strategies in a much simpler and informative manner than with qPCR; this could assist manufacturers in the development of more effective and reproducible formats.

Calibration may also be needed where different laboratories perform different procedures to prepare a sample for analysis. Because these steps can add both bias and variance, this may impact the final dPCR result and compromise reproducibility. As with reverse transcriptases, dPCR provides a simple method for interrogating specific extrinsic factors, such as the nucleic acid extraction method, in an "absolute" manner.

CONSIDERATIONS FOR THE FUTURE

dPCR is a powerful research tool that enables a number of unique measurements with high sensitivity and precision. In turn, it could push the frontiers of molecular microbiology by improving our understanding of microbial dynamics through potentially accurate quantification of DNA and associated mutations. However, while its sister technique qPCR has contributed a huge amount to clinical and biological research, including molecular microbiology, the technique has also led to much error due to its incorrect application (52) and difficulties associated with reproducibility, which were discussed above.

Such error is partly due to poor reporting of the basic details of experimental design and procedures in publications that apply the method, which has meant experiments may be very difficult to repeat. To rectify this, the Minimum Information for the Publication of Quantitative Real Time PCR Experiments, or MIQE, guidelines were published in 2009 (22), providing guidance on how to perform the methods and also a key set of experimental factors that should be described in a paper. This was followed in 2012 by the publication of the digital MIQE guidelines (53) to support the reproducible application of dPCR and increase the impact and, ultimately, the likelihood that findings can be translated into routine clinical applications.

Where dPCR may have an advantage in molecular diagnosis is that the final measurement of the DNA and its calibration are simpler. Thus, when applying dPCR to detect and quantify pathogens, detect mutations, or investigate linkage, not only may the method offer improved accuracy, but it could make it easier for two different laboratories measuring the same sample to get the same result. Furthermore, the simplicity of the dPCR output count will potentially make routine application simpler for the wider community of clinical scientists.

While dPCR may have considerable potential for routine clinical diagnostic applications, the reality is that instrument complexity and cost are likely to delay uptake. In the short term dPCR will have, and is having, a useful role in the value assigning of calibration materials needed for the other more common methods including qPCR (16, 54, 55).

Those who expound the benefits of dPCR must remember that comparisons that demonstrate improved performance with, for example, a clinically applied qPCR in a single laboratory are not considering a major factor that allows qPCR to be successfully applied clinically: namely, reproducibility. Until formal studies comparing interlaboratory performance of dPCR are undertaken, it will not be possible to provide a true assessment of dPCR as a diagnostic method.

CONCLUSION

The potential role of dPCR in diagnostic, predictive, or prognostic monitoring of microorganisms is now becoming apparent. dPCR has the potential to overcome one of the major shortfalls of other measurement techniques: "absolute" quantification without the need for calibration curves, thereby reducing measurement bias and potentially improving intra- and interlaboratory reproducibility. With the appropriate use of controls and continued development of the instrumentation, dPCR could improve microbial measurement and monitoring so that accurate routine molecular quantification may be facilitated.

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Massively Parallel DNA Sequencing and Microbiology

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5

Recent, dramatic increases in the throughput of DNA sequencing instruments provide opportunities for microbiology research that previously were limited to large genome centers. In sequencing the human genome, what once took years and hundreds of instruments and workers to complete can now be done in days on just one instrument. Moreover, such increases in throughput are likely to continue over the next few years. Here we review the different platforms for massively parallel sequencing (also called next-generation sequencing, or NGS) and compare those that are currently most popular. As evidenced by other chapters of this book, some of the exciting applications of NGS for microbiology research are the abilities to accurately deduce microbial population structure (also known as metagenomics), discover new pathogens, detect rare pathogens within a complex background, and elucidate the dynamics of drug resistance—even when culture of the microorganisms is impossible. These new technologies are directly applicable to and have significant ramifications for both research and clinical microbiology.

OVERVIEW OF METHODS

Most earlier generations of sequencing systems were based on slab or capillary gel electrophoresis of a nested series of DNA fragments produced by specifically primed, *in vitro* DNA polymerase reactions, i.e., Sanger sequencing (1). The sequencing templates for these reactions were prepared either by molecular cloning or PCR. Per sequencing instrument, up to a microtiter plate's worth of templates (96 or 384) could be run at one time using a corresponding array of capillary gels.

Many, but not all, of the technologies for massively parallel sequencing also use molecular cloning, not in bacterial hosts but by using limiting-dilution of DNA followed by the isolated PCR amplification of single DNA molecules. This is done in a highly parallel, automated, and high-throughput fashion with millions of such amplified templates being produced in a short time. The two most popular massively parallel DNA sequencing systems accomplish this clonal amplification in two ways, using either emulsion PCR (2, 3) or bridge PCR (4, 5), as described in more detail below. The two systems are the Illumina system (www.illumina.com) (6) and the Thermo-Fisher/Life Technologies Ion Torrent and Ion Proton system (http://www.lifetechnologies.com). In contrast to these methods, the sequencing technology of Pacific Biosciences (RS II) is based on sequencing of single native DNA molecules with no prior amplification (http://www.pacificbiosciences.com).

The sequencing reactions in earlier-generation systems were performed in microtiter plates, and the results were visualized after resolution of the nested DNA fragments upon gel electrophoresis. Massively parallel sequencing platforms perform reactions on millions of templates that are isolated from one another on nanometer-scale arrays. For each isolated template the sequence-specific incorporations of nucleotides are followed on a cycle (of incorporation)-by-cycle basis. The isolation and following of the sequencing reactions upon the array differ among the different platforms, as do the sequencing chemistries. In all these new platforms, the fact that hundreds of thousands to millions of reactions are followed in parallel accounts for the many orders of magnitude increase in sequence throughput per instrument.

As in earlier genome sequencing efforts, "shotgun sequencing" of randomly fragmented DNA and assembly of the sequences using the overlap between randomly distributed sequences into increasingly larger sequence "contigs" are required (7, 8). As before, this is handled by algorithms encoded in software. Particular challenges for such software in massively parallel sequencing include shorter read lengths for some of the technologies and therefore increased uncertainty with regard to the specificity of the overlaps, the higher error rates inherent in the sequencing methods employed, and the sheer number of sequence reads that need to be handled.

In this chapter, steps that are similar and shared in common among the massively parallel sequencing platforms will be described first. Following this, the differences among them and the advantages and disadvantages of each will be examined. Last, there will be a brief description of an application of NGS to metagenomic research.

METHODS BASED ON THE AMPLIFICATION OF SINGLE MOLECULES

A method for isolating and amplifying single DNA molecules in a thin, polyacrylamide gel layer was described in

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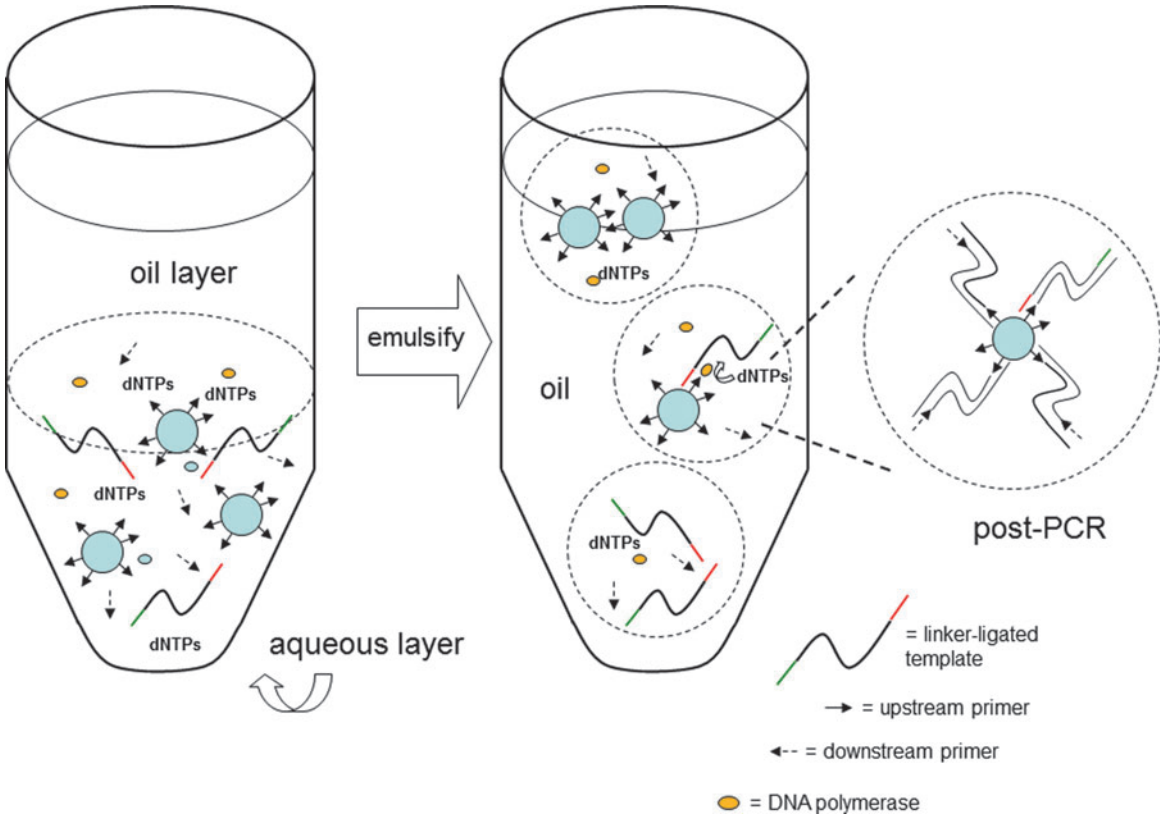

FIGURE 1 Emulsion PCR for single-molecule amplification. A two-phase (aqueous and oil) system in which PCR components are included in the aqueus phase. Also included are capture beads upon which one of the two PCR primers is attached. In the Ion Torrent system these beads are magnetic to facilitate their subsequent capture and washing. With vigorous agitation, emulsification takes place such that millions of tiny water droplets form in the oil. The concentration of DNA templates, beads, and water droplets is such that the frequency of there being one bead and one DNA template in one droplet is optimized. In this case, an amplicon attached to the bead as shown will form. Other possibilities include a droplet with neither bead nor template or a droplet with only a bead or a template (shown). In these cases no amplification can occur. The possibility of multiple templates and/or beads per droplet, which results in amplification, is minimized as much as possible by concentration. Multiple templates per droplet usually result in a mixed sequence read from the capture bead and are ultimately filtered out in software.

1999 (9). DNA molecules were diluted into the gel before it was formed, as were appropriate amounts of the ingredients for PCR—that is, primers, DNA polymerase, deoxynucleoside triphosphates (dNTPs), and the appropriate salts and buffer. The gel was subjected to thermocycling such that the individual template molecules were amplified. Because of the gel, the products of the amplification were localized to within a small area surrounding the initial template and formed molecular colonies, or "polonies," that generally did not overlap one another because of the level of dilution of the initial template molecules. Nonetheless, millions of such polonies could be contained in the area of a microscope slide. The gel-based polony method has been used to support high-throughput sequencing (10) using a scanning, fluorescence microscope but not in a commercially available system.

Another method of isolating and amplifying single DNA molecules is called emulsion PCR (2, 3), in which an oil-water emulsion is formed. As shown in Fig. 1, the aqueous phase contains the reagents for PCR, including primers matching the ends of template fragments (see "DNA Fragment Libraries" section, below) and the DNA template. Also present are beads to which are attached "capture" oligonucleotides. When emulsified with an excess of oil using controlled, vigorous agitation, aqueous droplets are formed that comprise millions of nanoscale "microreactors" in which PCR can take place. The DNA template and beads are dilute enough, and the number of microreactors large enough that if a microreactor contains both a template molecule and a capture bead, there is likely to be only one of each, ensuring that the amplification that takes place is clonal and that the clone will be captured on a single bead. Emulsion PCR is used in the 454 system (11) and the Ion systems.

Although originally used as a means of amplifying DNA on a solid phase irrespective of the number of starting template molecules, "bridge PCR" (4, 5) has been adapted to single molecule amplification in the Illumina system (Fig. 2). In bridge PCR both PCR primers are attached via the 5′ end to a solid support at sufficient density that a templated, 3′ extension can, after the template is denatured off, bend back and anneal to the reverse primer and allow extension off of that primer. The end result is that both strands are attached to the solid support in increasing numbers as PCR proceeds. In the Illumina system the solid support is a glass slide. The template molecules are flowed over the slide at low enough concentration that the primer extensions arise from a single template in a

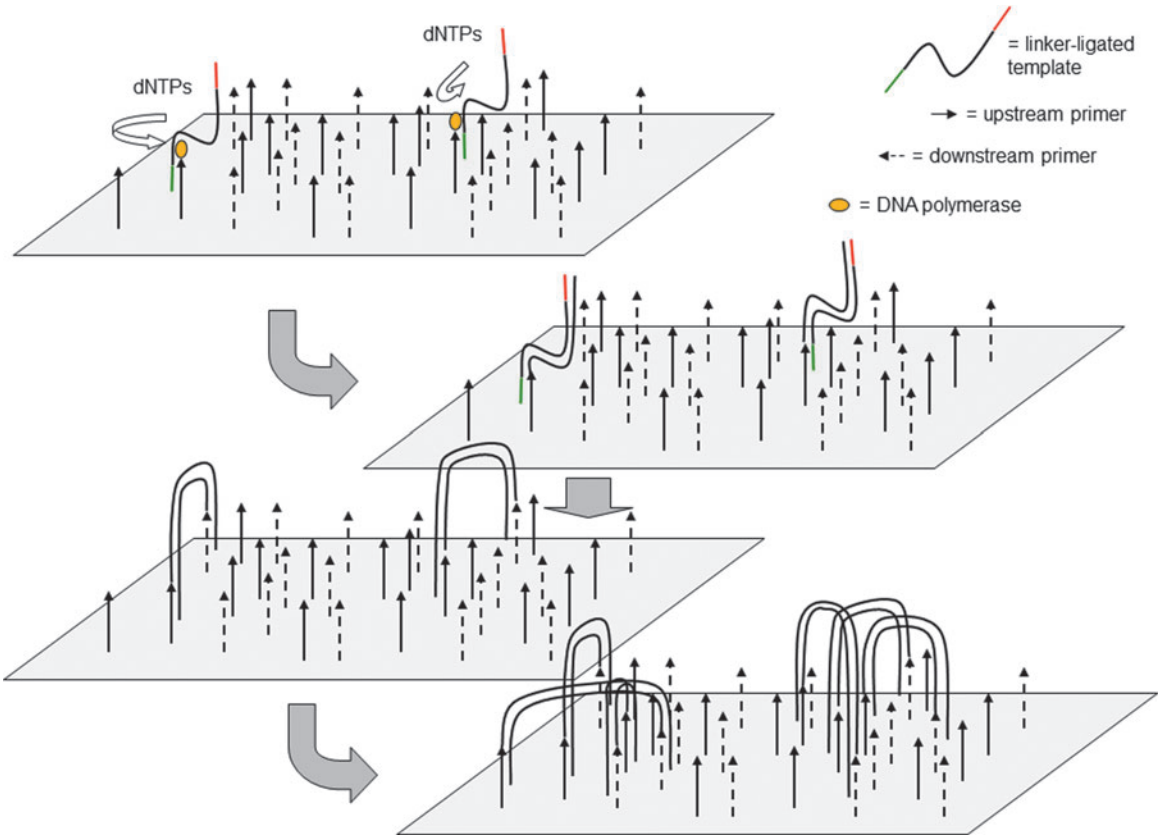

FIGURE 2 A representation of single-molecule, bridge PCR. Immobilized on a microscope slide are a high density of both forward and reverse primers with the 3′ ends free. Single DNA template molecules are flowed over the slide and captured by the PCR primers as shown. Rounds of replication and denaturation through thermocycling are allowed such that 3′ ends of primer extensions can anneal and reanneal to reverse primers, each time being copied again. The original template strands get released to solution in this process but are washed away. All the copies of the original template strand stay localized as shown.

localized, nanoscale area (referred to by Illumina as a "cluster"), usually not overlapping with another such area. For the purpose of this discussion, such single-molecule amplifications will be referred to as "clonal PCR," and the aggregate amplification product, however isolated and imaged in two-dimensional space, will be referred to as a "polony."

For the standard Illumina technology, the arraying of polonies on the surface of a glass slide is an inherent property of the bridge PCR used to originate them. In contrast, the Ion Torrent/Proton systems strive to capture single beads carrying amplified single DNA molecules from the emulsion PCR in single, nanoscale, indexed wells (Fig. 3). For both systems, careful titration of the number of seed molecules or beads relative to the slide or chip surface area is required to achieve an optimum density of enough polonies to maximize sequence throughput but not create overlapping (and thus unreadable) polonies. All of the above schemes require the appropriate modification of the ends of DNA fragments to be amplified, captured, and sequenced, as described in the next section.

DNA FRAGMENT LIBRARIES

"Library" is the term applied to a collection of DNA fragments—frequently obtained via random, physical shearing to facilitate sequence assembly by overlap—that form the basis for a sequencing run. Individual fragment molecules from these libraries are the single molecules that are either directly sequenced or amplified before sequencing as described in the previous section. For genomic sequencing, three types of DNA libraries are used: the fragment library, the paired-end library, and the mate-pair library. The standard fragment library is typically used in obtaining most of the unassembled sequences in whole-genome sequencing. Fragmentation is achieved by nebulization or sonication to a certain mean fragment size. After suitable "blunting" of the ends of the sheared double-stranded DNA molecules, DNA adapters are then ligated on. These provide universal, forward or reverse primer annealing sites for clonal PCR and subsequent sequencing. Unligated adapters are removed, and fragments of a size range appropriate for clonal PCR are isolated by gel purification. This sizing increases the percentage of meaningful sequencing reads.

More recently, an enzymatic means of performing DNA fragmentation and end sequence modification for library construction has been developed that requires fewer manipulations and is more efficient in its use of input DNA. The Nextera method uses an engineered transposase complex, referred to as a transposasome, that performs "tagmentation," that is, the simultaneous cleavage of high-molecular-weight DNA at somewhat random locations around 300 bp apart, and adapter sequence addition to the ends of the resultant fragments (12). Low-cycle PCR with

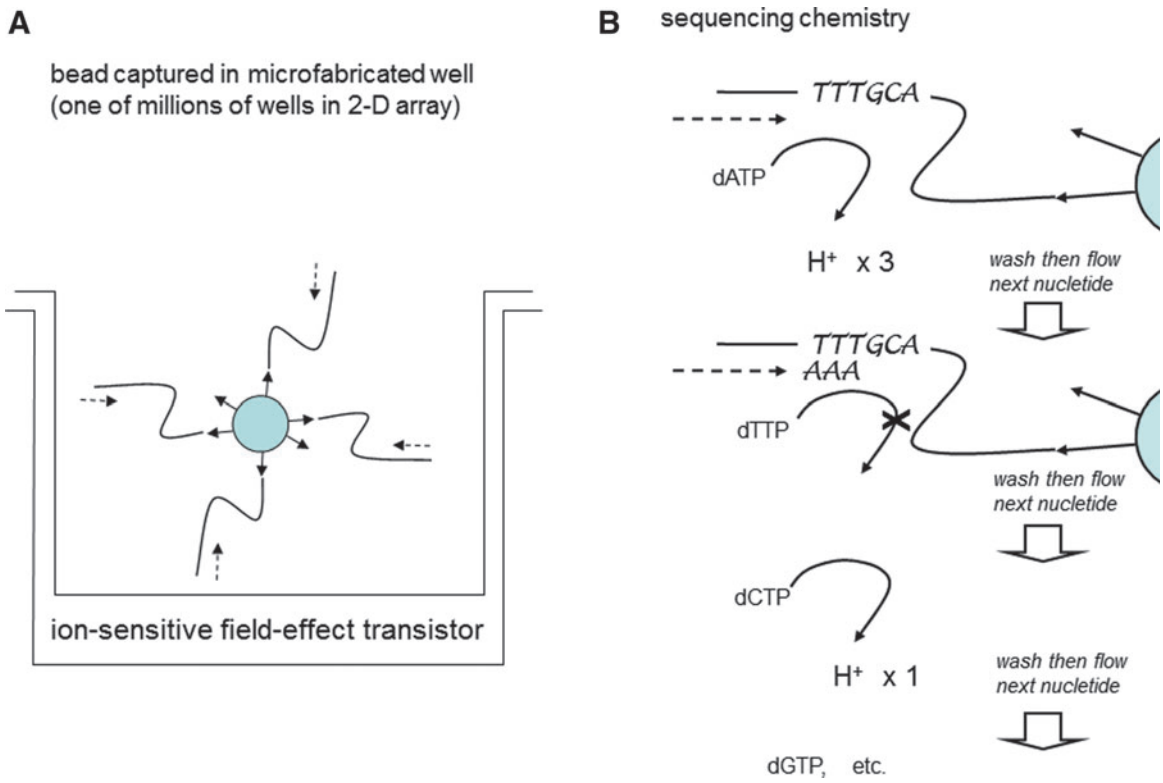

FIGURE 3 The capture of beads carrying amplicon and sequencing chemistry: Ion Torrent system. (A) A single bead in a single well of the array of wells on the sequencing chip. The beads—enriched for those that are carrying amplified template—are deposited in the chip wells by a short centrifugation step. The density of capture beads is adjusted such that there are more wells than capture beads and most wells with capture beads contain only one bead. Shown attached to the beads are single-stranded amplicon templates extended from a primer attached to the bead. Annealed to each template is a sequencing primer. (B) A closeup of the bead shows the sequencing chemistry employed. Sequencing reagents are flowed over the filled plate, including one of the four dNTPs at a time. For each incorporated nucleotide molecule, a hydrogen ion (H^+) is released. The sum of all such incorporations over all template molecules is recorded by the ion-sensitive field-effect transistor fabricated into the bottom of the well. The nucleotides are not modified or terminated such that if the template contains, for example, three sequential deoxyribosyladenines, three dTTP molecules are incorporated and three hydrogen ions are released. This is recorded as three times the signal as a single incorporation. If there is no base in the template complementary to the currently flowed dNTP, no signal is generated.

primers targeting the adapters amplifies the library to a level suitable for use.

The paired-end and mate-pair libraries both localize within the genome and subsequently facilitate the sequencing of the two end sequences of a larger DNA fragment. While paired-end sequencing involves determining the sequence of two ends of a fragment about 200 to 400 bp long, mate-pair sequencing involves sequences that are several kilobases apart. Although increasing steadily, current sequence read lengths for the Illumina and Ion Torrent systems—between 100 and 400 nucleotides (nt)—place limits on how large sequence contigs can be made using overlap assembly. Knowing that two sequences derive from the ends of the same large molecule of defined length allows the assembly of larger sequence contigs, particularly when the overlap assembly is interrupted by a repeated sequence. For the Illumina system, bridge PCR inherently captures both ends of a single molecule in a single polony; use of appropriate adapters and sequencing primers allows both ends to be sequenced.

Paired-end sequencing uses a larger bridge amplicon than normal sequencing—up to about the limit possible for efficient bridge PCR, which is around 300 bp. In mate-pair sequencing, DNA fragments of 2 to 10 kb are generated, end-repaired—with biotin labeled nucleotides—and circularized by ligation. Noncircularized DNA is removed by exonuclease digestion. The remaining circular DNA is then fragmented, and the fragments containing the ends that have been biotin-labeled during end repair and have become joined during ligation are affinity purified. Thus, the libraries consist of short fragments made up of two DNA segments that were originally separated by several kilobases.

Protocols have also been developed to obtain the longer sequence reads that to a large extent would obviate the need for paired-end and mate-pair libraries. This includes the Moleculo protocol, which can be used together with the Illumina technology (13). This method sequence-tags shorter reads from long (around 10 kb) PCRs to create "synthetic" long-read libraries. Longer reads are an inherent feature of Pacific Biosciences RS II single-molecule sequencing (see below) and the Oxford Nanopore technology (not yet on the market; http://www.nanoporetech.com). Long-range mapping technologies such as Irys (http://www.bionanogenomics.com) and Argus (http://opgen.com/) have been developed that are based on the location of sequence motifs (such as restriction enzyme sites) in very long single DNA molecules (100 kb and more).

These represent complementary technologies that are useful to bridge long highly complex repetitive regions.

PRE-ENRICHMENT OF PARTICULAR GENES AND "BARCODING"

In many instances, determining entire genome sequences is not required. Then one can take advantage of the massive throughput of NGS and direct it to multiple samples in parallel. This is facilitated by pre-enrichment of the desired target sequences before sequencing and by sequence tagging or barcoding DNA fragments arising from individual samples as described below.

In deep sequencing, the "bandwidth" of the sequencing experiment is focused only on certain key, subgenomic sequences such that low-level mutations in subpopulations of cells can be identified. This focus also allows the comparison of these subgenomic sequences among many individual organisms at once. The term "deep sequencing" was originally applied to the Sanger sequencing of up to thousands of bacterial clones of, say, a region of the 16s ribosomal gene but was most rapidly adopted once high-throughput *in vitro* molecular cloning and sequencing, that is, NGS, became available.

The most straightforward way to accomplish the enrichment of particular sequences is by using PCR. At first, multiplex PCR was limited in the number of amplicons that could be addressed, which was at most a few dozen (14). Subsequently, by limiting the number of cycles in PCR (low-cycle PCR), replication bias among amplicons, which would over- and underrepresent some sequences in the library, was prevented. Also, combinatorial methods of oligo synthesis were employed which allow the facile creation of primer sets with hundreds or even thousands of different targets. Examples of such multiplex PCR are in the Ion AmpliSeq human exome sequencing kit, which is based on multiplex PCR of 294,000 primer pairs across 12 primer pools (http://tools.lifetechnologies.com). The primers also feature proprietary modifications that allow their subsequent removal because they would interfere with the sequencing process. Another method of using primers to enrich specific target sequences is used by Illumina and called the TrueSeq targeted or enrichment system and features target-specific primer extension and ligation followed by PCR to 5′ and 3′ generic extensions of the targeting primers.

Larger subgenomic regions can be enriched by sequence-specific capture on microarrays of probes or through solution hybridization to probes on beads (15–18). The ability to make many different probes in a facile manner on microarrays is exploited to be able to select large fractions of the genome for enrichment. In the case of solution hybridization to probes on beads, probes are first made on a microarray, amplified and linked to an RNA polymerase promoter, and transcribed into single-stranded, biotinylated RNA. The biotinylated RNA is then coupled to streptavidin magnetic beads. Such enrichments have been demonstrated to work with good efficiencies such that the majority of the recovered sequences are those desired, even from genomes of high sequence complexity. These kinds of enrichments have been performed both prior to and after library preparation.

Since all the systems feature added adapter sequences to facilitate priming of the sequencing reactions, it is also possible to add immediately downstream of the primer annealing sequence a short identifier tag or barcode sequence (19). Such adapter sequences with unique barcodes can be added to individual DNA samples; when read out during a sequencing reaction, the barcode can identify the sample. This allows the pooling of multiple samples, which simplifies sample preparation, particularly for the systems employing emulsion PCR and bead capture. A four-base code in theory would be adequate for 256 samples, although the impact of a misread of the barcode—the result of which is the misassignment of a sequence read—needs to be considered.

SEQUENCING CHEMISTRIES

The sequencing chemistries applied to the two-dimensionally arrayed templates include either generation of light (through fluorescence or luminescence) in response to the incorporation of specific single nucleotides by polymerases (Illumina, Pacific Biosciences) or release of a proton in connection with the incorporation (Ion system). The light-generating reactions are followed in two-dimensional space and time using imaging, and the proton releasing reactions using pH change in a microfabricated well/detector. Fluidic systems deliver nucleotides and other reagents as well as wash away unincorporated nucleotides in a laminar flow over the arrayed polonies. This is repeated in cycles to build up the template DNA sequence. The Illumina system uses a terminated nucleotide much as Sanger sequencing does, but as a complete rather than partial block to further extension until the terminator is removed. A sequencing cycle consists of the flow of reagents for DNA polymerization (dNTPs, polymerase, sequencing primer, etc.) over the arrayed templates (Fig. 4). The four dNTPs are reversibly blocked to further extension as well as fluorescently labeled, each dNTP with a fluorophore of distinguishable wavelength. A color charge-coupled-device image of the two-dimensional array records for each polony (also known as cluster) the color, and hence identity, of the base incorporated. For optimal signal to background, a scanning laser microscope is used to generate the image (this is the most time-consuming part of the process). The block is then removed in a subsequent flow, and the cycle begins again. Read lengths in the range of 2×150–300 nt (paired end) are achieved.

The Ion Torrent/Ion Proton sequencing chemistry is based on incorporation of native nucleotides and the release of a proton (H^+) for each molecule of nucleotide incorporated (20). The pH change induced by the release of such protons is detected at the bottom of the nanowell and converted to an electric signal. By flowing each of four nucleotides in succession over the array of wells (separated by washes) and monitoring the proton release in individual wells in relation to the order of bases introduced, the template sequence can be determined (Fig. 3). There will be proton release in a given well when, say, dATP is flowed only if the next base in the template is deoxyribosylthymine. Since native, nonterminated nucleotides are used, multiple incorporations of a single base occur in response to homopolymer runs in the template. These are seen as a proportionately larger electronic signal in response to a release of proportionately more protons. In this way, homopolymer runs of two, three, four and so on bases can be discerned, although the accuracy of discriminating among longer runs is diminished as the relative increase in signal declines. Read lengths in the range of 200 to 400 nt are achieved.

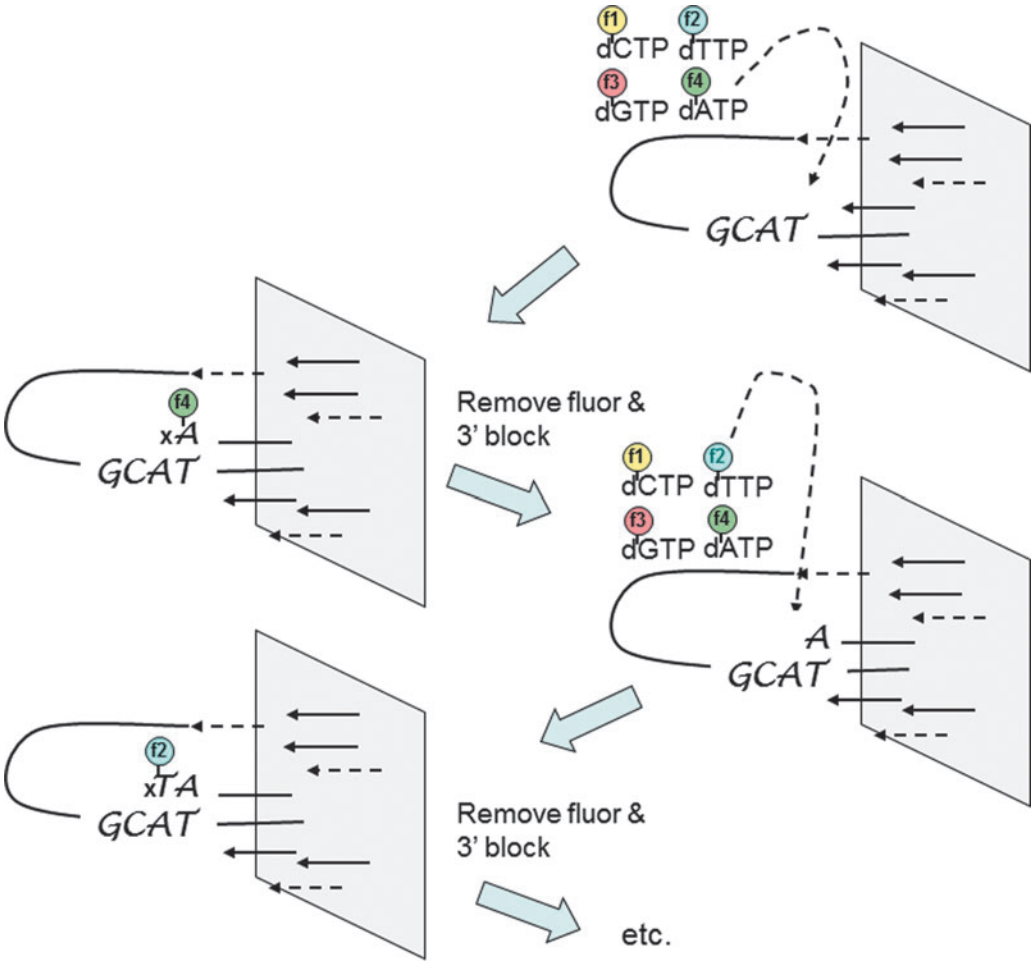

FIGURE 4 Illumina sequencing chemistry. Different fluorophore-labeled nucleotides (f1 through f4) with blocked 3′ termini are added specifically to the end of a primer one at a time. After each addition the fluorophore color is recorded by the imaging system, the block and fluorophore are removed, and the next addition is made.

SINGLE-MOLECULE, LONG-READ DNA SEQUENCING TECHNOLOGY

As in other systems, the Pacific Biosciences RS II technology distributes upon a two-dimensional array (the bottom surface of the "SMRT cell") massive numbers of individual "sequencing-by-synthesis" reactions that are followed in parallel in real time. In contrast to the Illumina and Ion Torrent/Proton systems, the Pacific Biosciences RS II technology is based on such reactions performed upon single, long, native DNA molecules (21). This is possible through the use of several proprietary technologies, including the zero-mode waveguide, which allows for monitoring of the incorporation of individual nucleotides by a single DNA polymerase molecule (Fig. 5). The microfabricated zero-mode waveguide confines the single polymerase molecule and DNA template within a nanometer-scale hole in an aluminum film deposited on a silica substrate. There are 150,000 such wells in the SMRT cell. This well is smaller in all dimensions than the wavelength of the illuminating laser light, such that only light generated very close to the bottom of the well is detected, e.g., the light generated from the incorporation of a specific, modified dNTP. Thus, interference from background light and from unincorporated nucleotides is greatly reduced. Each of the four dNTPs is labeled with a different color fluorescent dye. Colocalization of the incorporating dNTP and the polymerase/template complex in the well results in readable fluorescence of a specific color attributable to the specific incoming base. Completion of the incorporation reaction results in cleavage of the fluorescence tag and its diffusion away from the polymerase such that it will not interfere with the detection of the next, incoming dNTP.

In the RS II system, DNA libraries are generated by fragmenting DNA to a chosen size between 2,000 and 40,000 bp, repairing the ends, and ligating on oligonucleotides to generate circular DNA molecules. The advantage of circular DNA templates is that by using a polymerase with the ability to both displace preexisting complementary DNA and synthesize a new strand of complementary DNA (displacement synthesis), the same sequence can be "read" many times, providing an error correction mechanism that can produce a sequence with 99.999% accuracy, which is higher than the previous "gold-standard" Sanger sequencing method. These circular sequencing templates (SMRT bells) are mixed with DNA polymerase in such a proportion that on average one DNA polymerase is bound to each DNA template. The polymerase/template mix is

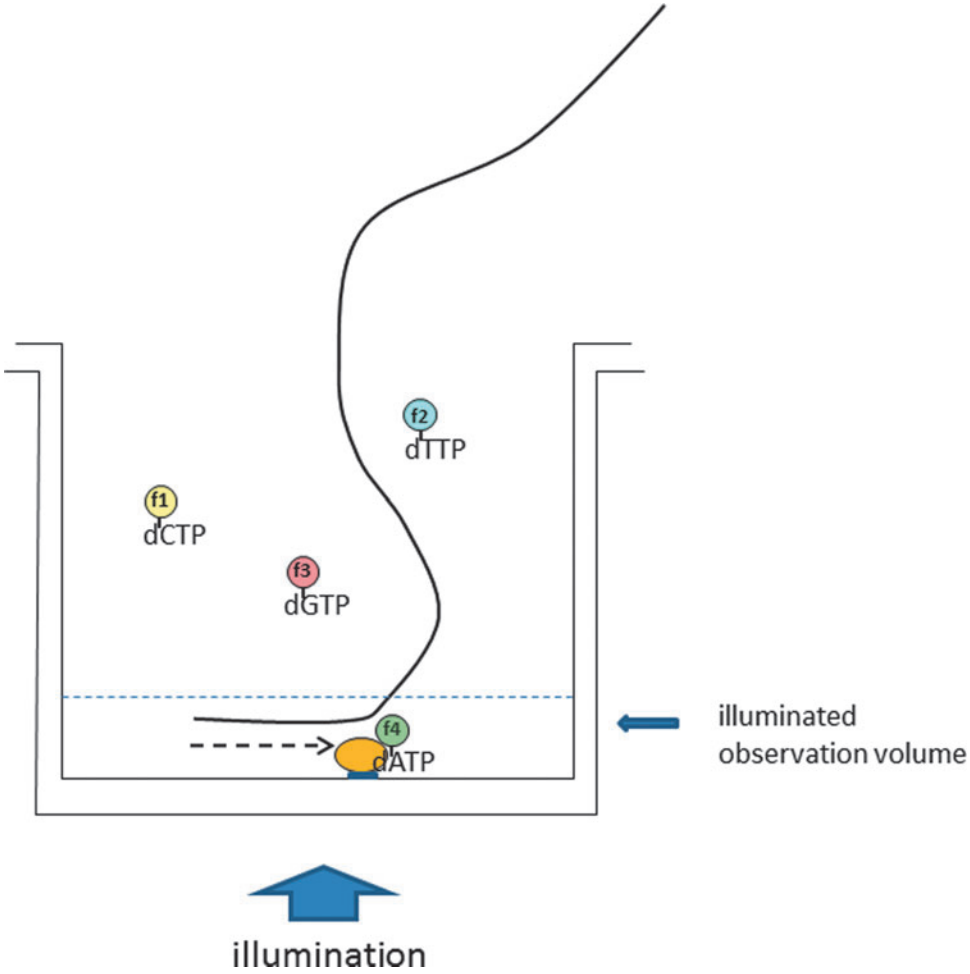

FIGURE 5 Single-molecule sequencing: the Pacific Biosciences system. Shown is a single, microfabricated nanowell with a light-transmitting bottom and with dimensions smaller than the wavelengths of light used to illuminate the well. This results in a "zero mode waveguide" with a narrow zone of illumination as shown. The nanowell has a single DNA polymerase molecule attached to its bottom within this zone. When overlaid with a solution of prepared DNA templates (also known as a library) at a suitable concentration, the polymerase is able to capture and bind a single primer-template molecule, as shown. Present in the solution are the four dNTPs, each labeled with a different color of fluorescent dye as shown and mostly outside the zone of illumination. Also shown is one of the four dNTPs bound to the polymerase-template-primer complex. This is the next base to be incorporated opposite its complementary base. The fluorescent tag is bound long enough for its presence and color to be detected. Subsequently, completion of the incorporation reaction results in cleavage of the fluorescence tag and its diffusion away from the polymerase such that it will not interfere with the detection of the next, incoming dNTP.

then loaded onto a SMRT cell in such a way that each well should contain on average one DNA template/one polymerase molecule. The polymerase is bound to the bottom of the well, and DNA sequencing is initiated by flowing the cell with the four fluorescent nucleotides and monitoring their incorporation as described above. Templates with, by chance, multiple bound polymerases give a "mixed" sequence read which can be discriminated from a valid read. When using passive loading of the polymerase/DNA template to the SMRT cell, the number of wells with a single (as opposed to multiple) polymerase/DNA template complex follows the Poisson distribution. This results in a maximum of about 50,000 wells with a readable DNA sequence. Systems for active loading of the SMRT cells are under development and will significantly increase the number of wells containing a single polymerase/DNA template complex. All 150,000 wells are followed in parallel to generate sequence reads of 2,000 to 40,000 nt in 0.5 to 2 hours.

In contrast to the other massively parallel sequencing technologies, the RS II technology can be used to sequence very long individual DNA strands (see discussion below). The reaction proceeds as long as there are nucleotides available and the DNA polymerase is active.

COMPARISON OF NGS PLATFORMS

The Illumina technology currently dominates the market. Illumina provides a range of instruments with different throughput, sequencing run times, and read lengths. The

larger Illumina instrument (HiSeq) runs flow cells containing eight independent lanes. An individual sample can be run on each lane in parallel with others. The Illumina system, since it does not use emulsion PCR and beads, has a less cumbersome and time-consuming protocol for preparing samples for the sequencing run than does Ion Torrent's system. Although the shearing, adapter ligating, and sizing of input DNA required for genomic library creation are a day's worth of intensive work for all the systems, the creation of a two-dimensional array of polonies takes less than 30 minutes of hands-on time in the Illumina system.

Illumina has recently introduced a novel development of its sequencing technology, initially only implemented in a system called HiSeq X Ten. The throughput of this instrumentation has been increased dramatically using dual-surface imaging (using both sides in a flow cell) enabled by a two-camera, four-sensor, epifluorescence system with a novel scanning technology. Also, the HiSeq X Ten uses a patterned flow cell with billions of ordered nanowells that allows the generation of sequencing clusters in an ordered arrangement, which increases the density of clusters and thus the sequence reads. Although initially dedicated to whole human genome DNA sequencing, this technology is likely to become available for a wider range of applications and enables terabases of DNA to be determined in a single instrument run.

The Ion Torrent/Ion Proton system has lower sequence throughput but is suited to projects in microbiology because of the rapid generation of data. Although the library preparation involves the somewhat cumbersome emulsion PCR, this can now be partly automated. The sequencing time is relatively short, involving only a couple of hours on the instrument, as opposed to days on the Illumina platform. Because the imaging systems of the Illumina system require a scanning microscope, completing the image takes considerable time per cycle. The entire time for a run on the larger HiSeq instruments amounts to between 1 and 6 days, depending on the throughput required. The smaller MiSeq has a run time of about 5 to 55 hours. The Ion system's read length is on par with the Illumina system's, up to about 400 bp per fragment. Methods for emulsion PCR-free library preparations are under way (Avalanche). One weakness of the Ion Torrent/Proton system has been the higher error rate in homopolymer nucleotide stretches. This has been largely corrected with successive updates in chemistry and software.

The Pacific Biosciences RS II system is unique in that it does not require amplification of the original DNA template. Prior amplification has a number of advantages, in particular, reducing the ambiguity and complexity of the sequence analysis, but it also may result in biases due to differences in amplification efficiency of individual targets. Also, without amplification, DNA modifications such as methylations, which would not be copied and would be lost in amplification, can be studied. This is because the average time it takes for incorporation of a base opposite to a modified base is significantly longer and can vary depending on the specific modification (22). It is also the only commercially available system that allows for very long DNA strands to be sequenced, dramatically extending the length of a sequence read beyond that obtained even by Sanger sequencing. Such long reads of up to 40,000 bases result in more efficient assembly of contigs both because of certainty of overlap and because of the ability to span short interspersed repeat sequences that otherwise prevent unambiguous sequence assembly. The simple procedure for library preparation and the very short time on the instrument also position the RS II system as overall the fastest sequencing technology for small or medium size genomes. This includes the faster turnaround time for the software to assemble microbial genomes. The drawback of the RS II system is the relatively low throughput (presently about 1 Gb per SMRT cell) and the per-nucleotide high error rate. The latter, however, can be handled effectively with sufficient coverage, since errors are random relative to nucleotide position and type. Also, improvement in loading efficiency and polymerase performance is projected to triple the sequence throughput in the coming year, making this technology also suitable for medium size genomes. See the section below on application of the RS II system to microbiology.

APPLICATION OF LONG-READ SEQUENCING TECHNOLOGY TO MICROBIOLOGY

The ability to exhaustively analyze nucleotide sequence content (base by base, single molecule by single molecule), even for sequences present in very low abundance, means that deep sequencing is an ideal tool for pathogen discovery and for evaluating the population structure of complicated mixtures of bacterial and viral organisms. The most economically feasible way to perform deep sequencing is by targeting subgenomic sequence elements, such as 16S ribosomal DNA. 16S ribosomal DNA is highly conserved among species in general, allowing it to be targeted, yet variable enough that it can distinguish even closely related species. However, the throughput and read lengths of NGS have increased to the point where the assembly of viruses, plasmids, and even whole bacterial genomes from complex populations of genomes has become feasible. Here and in general, deep sequencing has used computational algorithms and electronic subtraction to make sense of the massive amounts of data generated by each run. Such computer-intensive approaches represent the general trajectory of this technology in the future.

The Pacific Biosciences RS II long-read sequencing technology provides unique opportunities for generating complete genome sequences with no gaps. The sequence throughput per SMRT cell is ideal for sequencing microbial genomes with sufficient coverage. Also, the technology has a short turnaround time, making it suitable for clinical applications. For example, sequencing of the complete genome of multiresistant bacteria can be performed in 2 days, from DNA extraction to delivery of a complete microbial genome (Ulf Gyllensten, unpublished). Also, the long sequence read length provides advantages in metagenomic sequencing, since individual reads can encompass the entire genomic sequence of viruses or plasmids, increasing the accuracy of a genome assembly. An example of the application of this technology is the study by Conlan et al. (23). They isolated a repertoire of carbapenemase-encoding *Enterobacteriaceae*, including *Klebsiella pneumoniae*, *Klebsiella oxytoca*, *Escherichia coli*, *Enterobacter cloacae*, *Citrobacter freundii*, and *Pantoea* species. Long-read genome sequencing using the RS II system resulted in full genome, end-to-end assembly and revealed that these organisms carry the carbapenem resistance genes on a wide array of different plasmids. They found evidence of horizontal transfer of carbapenemase-encoding plasmids between *K. pneumoniae*, *E. cloacae*, and *C. freundii* in the hospital environment. By enabling full plasmid identification, their

data challenge assumptions about horizontal gene transfer events within patients and identify possible connections between patients and the hospital environment. In addition, they identified a new carbapenemase-encoding plasmid of potentially high clinical impact carried by *K. pneumoniae*, *E. coli*, *E. cloacae*, and *Pantoea* species in unrelated patients and in the hospital environment. Because plasmid sequences contain complexities such as repeats and mobile genetic elements, previous attempts to sequence them with short-read technology have not been able to discriminate and resolve plasmid from chromosomal genes.

CONCLUDING REMARKS

Massively parallel sequencing technologies have continued to make remarkable advances. The cost of sequencing has come down steadily and at a faster rate than Moore's law, which is used to predict the cost versus performance of computer chips and which promises a doubling in speed at the same cost every two years. The complexity of the technology has also been reduced, making it available to individual laboratories as well as core facilities. In addition, advances in computational support have made the systems faster and easier to use. The systems have also been made easier to use by integrating sample preparation with the automated steps of the sequencing reactions themselves. A major breakthrough has been the emergence of technologies that enable very long, contiguous stretches of DNA to be read. At present only one such technology, that of Pacific Biosciences, is available, but several others are in development and are likely to come to fruition in coming years. A promising development is the use of molecular (or nano-) pores for sequencing. This technology infers a DNA sequence based on the rate of passage of individual or groups of nucleotides as the whole DNA molecule passes through the pore. In principle such a sequencing technology could be much faster, simpler, and more economical than current NGS. One implementation of nanopore sequencing (Oxford Nanopore) has been made available for early testing, but initial results indicate that it requires further improvement. Overall, given the rapid rate of technology development and the decrease in cost and time, massively parallel sequencing is likely to eventually become the method of first choice for accurate identification of bacterial and viral infections both in experimental science and clinical microbiology, replacing most of the current laboratory methods.

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Next-Generation Sequencing

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6

Next-generation sequencing (NGS), otherwise known as deep or massively parallel sequencing, refers to the technological advances in DNA sequencing instrumentation that enable the generation of hundreds of thousands to millions of sequence reads per run. Sequencing of the human genome, which was once a >10-year endeavor by the NIH at the cost of approximately $3 billion (1), can now be done routinely on a single instrument. Rapid advances in technology led to the first-ever FDA clearance of an NGS instrument, the Illumina MiSeq, in 2014 (2), and the development of rapid, miniaturized sequencing devices such as the Oxford Nanopore are ongoing (3). The applications of NGS are wide-ranging and include (i) whole-genome sequencing, (ii) pathogen discovery, (iii) metagenomic/microbiome analyses, (iv) transcriptome profiling, and (vi) infectious disease diagnosis. Here we will focus on NGS technology and the last three applications, because the first two topics are described in detail elsewhere.

OVERVIEW OF NEXT-GENERATION SEQUENCING METHODS

Prior to the 1980s, Sanger sequencing, based on slab or capillary gel electrophoresis of individual DNA fragments (4), was the only available sequencing technology. The technique was laborious, with a turnaround time of 6 to 24 h, and capacity was limited to the sequencing of fragments in 96 or 384 microtiter wells at a time. The approach taken by NGS technologies, on the other hand, is based on preparation of a "library" of DNA fragments to be sequenced (5). The library is typically produced by the clonal amplification of millions of amplified DNA templates at a time, followed by some method to determine the sequences in a massively parallel fashion. The first available NGS system was the Roche 454 pyrosequencing instrument (6), followed by the emergence of "second-generation" systems (7, 8), including the Illumina (formerly known as Solexa) HiSeq/MiSeq/NextSeq, ABI SOLiD, Life Technologies Ion Torrent, and the PacBio RX system. Currently, the Illumina instruments are used in most published NGS studies, including those in the microbiological field, although new "third-generation" platforms, such as those based on nanopore sequencing (9), are now available and being increasingly used.

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Roche 454 Pyrosequencing and SOLiD Sequencing

Both the Roche 454 instrument and SOLiD systems isolate and amplify single DNA molecules to construct a library for sequencing by a process known as emulsion PCR (Fig. 1A) (10). Emulsification of an oil-water interface leads to the formation of droplets, with each droplet, referred to as a microreactor, containing a bead that is covalently bound to a single DNA template. PCR amplification is then performed across the surface of the bead to generate clonally amplified fragments. For Roche 454 pyrosequencing, the beads are then deposited into individual wells on picotiter plates, and sequencing reagents containing DNA polymerase are added into the wells (Fig. 1A, left). As the complementary strand is synthesized by nucleotide incorporation, pyrophosphate release produces a fluorescent signal that can be recorded by a CCD (charge coupled device) camera for base calling. For SOLiD, after emulsion PCR, the 3′ ends of the DNA template on the bead are modified to permit chemical linkage to the surface of a glass slide (Fig. 1A, middle). When sequencing reagents containing DNA ligase are flowed over the slide, a fluorescent signal is generated that is captured by a CCD camera for base calling. Roche 454 pyrosequencing is classified as sequencing-by-synthesis, because the sequence is being read concurrently with synthesis of the complementary strand by incorporation of fluorescent-labeled nucleotides (11), whereas SOLiD sequencing is classified as sequencing-by-ligation, because sequencing is determined according to the selective mismatch sensitivity of DNA ligase to fluorescently labeled probes (12).

Ion Torrent Sequencing

For the Ion Torrent, which similar to the Roche 454 uses a sequencing-by-synthesis approach, a semiconductor chip is used to detect hydrogen ions released during DNA polymerization (Fig. 1A, right). A library is prepared by emulsion PCR, and amplified fragments are coupled to beads that are individually deposited in sequencing wells. Nucleotides are then added to the chip, with each of the four bases (A, C, T, and G) being introduced one at a time in a predetermined order. As each nucleotide is incorporated during strand synthesis, a hydrogen ion is released that alters the pH value. Changes in pH are converted and measured in voltage values, which are directly proportional to the number of nucleotides that are incorporated during each cycle.

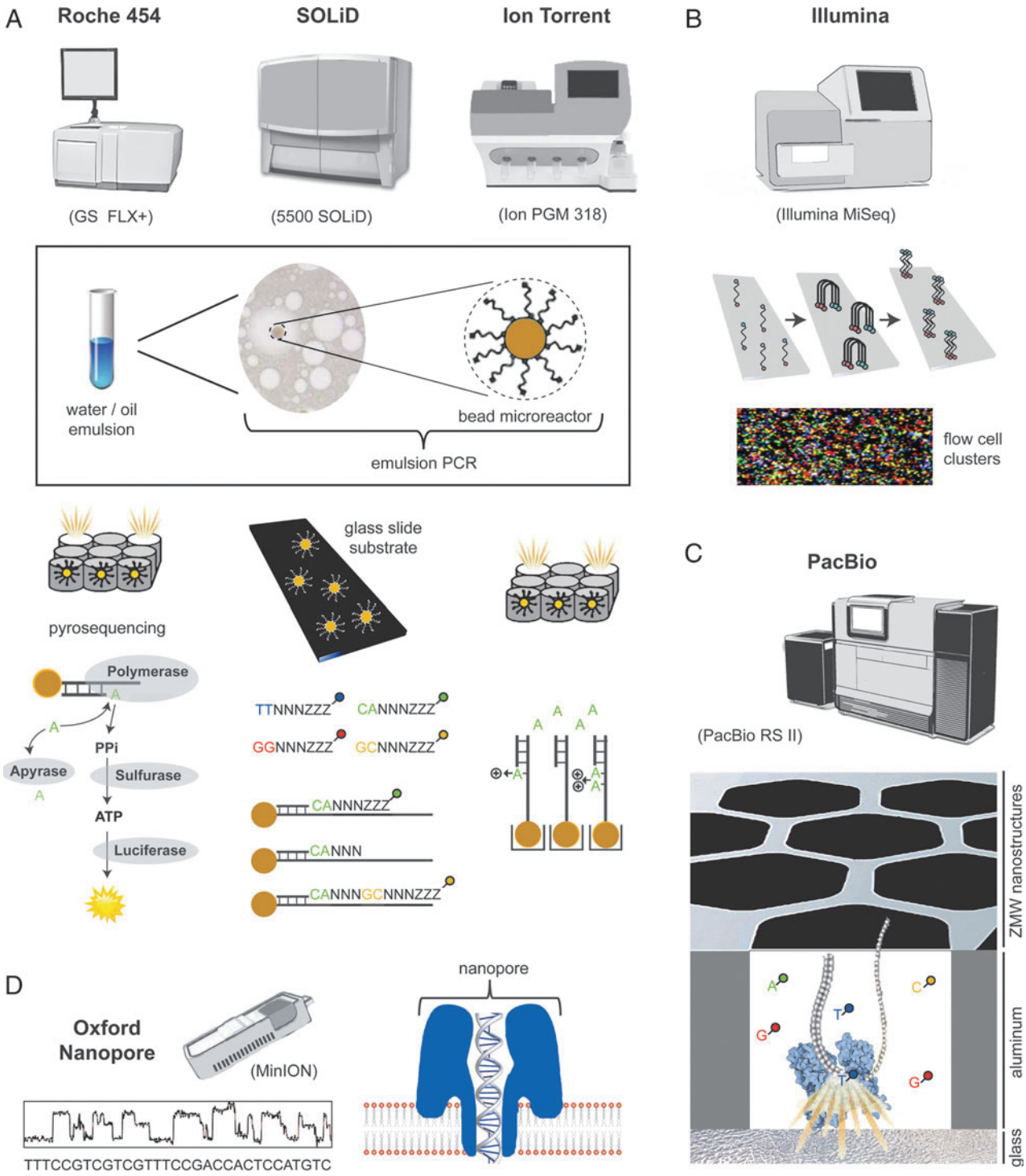

FIGURE 1 Sequencing methods for currently available NGS platforms. (A) The sequencers manufactured by Roche/454 (left), SOLiD (middle), and Ion Torrent (right) all use bead-based emulsion PCR (rectangular inset) in the library generation process, followed by different approaches to fluorescent-based sequencing. (B) Illumina sequencing involves library generation on a flow cell via a sequencing-by-synthesis approach and the imaging of millions of fluorescent flow cell clusters. (C) PacBio sequencing is performed by a DNA polymerase enzyme affixed to a glass substrate in a zero-mode waveguide nanostructure. Each nanostructure generates an individual sequence. (D) Nanopore sequencing, as performed by the Oxford Nanopore MinION instrument, leverages the voltage conductance changes (left) that occur in response to passage of DNA through a nanopore (right), a protein in the lipid-bilayer membrane containing a single hole that allows a single molecule of DNA to pass through.

Illumina Sequencing

The library preparation is simpler than emulsion PCR for Illumina sequencing (Fig. 1B). Two unique primers (adaptors) are attached to the ends of each DNA fragment by ligation, PCR, or transposon switching (Nextera technology) and then affixed to the surface of a flow cell in the form of hairpin loops. "Bridge amplification" using PCR is then performed on the flow cell surface by denaturing the 3′ end of the DNA fragment and replicating the complementary strand. Successive rounds of replication and denaturation by PCR thermocycling result in the generation of thousands of copies of clonally amplified fragments in a tightly circumscribed cluster. Sequencing reagents, including DNA polymerase and a sequencing primer, are then passaged across the flow cell. For each cycle, a single fluorescently labeled nucleotide containing a reversible terminator is added to the complementary strand within each individual cluster in a sequencing-by-synthesis approach. After CCD imaging, cleavage of the fluorescent label permits the next nucleotide to be added. The number of cycles producing the final read length is specified in advance, and sequencing can also be done from both ends (paired-end sequencing) using a second primer to the newly synthesized DNA strand.

PacBio Sequencing

In PacBio single-molecule real-time sequencing technology, individual molecules of DNA template are affixed to the bottom surface of the chip in an optical waveguide called a zero-mode waveguide (Fig. 1C). The zero-mode waveguide creates an illuminated volume within which to observe the incorporation of single nucleotides of DNA. The four nucleotide bases are labeled with different fluorescent dyes and added simultaneously to synthesize the complementary DNA strand. During nucleotide incorporation, the fluorescent tag is cleaved off and a base call is made according to the corresponding fluorescence of the dye (sequencing-by-synthesis). Each single-molecule real-time cell contains approximately 150,000 zero-mode waveguides (13).

Nanopore Sequencing

The sequencers manufactured by Oxford Nanopore use arrays of specialized nanopores that allow a single DNA molecule to pass through at a typical rate of 30 bases per second (range of 0 to 250 bases per second) (14). Current versions of the Oxford Nanopore MinION sequencer, a miniaturized device about the size of a USB stick, contain arrays of 512 nanopores (15), although greater capacity can be achieved with instruments in development including the GridION and PromethION. The current passing through the pores changes in response to the different nucleotide bases as they pass through (Fig. 1D), allowing the sequence to be determined without synthesis, ligation, or other enzymatic steps. Library preparation is simple, but the rate at which a nanopore can capture and sequence a diffusing DNA molecule is limited by concentration, thus requiring relatively high input concentrations of target DNA. At present, error rates in practice are high compared to the more mature sequencing-by-synthesis methods (20 to 40%) (16), but quality consensus sequences can be generated given adequate sequencing coverage.

DIFFERENCES BETWEEN NGS PLATFORMS

Table 1 shows a comparison of the different NGS technologies. The choice of which NGS platform is best suited for any particular application depends on a number of factors including cost, sequencing read lengths, sequencing depth (number of reads per clinical sample) and coverage, and sequencing quality.

Cost

The costs of sequencing have decreased significantly in recent years. Nevertheless, an NGS run is still typically at least an order of magnitude more expensive than that of conventional microbiological assays. Often samples must be individually barcoded and pooled into single runs to decrease costs. As the costs continue to decrease, however, increasing consideration should be given to the cost attractiveness of NGS relative to other recent technologies such as mass spectrometry and microarrays.

Read Length

Longer read lengths are more desirable than shorter read lengths for many applications. For example, in pathogen discovery, longer read lengths facilitate detection of sequences from highly divergent microorganisms such as novel emerging viruses that may be only identifiable on the basis of weak homology in their translated amino acid, rather than nucleotide sequence (17). For metagenomic sequencing, longer reads can also be more accurately classified according to their origin (e.g., human, virus, bacteria, fungus, or parasite), because they are more likely to be uniquely identifying than shorter reads. In addition, longer reads can provide genomic scaffolds that are critical in the *de novo* assembly (joining together of individual reads on the basis of overlapping sequences) of novel microbial genomes for which there is no closely related reference in the database (18). Indeed, many *de novo* assembly approaches combine two technologies: one technology that employs longer but fewer reads (e.g., PacBio, Oxford Nanopore) to enable genomic scaffolding and another technology that employs many more short reads (e.g., Illumina) that can be subsequently mapped onto those scaffolds (3, 19).

Sequencing Depth and Coverage

Sequencing depth and coverage are important parameters for many NGS applications. As a rule of thumb, at least 20× coverage of the genome is generally thought to be needed for accurate *de novo* assembly of a novel organism from short NGS reads (20). In metagenomic "needle-in-a-haystack" applications, a minimum sequencing depth is needed to detect sequences from a target pathogen with high sensitivity amidst a large number of human or animal host background reads (21). The required depth depends on the relative copy number of microbial versus host nucleic acid in the library, with acellular fluids such as serum/plasma, cerebrospinal fluid, and respiratory secretions typically requiring much less sequencing depth at a given level of sensitivity than tissue samples, for which host background sequences are predominant.

Sequencing Quality

Some technologies, such as Roche 454 and Ion Torrent, have difficulty sequencing long homopolymers (22, 23). Other technologies, such as PacBio and Oxford Nanopore, have inherently low individual sequence quality. The low, per-read sequencing quality can be compensated for in PacBio by resequencing the same fragment multiple times

TABLE 1 Comparison of NGS platforms

Platform	Sequencing method	Instrument	Typical read lengths	Accuracy	Throughput (reads per run)	Run time	Instrument cost	Sequencing cost	Key advantages	Key disadvantages
454 Roche	Pyrosequencing	GS FLX+	Up to 700 bp	99.9%	Up to 1 million	20 h	++	+++	Long reads; fast run times	Low throughput; homopolymer errors
SOLiD	Sequencing by ligation	5500 SOLiD	35–50 bp	99.9%	1.0–1.5 billion	1–2 weeks	++++	+	Low cost per base	Very short reads; slow
Ion Torrent	Ion semiconductor	Ion PGM 318	100–200 bp	98.0%	4–5.5 million	2 h	++	++	Fast run times	Homopolymer errors
		Ion Proton I	200–400 bp	98.0%	60–80 million	8 h	+++	++	Fast run times	Homopolymer errors
Illumina	Sequencing by synthesis	HiSeq 2500	50–300 bp	98.0%	0.6–4 billion[b]	6 h to 11 days[b]	++++	+	Highest yield; low cost per base	Instrumentation expensive
		MiSeq	50–300 bp	98.0%	20–30 million	6–40 h	++	+	FDA cleared; low cost per base	Lower throughput
		NextSeq	50–300 bp	98.0%	Up to 800 billion	6–40 h	+++	+	Intermediate yields; low cost per base	Lower throughput
PacBio	Single-molecule real-time (SMRT)	PacBio RSII	10–15 kb	87% or >99.9%[c]	50,000	2 h	++++	+	Long read	Instrumentation expensive
Oxford Nanopore	Nanopore sequencing	MinION	100 bp–10 kb	60–80% or >99%[c]	10,000–50,000	6 h[d]	+	?	Real-time sequencing; portable; long reads	High error rate; low throughput
Sanger[a]	Chain terminator	3730xl	400–900 bp	99.9%	N/A	2 h	++	++++	Long reads; fast run times	Lowest throughput

[a]Not an NGS method; included for purposes of comparison.
[b]Dependent on whether run is rapid-mode or standard-mode.
[c]Individual read or consensus read accuracy.
[d]Can be run until sufficient data are collected; lifetime of flow cell currently 24 to 48 h.

to generate consensus reads (24), while Oxford Nanopore relies on having redundant coverage to compensate for the high error rates (75). The sequencing quality can also vary with length. For example, the quality of Illumina reads deteriorate gradually toward the end of the read (25).

OTHER NGS CONSIDERATIONS

Sample Selection

The NGS approach in microbiology is compatible with a wide range of samples, including clinical human, animal, and even environmental samples, and the choice of sample type is highly dependent on availability and the desired application. When applying unbiased metagenomic techniques that do not rely on specific primers or probes, acellular fluids are preferable to tissues because they have much less host background (21). Metagenomic detection of pathogens is generally less sensitive in whole blood, for example, than in acellular serum or plasma samples. When available, freshly frozen samples are generally superior in quality for NGS applications than formalin-fixed, paraffin-embedded samples or samples allowed to sit at room temperature or 4°C, due to the risk of nucleic acid degradation (26). For applications involving labile RNA such as detection of RNA viruses or transcriptome profiling of mRNA, the use of stabilization reagents at initial sample collection (e.g., PaxGene tubes) should be considered (27). For applications such as infectious disease diagnostics, analysis of more sterile samples such as blood or cerebrospinal fluid is preferred given the increased likelihood of finding a sole causative agent (17), as well as the difficulty in bioinformatics analysis and interpretation of more complex, "environmental" microbial samples such as stool (21). On the other hand, metagenomic and microbiome analyses typically require the presence of a diverse polymicrobial community, such as those found in stool or respiratory secretions. These analyses may not be meaningful for more sterile samples such as blood or cerebrospinal fluid, for which a virome (28), but probably not bacteriome, exists in the healthy state.

For diagnostic NGS, several other considerations need to be taken into account. Collection of noninvasive samples (e.g., sweat, saliva, stool, and urine) is easier than collection of blood or tissue biopsy samples. However, any detected association with NGS is much stronger if made from invasive samples such as tissue biopsy, especially if there is concurrent pathology such as inflammation. Another key consideration is whether to focus on sequencing of library DNA or cDNA generated from RNA. RNA-based NGS is obviously required for RNA virus detection or mRNA transcriptome profiling. For bacterial, fungal, or parasitic identification by 16S/18S rRNA (see below) (29), it may also be preferable to detect transcribed rRNA molecules rather than the rRNA genes to maximize sensitivity, because 10^4 to 10^5 rRNA molecules can be present per microbial cell versus only 1 to 10 copies of the rRNA gene (30). It is also worth noting that RNA-based NGS detection is still capable of detecting DNA-based organisms such as DNA viruses and bacteria by detection of their corresponding host or pathogen mRNA transcripts, respectively. However, RNA is significantly more labile than DNA, and NGS libraries constructed from RNA are also more prone to contamination from exogenous bacterial rRNA from laboratory reagents and the environment (31), which can confound interpretation of the sequencing results.

Disease and Host

Most NGS applications in microbiology are based on direct detection and/or sequencing of microbes. Thus, acute diseases such as febrile illness, which can be associated with high titers of the causative agent (32), are generally more amenable to NGS analysis than chronic diseases. In chronic diseases such as cancer or chronic autoimmune disease, NGS for pathogen detection and discovery relies on the infectious agent still being present at detectable levels in tissue at the time of clinical sample collection. NGS studies in animals can also be more problematic than those in humans, especially if the genome of the animal or a close relative has not yet been sequenced, precluding computational host subtraction approaches to simplify the data analysis (see below).

Sample Preparation Methods

Clinical and environmental samples for NGS are prepared in a series of steps beginning with nucleic acid extraction followed by library preparation (+/− sample barcoding) and loading onto the instrument for sequencing (Fig. 2, left). Initial sample preparation and nucleic acid extraction methods vary depending on the assay type, sample matrix, and pathogen type being detected. Methods to reduce host background or enrich for microbial sequences include ultracentrifugation, nuclease treatment (either pre- or postextraction), and probe-based enrichment. Ultracentrifugation allows for enrichment of virus-like particles, enhancing viral detection (33, 34). Treatment with DNase or RNase will enrich for RNA or DNA targets, respectively, and can substantially reduce host background (33, 34). Probe-based enrichment can be performed using a panel of targets to recover specific organisms from low-titer samples (35).

Sample Barcoding

To multiplex analysis of specimens in a single NGS assay, each sample can be barcoded by adding a short oligonucleotide tag 6 to 12 base pairs (bp) in length to each end of the DNA molecule. Barcoded libraries are then mixed and sequences classified bioinformatically based on the sequenced barcode. To reduce barcode switching, the barcodes are designed to be different by more than one base pair change in a single sequencing run. The use of Hamming code-based designs can preserve minimal distance (in number of base pair changes) between barcodes, and also enable error correction (36). Separate barcodes can be attached to each end of the sequencing target (dual-index barcoding), and barcodes can be rotated over time, reducing the risk of carryover contamination.

Library Preparation Methods

Once the samples have been prepared and nucleic acid extracted, the library is constructed. Each instrument method requires an optimal input amount, which can be generated by preamplification. The final library is generated using emulsion PCR or sequencing adaptor ligation specific to each method (Fig. 1). Library quality control is performed by determining the concentration and size distribution using capillary electrophoresis or real-time PCR. Individually prepared libraries with different barcodes can be pooled for sequencing on a single run, depending on the desired number of sequences per sample.

Contamination

Due to the high sensitivity offered by sequencing large numbers of reads, NGS approaches are extremely vulnerable to

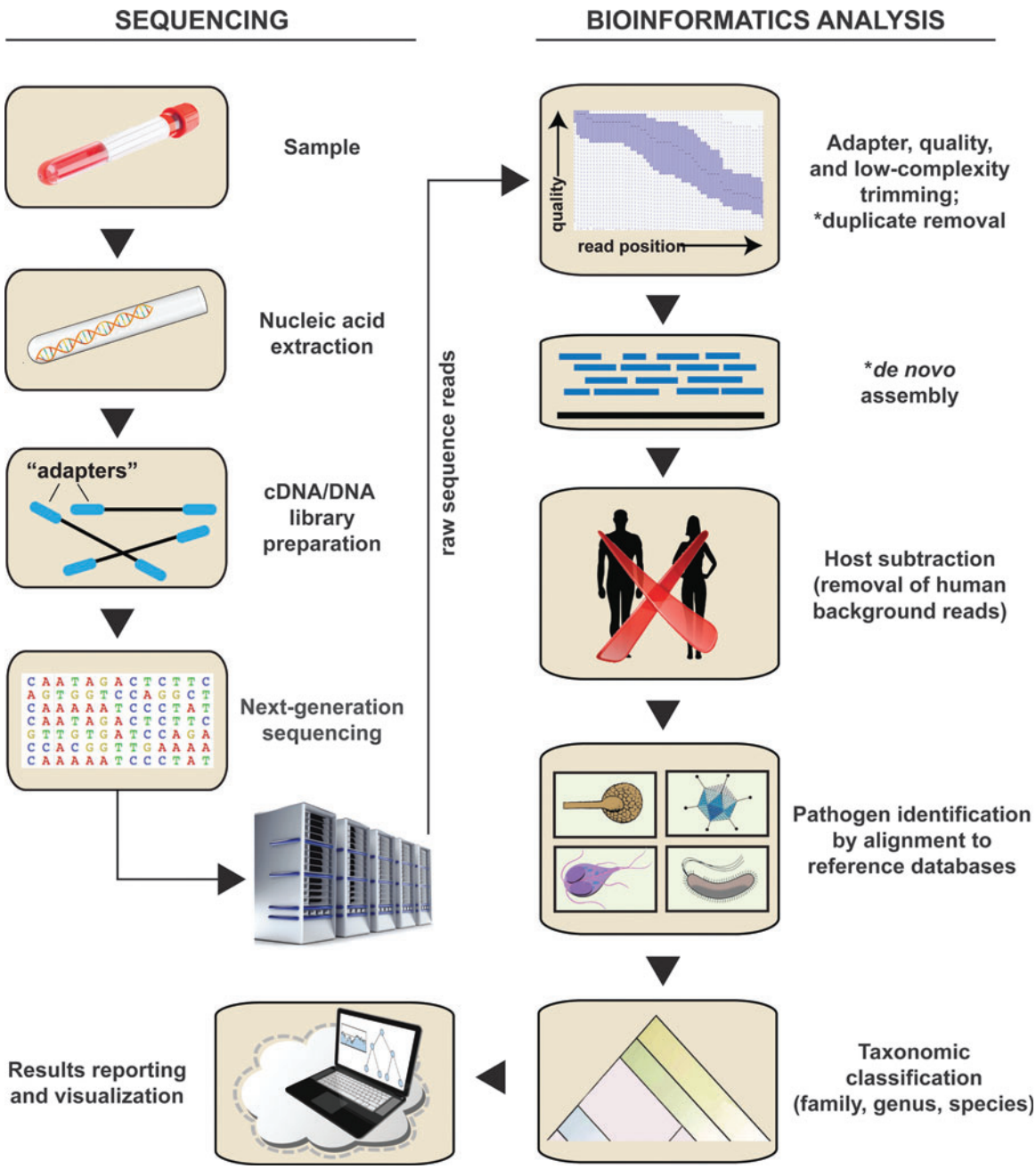

FIGURE 2 Schematic overview of an NGS pipeline. Sample processing for NGS involves a stepwise process of nucleic acid extraction, library preparation, and sequencing on a dedicated instrument (left). Following generation of raw data, bioinformatics analysis of metagenomic or microbial NGS data includes preprocessing, *de novo* assembly, host subtraction, pathogen identification, taxonomic classification, and results reporting/visualization (right). The asterisks denote optional steps in the procedure.

contamination (31). There are multiple points where contaminating organisms or nucleic acid may be introduced into the system, including sample collection, sample processing, and on the sequencing instrument. NGS traditionally requires handling of libraries in an open environment with multiple steps, so amplified material may cross-contaminate samples prepared simultaneously or during subsequent sequencing runs. Reagents used for NGS analysis may be contaminated with microbial nucleic acid, because it is difficult to completely remove DNA from recombinant enzymes. Even commonly used supplies can harbor microbial contamination, such as silica-based DNA purification columns containing what is now thought to be an algal virus (37). Instrument carryover can also occur and is seen both within a run and between runs. Finally, cross-contamination of barcoded samples that are multiplexed in a single run can occur, especially if an individual sample contains a high titer of a specific microbial agent, reads from which can "spill over" into adjacent barcoded samples. This can be mitigated, but perhaps not eliminated entirely, with the use of dual-indexed barcodes at both ends of library amplicons. Thus, careful handling, unidirectional

sample flow, proper quality control, and careful measurements of levels of background contamination are necessary to reduce the risk of false-positive identifications using NGS.

Quality control of NGS reagents is yet another key step to minimize false-positive identifications, particularly with low-input samples having minimal titers of target nucleic acid. Despite efforts to produce ultra-pure reagents (38), there will likely never be assurances that reagents are truly nucleic acid–free. Thus, each new lot of reagents should always be tested with negative controls, and laboratories need to understand the expected frequency and distribution of reagent-derived contaminating sequences and establish appropriate threshold levels of detection to avoid false-positive calls. Common laboratory water supplies often contain bacterial DNA from organisms such as environmental *Burkholderia* and *Ralstonia* species (39, 40), making it difficult to distinguish a true positive identification from background levels of contamination. Also, aerosolized nucleic acid has the potential to contaminate sample hoods and can become a major component of libraries prepared in the hood, requiring extensive cleaning. For certain NGS applications such as pathogen discovery and clinical detection of unusual or unexpected agents, it may be desirable to confirm the results using different extraction methods or reagents or even running the NGS assay in separate laboratories. Confirmation using an orthogonal method such as specific PCR testing from the original sample may also be necessary to exclude the possibility of contamination.

NGS BIOINFORMATICS WORKFLOWS

The sheer number of NGS reads generated by existing instruments and rapid increases in sequencing capacity pose a major computational challenge for analysis of NGS data. A number of bioinformatics software choices are now available, both commercial and open source. For the most part, some degree of computational expertise is needed to take full advantage of these algorithms and workflows, although user-friendly options for NGS analysis exist, such as Geneious (41) and Galaxy (42). Although the details can vary significantly, a computational pipeline for processing and analyzing NGS data follows a general schema (Fig. 2, right). First, sequencing reads are preprocessed by trimming of adapter, low-quality, and low-complexity sequences, with optional removal of duplicate reads. With the exception of host transcriptome profiling using RNA-Seq (43, 44), which deals with alignment and classification of human mRNA genes and isoforms (see below), the next step is to computationally subtract background host sequences (45). For human clinical samples, NGS reads are aligned to the human genome and then removed from the dataset, which decreases the number of remaining reads that need to be analyzed using more computationally intensive downstream algorithms. Next, microbial sequences are identified by alignment to pathogen-specific reference databases such as the National Center for Biotechnology Information (NCBI) bacterial or viral RefSeq databases. Specialized applications such as 16S rRNA sequencing for microbiome analysis classify reads on the basis of alignments to the rRNA gene sequences in the Ribosomal Database Project database (46). Recent advances in the speed and efficiency of alignment algorithms have even made simultaneous alignment to all nucleotide sequences in the NCBI nucleotide (NT) database, including all of GenBank NT (~160 gigabases of sequence as of February 2014; ftp://ftp.ncbi.nlm.nih.gov/genbank/release.notes/), computationally feasible (21).

In addition to sequence alignment, either *de novo*, seed-based, or mapped (using a discrete reference) assembly can be performed to join NGS reads together into contiguous sequences (contigs) and recover partial or even full genomes (47). With metagenomic data, the use of an ensemble method that partitions the data beforehand and combines the use of multiple assembly algorithms may be preferable to maximize contig lengths (48). Translated nucleotide alignment to a protein database or remote homology detection using hidden Markov models (49) can be useful in identifying sequences corresponding to highly divergent pathogens, such as novel viruses. Finally, for NGS applications such as infectious disease diagnosis, precise taxonomic classification of reads to the species level is a necessary step in the analysis (50–52). For example, it is often clinically relevant to be able to distinguish *Staphylococcus* species (e.g., *Staphylococcus aureus* versus coagulase-negative staphylococci) or influenza subtypes (e.g., influenza A [H3N2] versus 2009 pandemic influenza A [H1N1]).

Especially for clinical applications, the development of visualization tools and cloud-computing-compatible platforms will be critical in providing interpretation and context to the NGS data analysis. Software that is user-friendly and produces results that are understandable by microbiologists who lack bioinformatics expertise is greatly needed to enable communication of accurate NGS results to clinicians. A key aspect of NGS for clinical microbiology laboratories will also be not only standardization of the bioinformatics analysis workflows but also standardization of the reference databases. There is currently no consensus as to what standard reference databases will be needed for microbial NGS applications and who would be responsible for developing and maintaining such a database. Nevertheless, working groups consisting of the FDA, NCBI, CDC, and other institutions have been formed to discuss and implement standardized microbial reference databases for NGS (75) as part of a larger effort to ensure the quality of next-generation sequencing in clinical laboratory practice (53).

NGS APPLICATIONS

Amplicon Sequencing

NGS is suitable for sequencing of PCR amplicons in a massively parallel fashion. Applications include determination of minority sequence variants or viral quasispecies and targeted metagenomic analysis. NGS analysis of specific amplicons can deconvolve multiple species in mixed infections, allowing each component to be recognized, whereas Sanger sequencing requires the majority sequence to comprise at least 75% of the total.

Universal Bacterial Identification by 16S PCR

Although the 16S small rRNA gene is found in all bacteria and is highly conserved, the presence of hypervariable regions in the gene sequence allows it to be useful for specific diagnostic identification to the genus and even species level (54). The 16S rRNA gene is 1.5 kB in length and consists of nine hypervariable regions flanked by highly conserved regions. Universal bacterial primers targeting

the conserved regions enable amplification and subsequent sequencing of the hypervariable regions.

A clinically validated assay based on 16S rRNA PCR followed by NGS has been shown to be useful for universal diagnostic identification of bacterial pathogens directly from clinical samples (55). This approach has the advantage of not relying on "gold-standard" culture-based identification, which requires that organisms are capable of growing and replicating *in vitro*. Such an assay based on 16S rRNA PCR would be able to detect fastidious or slow-growing organisms or those rendered nonviable by prior antibiotic treatment or processing (e.g., formalin-fixed paraffin-embedded tissue samples). With the sequencing depth provided by NGS, the presence of even low-titer microorganisms in a highly diverse, polymicrobial sample can potentially be identified. The 16S rRNA gene is also used in most environmental metagenomic studies (56), because it can reveal the phylogenetic relationships among complex bacterial populations at very high resolution. Other targets in bacteria that have been used for these applications include the 23S gene and the intergenic spacer region located between 16S and 23S (57).

UNIVERSAL EUKARYOTIC IDENTIFICATION BY 18S AND/OR ITS PCR

Analogous to the 16S rRNA gene in bacteria, eukaryotic microorganisms that lack a backbone (nonchordate eukaryotes) such as fungi and parasites are identifiable on the basis of 18S or 28S rRNA sequences (58). For fungi, the internal transcribed spacer (ITS) regions can also be used. The hypervariable regions within these sequences can be used to classify fungi and parasites to the species level, and NGS can be readily used for metagenomic analysis as well as provide high sensitivity for detecting low-titer organisms in mixed infections. Because the 18S and 28S rRNA genes are also found in high-order eukaryotes such as animals and humans, inadvertent host background amplification can be significant, generally requiring higher sequencing depths for successful microbial identification.

Pathogen versus Commensal

Many microorganisms are commensals that colonize various body niches of their host and are only associated with disease in the setting of invasion. For instance, fungi such as *Malassezia* spp. and bacteria such as *Staphylococcus* spp. and *Propionibacterium acnes* colonize the skin of healthy adults (59). Therefore, the presence of microbial sequences from nonsterile body sites needs to be interpreted in the context of the infectious disease being studied. A positive detection from a sterile body site is more likely to be associated with true infection but requires differentiation from potential contamination. Also, microbial nucleic acid does not necessarily indicate the presence of live microorganisms but could simply indicate prior colonization. Assessment of the patient's symptoms and clinical presentation, along with the sequencing results, is necessary to determine the pathogenic significance of any microorganisms detected by NGS analysis.

METAGENOMIC AND MICROBIOME ANALYSES

Metagenomic sequencing is targeted (e.g., 16S) or shotgun sequencing of clinical or environmental samples and is now being largely performed by NGS given the depth of coverage that can be achieved. The microbiome, the totality of microorganisms that reside in diverse niches of the human body (60), can be assayed using metagenomic sequencing. The Human Microbiome Project, started in 2008, used 16S sequencing to profile microbial communities at different body sites and thus characterize the baseline microbiome responsible for the maintenance of human health (61). 16S metagenomic or microbiome sequencing can now be routinely performed using customized workflows such as QIIME to classify reads into operational taxonomic units and assess sample diversity (62). Similarly, 18S/ITS or shotgun metagenomic sequencing can be done to analyze fungi for high-resolution species identification and overall profiling of complex microbial communities.

TRANSCRIPTOME PROFILING

Transcriptome profiling by NGS, otherwise known as RNA-Seq, has many applications to microbiology. Transcriptome profiling by NGS is the sequencing of all of the mRNA molecules from either the host or the microorganism to obtain a global view of the gene expression pattern in a clinical sample (43, 44). For full coverage of the human transcriptome, approximately 30 to 50 million short reads are needed. Previously, only microarrays were available to conduct comprehensive gene expression analyses. By transcriptome analyses of the human host response to infection, microarray-based methods have proven effective in the diagnosis of staphylococcal bacteremia (63), active versus latent tuberculosis (64), and acute respiratory infections such as influenza (65). RNA-Seq using NGS has been shown to be more sensitive for detection of low-abundance transcripts, with a broader dynamic range in detecting fold-changes in gene expression at the cost of greater complexity of analysis and current lack of standardization (66, 67).

Transcriptome profiling of the microorganism is also possible, either in pure experimental cultures *in vitro* or directly from clinical samples (68). The data from mRNA gene expression is compared to that from the DNA genome. Microbial transcriptional profiling may yield insights into the overall activity of the organisms (latent versus active metabolism), growth characteristics (aerobic versus anaerobic growth), or expression of resistance and virulence elements.

INFECTIOUS DISEASE DIAGNOSTICS

There is much excitement about the potential of NGS to cause a paradigm shift in microbiology by complementing or even replacing existing diagnostic tests in the clinical laboratory. Metagenomic NGS in particular is promising for diagnosis because this unbiased approach does not target any individual microbial agent but, rather, identifies any and all potential pathogens simultaneously on the basis of sequence homology (17, 21). The capacity of metagenomic NGS to generate clinically actionable data was recently demonstrated in its use to diagnose a case of neuroleptospirosis in a critically ill child that had eluded all conventional diagnostic testing for 4 months (69). Once the diagnosis was made, appropriate targeted therapy resulted in a prompt recovery and cure.

However, translation of NGS assays from research tools for microbial characterization, pathogen discovery, and

epidemiological investigation to actionable clinical diagnostic tests introduces a number of new challenges. Reproducibly generating acceptable libraries from a variety of specimen types that vary by orders of magnitude in human and microbial nucleic acid content is difficult and currently requires multiple parallel strategies. Samples with low organism loads may require pathogen enrichment or amplification, while tissues with high human DNA content may need host subtraction techniques. Each additional step must be controlled for quality and has the potential to introduce contamination, so it is preferable to minimize processing steps where possible. To date, we are unaware of any universal library preparation protocol that can be used to detect all pathogen types in clinical samples with high sensitivity and specificity. One potential workaround is to bias the detection for specific pathogens using a targeted probe enrichment or amplification panel approach followed by NGS instead of relying on shotgun metagenomic NGS for diagnosis (35).

Even with technical hurdles cleared, it remains to be seen whether NGS allows for improved efficiency when compared to conventional clinical diagnostic testing. Certainly the promise of enhanced breadth of detection and genomic characterization is compelling, since it could allow for more personalized medicine and individualized treatment regimens. NGS-based analysis of the host transcriptome response using RNA-Seq may provide complementary information that can be used to guide or modify the approach to patient management and treatment. Furthermore, many studies are now describing how the human microbiome and pathogen genotype influence disease progression, but our knowledge in this area is far from complete. However, we expect that ongoing findings and insights from NGS in microbiology will enable a more comprehensive perspective regarding health and disease states and eventually lead to treatments targeted to specific aberrations in the host and microbial genomic profile.

CLINICAL VALIDATION

Clinical validation of a metagenomic or targeted NGS assay is a substantial undertaking, designed to demonstrate acceptable performance characteristics for an essentially unlimited number of pathogen targets and sequence variants. The assay should be shown to be significantly robust with valid limits of detection, accuracy, specificity, and reproducibility (70). Here, the traditional approach to single-analyte validation fails, because it is impossible to confirm the presence or absence of all possible organisms using standard reference methods. Instead, a validation approach that aims to identify and reduce potential sources of error in the test may be a practical alternative. For infectious disease NGS, this can be done using representative pathogen types in clinical matrices of interest, along with a thorough analytic evaluation to identify error-prone steps and introduce specific quality controls designed to detect errors when they occur. Controlling for sources of contamination is particularly important and should be addressed in the workflow and implementation of routine internal and external controls. Additionally, the NGS data analysis pipeline and reference databases will need to be separately validated. Establishment of curated standard reference databases will likely to be needed, in parallel with the use of additional bioinformatics analysis and review steps to identify misannotated or incomplete database entries. Finally, the reports must be interpretable by clinical microbiologists, be understandable to treating physicians, and provide clinically relevant and actionable results.

REGULATORY AND OTHER CONSIDERATIONS

Currently, no NGS assays for infectious disease diagnosis have been approved by the FDA, though clinical laboratories are starting to offer them as laboratory-developed tests. Proposed regulatory changes initiated by the FDA would likely establish a mechanism for review of newly developed NGS assays, and additional requirements may be instituted in the future to ensure that these clinical tests are safe and efficacious (71). While clinical laboratories are familiar with the requirements under the Clinical Laboratory Improvement Amendments for test validation, quality control, and proficiency testing, they do not typically establish *de novo* clinical utility for these assays. Indeed, the clinical trial design, outcome measures, and statistical confidence needed to demonstrate clinical utility are unknown. It will likely take a coordinated effort between academia and industry as well as stepwise guidance by the FDA to bring NGS for infectious disease to regulatory approval.

The validation of bioinformatics pipelines and databases is another challenge that is beginning to be addressed, but a suitable solution is not yet available. Analysis tools are being continually refined for speed and accuracy, but there is no standardized method to compare them or benchmark their performance. Curated databases typically have a limited number of microorganisms represented, and large public databases such as NCBI NT contain many misannotated sequences that could lead to erroneous results and interpretation (72). Curated 16S ribosomal databases are available for bacterial amplicon sequencing, but databases for other targets and whole-genome sequences are less well characterized. On the other hand, if a standardized reference database is successfully established, it is possible that it can be used as a sole comparator to establish the performance of an NGS assay, forgoing the need for traditional confirmation by orthogonal testing. Given the risk of contamination with metagenomic NGS, multisite evaluation would likely be a requirement for regulatory approval. Development of a panel of representative microorganisms that would function as microbial reference standards, under way at National Institute of Standards and Technology, would also likely be needed for NGS validation (73).

Due to the complexity and data storage requirements for high-throughput NGS analysis, cloud computing and remote storage are attractive options. However, demonstrating HIPAA (Health Insurance Portability and Accountability Act) compliance may be difficult, and there is a risk of data loss during transfer or storage. Most clinical laboratories are unfamiliar with the establishment or maintenance of large computational servers and databases, and the requirements for remote systems are not always clear. The use of bioinformatics tools for NGS analysis and interpretation of results are also not part of the routine skill set of most microbiology laboratories, so simpler graphical visualization interfaces and additional training in bioinformatics may be needed to enable these tests to be more broadly accessible to laboratory personnel. Standards will need to be established for the storage of clinical and technical metadata in addition to the sequence data. Finally, advances in health information technology and electronic medical records software will be required to determine how best to incorporate NGS information into the patient medical record.

CONCLUSIONS AND PERSPECTIVE

NGS assays hold great promise for the broad identification and genomic characterization of infectious disease pathogens. A variety of NGS technologies are now available, each with specific advantages and disadvantages. Sequencing assays incorporating pathogen detection, microbiome analysis, and host transcriptome profiling may lead to more personalized treatment approaches in the future. Several technical hurdles remain to be overcome prior to routine use, including optimal library preparation techniques for different microorganism and sample types, choice of bioinformatics pipelines, and suitable reference databases for comparison. The pathologic significance of microbial detection requires interpretation within the clinical context and may need additional confirmatory testing, particularly for detection of unexpected and/or novel agents. A multifaceted approach involving clinical and research laboratories, bioinformatics scientists, biotechnology companies, and regulatory agencies will likely be needed to take advantage of the large and complex sequence datasets that are currently generated by NGS analysis.

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Pathogen Discovery

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7

There are many clinical syndromes that pose diagnostic conundrums. Despite clinical symptoms consistent with an infectious etiology, many of these cases are negative by all clinical diagnostic assays. Specimens from such cases are ideal substrates for pathogen discovery efforts, which entail a range of additional technical methods beyond those routinely used in the diagnostic laboratory. Furthermore, the number of emerging infectious diseases has greatly increased in the last 2 decades. Many emerging infectious diseases originate from cross-species transmissions from animals to humans. Recent environmental and ecological changes are leading to a greater frequency of human and wildlife interactions that catalyze pathogen emergence. Furthermore, globalization and improvements to travel have made it easier for infectious diseases to spread rapidly. Therefore, there is an urgent need to identify novel pathogens in a rapid and efficient manner. Thus, pathogen discovery is a critical component of modern public health care.

The goal of this chapter is to review methods and approaches to identify novel microbial agents with an emphasis on viral discovery. We will illustrate this with examples of pathogens noted for their discovery method (Table 1) and highlight new trends in the field that have been driven by next-generation sequencing (NGS).

PATHOGEN DISCOVERY TECHNIQUES

Over the last few decades, pathogen discovery methods have evolved dramatically, largely driven by significant technological breakthroughs. Traditionally, pathogen discovery involved culturing the pathogen in animals or in cell cultures. For example, poliovirus was isolated by passaging the virus through rabbits, guinea pigs, mice, and monkeys in 1908 (1). Culture remains an important component of viral discovery efforts as exemplified by the isolation of Middle East respiratory syndrome (MERS) coronavirus after passaging in LLC-MK2 and Vero cells (2). However, not all pathogens can be cultured under standard laboratory conditions. Therefore, many culture-independent methods have been developed to complement traditional approaches. These methods can be used directly on clinical specimens or in many cases in conjunction with culture-based approaches to significantly speed up the identification process and increase its accuracy.

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IMMUNOSCREENING

One of the early milestones in pathogen discovery methodology was the application of immunoscreening, which utilizes antibody screening against a cDNA library derived from the infected specimen to identify pathogens. While this method has not been used as widely in recent years, it is worth noting its approach in several seminal discoveries tracing back to 1989 with the discovery of hepatitis C virus (initially named non-A, non-B hepatitis virus) (3). Hepatitis C virus is an enveloped RNA virus that encodes a 9.6-kb single-stranded positive-sense RNA genome. It is estimated that up to 150 million people worldwide are chronically infected with hepatitis C virus, accounting for more than 350,000 hepatitis C-related deaths each year. Previous efforts leading up to the study failed to identify specific viral antigens or antibodies to the non-A, non-B hepatitis agent. The primary hypothesis that led to this approach was that the levels of viral antigen were below the detection limit of standard immunological methods at that time. Therefore, a random cDNA library was constructed from infectious material whereby efficient cDNA expression could be driven by the vector. The library was screened for clones that expressed cDNA-encoded polypeptides that cross-reacted with sera from a patient with chronic non-A, non-B hepatitis. This led to the identification of a single positive clone (clone 5-1-1) out of approximately one million libraries. Additional biochemical experiments supported that clone 5-1-1 was derived from the genome of the etiologic agent, and this led to the sequencing and identification of hepatitis C virus. Immunoscreening was also used subsequently in the identification of Norwalk virus and GB virus C (4, 5).

REPRESENTATIONAL DIFFERENCE ANALYSIS

Representational difference analysis uses subtractive DNA hybridization coupled to PCR amplification to identify genomic or cDNA sequences that differ between two samples (6). Developed in 1993, this method was originally used for genome studies and was shown to be able to detect a single copy of adenoviral DNA in human tissue. Representational difference analysis was used in the identification of human herpesvirus 8, also called Kaposi's sarcoma-associated herpesvirus, in Kaposi's sarcoma (7). Human herpesvirus 8 is a large double-stranded DNA virus that encodes an approximately 165-kb genome. Seroprevalence rates of human herpesvirus 8 range from 20 to 80% in

TABLE 1 Summary of molecular approaches to pathogen discovery

Pathogen discovery method	Human pathogen(s) identified	Reference(s)
Immunoscreening	Hepatitis C virus, Norwalk virus, GB virus C	3–5
Representational difference analysis	Human herpesvirus 8	7
Random arbitrary primer binding PCR (RAP-PCR)	Human metapneumovirus	35
DNase-SISPA	Parvovirus 4	41
DNA microarray	SARS-coronavirus	42
Virus discovery cDNA-AFLP (VIDISCA)	Human coronavirus NL63	53
Direct high-throughput sequencing	Human bocavirus, KI polyomavirus, WU polyomavirus	40, 57, 58
Next-generation sequencing	Merkel cell polyomavirus, MERS-coronavirus, Heartland virus, Bas-Congo virus	2, 62, 66, 69

African and Mediterranean regions but are lower (<10%) in the United States and Northern Europe (8). Kaposi's sarcoma is the most common neoplasm in AIDS patients. To identify the pathogen, Kaposi's sarcoma tissue and tissue from a nondiseased site were analyzed. Nucleic acid from the diseased samples was PCR amplified and ligated to a specific priming sequence adaptor. The DNA was then hybridized to an excess of nucleic acid from the normal nondiseased sample that lacked the priming sequence adaptor. Doing this blocked homologous nucleic acid fragments from the subsequent PCR amplification step, thus enriching for DNA fragments that are unique to the diseased sample. As a result, four unique PCR bands were identified. Subsequent cloning and sequencing led to the identification of a novel herpesvirus. While successful in this instance, a major technical limitation is that it is difficult to obtain a well-matched negative control for the specimen of interest, and frequently, the amplified differences between the samples reflect differences in host gene expression rather than foreign microbial sequences.

TAXON-SPECIFIC CONSENSUS/ DEGENERATE PCR

Since its advent in the 1980s, PCR has become a common method for microbial detection. Once the genome sequence for a given microbe is available, it is a straightforward endeavor to design primers that detect the target microbe. Application of PCR to highly conserved regions of microbes has enabled the identification of many novel bacteria, fungi, and viruses. For bacteria and fungal detection, universal loci (e.g., 16S, 18S, or ITS) can be targeted. However, because there is no universally conserved site in viruses, primers must be designed to detect any given specific taxon (viral family or genus) of interest.

BACTERIAL IDENTIFICATION BY 16S PCR

Bacterial pathogens can be difficult to routinely culture. Hence, sequencing-based bacterial identification has emerged as an attractive strategy. Ribosomal gene sequences are commonly used for bacterial pathogen identification and phylogenetic analyses. The 16S small ribosomal subunit (16S rRNA) gene is widely used because the gene is ubiquitous to all bacteria. As applied to pathogen discovery, pioneering studies by Relman et al. in the early 1990s led to the identification of *Bartonella* as the cause of bacillary angiomatosis and *Tropheryma whipplei* as the etiologic agent of Whipple's disease (9, 10).

Use of the 16S locus has expanded in recent years beyond discovery of novel taxa to studies of bacterial populations. In addition, its clock-like rate of evolution supports robust phylogenetic resolution (11). The 16S rRNA gene is approximately 1.5 kb long and contains nine hypervariable regions, each flanked by highly conserved regions. Universal bacterial primers designed to the conserved regions are used to amplify the hypervariable regions. A sequence from one or more hypervariable regions, such as the V2 and V3 region, is sufficient to distinguish genus and species taxa (11, 12). Analysis of the entire 16S gene sequence can even distinguish between strains. Sequences are compared to curated 16S reference databases such as the Ribosomal Database Project, EzTaxon, and Silva (13–15). One caveat is that intracellular polymorphisms can exist from multiple copies of 16S rRNA gene in the genome (16). Other alternative genes used for molecular identification include the 23S rRNA and the intergenic spacer region between 16S and 23S (17). However, these sequence regions are longer, and variations are generally marked by insertions and deletions (18).

FUNGAL IDENTIFICATION BY CONSENSUS PCR

Despite being found in almost all habitats on Earth, it is estimated that more than 90% of *Fungi* have yet to be identified (19). Sequencing-based identification of fungal pathogens is particularly beneficial for its fast workflow. Standard microbiological tests can take more than a week because fungal organisms can be slow-growing. Initially, a mitochondrial locus for the cytochrome oxidase I (COI) gene was used for fungal classification. COI has been shown to accurately resolve *Ascomycota*, *Penicillium*, and *Neohumicola* species (20, 21). However, COI is less effective in other fungal genera such as *Aspergillus*, *Basidiomycota*, and *Fusarium* due to higher amplification failures from "universal" primer mismatches and because the gene can be present in multiple copies in some species (22). These multiple gene copies often vary in length and sequences, making its use phylogenetically ambiguous. In fact, some fungal clades such as *Neocallimastigomycota* lack mitochondria altogether (23). Hence, use of nuclear rRNA gene sequences has since been favored in fungal identification.

Two internal transcribed spacer (ITS) regions of the nuclear rRNA genes are most commonly used and are the fungal typing loci recommended by the Fungal Barcoding Consortium (24). The first region, called ITS1, lies between 18S and 5.8S rRNA genes, and the second region, called ITS2, lies between the 5.8S and 28S rRNA genes. ITS regions have a high PCR amplification success rate, and their sequences are able to discriminate between species taxa across the 17 major fungal lineages (21, 24). This has been applied in metagenomic studies that analyze the fungal community at the mouth, skin, and mucosal sites (25, 26). However, some disease-causing fungi such as *Candida* and *Malassezia* species can be commonly found on mucosal surfaces and the skin of healthy adults (26–28). It is believed that they represent low-level commensals and that they cause disease when they are able to establish an outgrowth niche such as when there is physical disruption at the site, antibiotic treatment, or host immunosuppression (29).

VIRAL IDENTIFICATION BY CONSENSUS PCR

The first example of viral discovery using conserved PCR primers was during the emergence of hantavirus pulmonary syndrome in 1993 (30). Initial serologic tests cross-reacted with known hantaviruses, suggesting infection by a virus in that family. Based on this information, conserved PCR primers derived from known hantavirus genomes were designed and used to interrogate specimens from the patients. Subsequent analysis of the amplicons yielded sequences phylogenetically distinct from the known hantaviruses. This virus was demonstrated to be the causal agent and was ultimately named *Sin Nombre hantavirus*. There have since been many additional novel viruses identified by consensus PCR approaches. Due to the broad utility of this approach, various computational strategies for design of consensus PCR primers for novel virus identification have been described (31, 32).

PCR screening for novel viruses using primers designed to conserved features of viral families offers a powerful approach to virus identification, provided there is adequate rationale to select a given candidate viral family or taxon to test. In the absence of a logical set of candidates to screen, it is typically not feasible to target every viral family individually, which would be prohibitively laborious, time-intensive, and reagent-intensive. Therefore, the development of methods in which nucleic acids could be amplified in the absence of primers designed to a specific target sequence has played a critical role in the latest methods for virus discovery. These sequence-independent amplification methods are capable of increasing the quantity of an unknown agent to detectable levels. In addition, various postamplification detection strategies have been devised to "visualize" the products of interest, which include gel electrophoresis-based differential display approaches (e.g., VIDISCA), hybridization to pan viral microarrays, and high-throughput mass sequencing.

RAP-PCR

In 2001, random arbitrary primer binding PCR (RAP-PCR) was adapted for pathogen discovery. This method was initially used in biochemical studies to compare genomic fingerprints between polymorphic genomes (33, 34). Generic primers are used in a PCR reaction performed initially under low stringency conditions, followed by high-stringency PCR amplification. The initial low-stringency conditions accommodate more mismatches during primer annealing, while the high-stringency conditions selectively amplify the PCR products. PCR products are then analyzed by gel electrophoresis to visualize the genomic fingerprint. In this way, differences in genomic content between samples such as those derived from pathogen infection can be distinguished as unique bands in the genomic fingerprints. This method was used in the identification of human metapneumovirus, a negative single-stranded RNA virus encoding an approximately 13-kb genome, in 2001 (35). RAP-PCR analysis was performed on cell culture that was infected with the virus and compared to a control culture. By cloning the unique PCR bands, the authors obtained sequences spanning different regions of the genome and were able to verify that the virus isolate was distinct from known members of the *Paramyxoviridae* family. Human metapneumovirus has since been detected worldwide and found to cause acute respiratory illness in infants, older adults, and patients with underlying chronic conditions (36, 37). Human metapneumovirus infections can be typically detected in infants from around 6 months of life onward, and the seroprevalence rate of human metapneumovirus is 100% by 5 years of age (35, 38).

DNASE-SISPA

Another approach, known as sequence-independent single primer amplification (SISPA), involves the ligation of a common primer binding sequence to both ends of cDNA molecules, which can then be amplified with a cognate primer (39). A refinement of this approach, called DNase-SISPA, involves the treatment of samples with DNase prior to nucleic acid extraction (40). The theory behind DNase-SISPA is that the DNase should degrade host DNA, but viral nucleic acid should be protected by the protein capsid and for some viruses also by the lipid viral envelope. Therefore, nucleic acids that survive the DNase treatment step should be enriched for sequences of viral origin. In the initial description of the method, the extracted nucleic acid was digested using restriction enzymes, and the resulting restriction fragments were then subjected to SISPA. The SISPA products were visualized by gel electrophoresis, and prominent bands were sequenced. Amplicons derived from novel bovine parvoviruses were identified in this initial proof of concept. Subsequently, DNase-SISPA has been used to identify novel viruses from human plasma (parvovirus 4 and two TTV-like anelloviruses) (41). Parvovirus 4 has since been detected in human serum in multiple studies, but its role in human disease is not currently known.

DNA MICROARRAY

In 2002, the first large-scale DNA microarray for pathogen discovery was developed (42). Prior to this, DNA microarrays were used primarily to measure gene expression or changes in genomic content in a massively parallel DNA hybridization assay. Pathogens often share stretches of nucleotide sequences that are conserved among members of the same taxa. These sequences are usually constrained by strong purifying selection to maintain an essential molecular function. For example, the untranslated regions of picornaviruses involved in transcription regulation are highly conserved across genera. Capitalizing on such features, the DNA microarray was designed to capture novel pathogens

by maximizing cross-hybridization to highly conserved sequences (42). A defined collection of pathogen-derived 70-mer oligonucleotide probes were printed onto a solid-surface microarray. In principle, the number of probes can vary up to several hundred thousand probes per slide. Multiple probes were often designed for each pathogen of interest for redundancy, and repetitive elements are avoided during probe design because they might reduce the specificity of the probes. Next, viral nucleic acid from the target specimen was randomly PCR amplified, fluorescently labeled, and hybridized to the probe microarray. The microarray was then visualized to quantify the probe signal intensities. This generated a hybridization profile specific for features that were conserved among viral families and genera. In certain cases, the microarray was also able to distinguish between serotypes (42).

Microarray-based pathogen discovery was applied in the identification of severe acute respiratory syndrome (SARS) coronavirus during the global outbreak of SARS in 2003 (43, 44). SARS coronavirus is a single, positive-stranded RNA virus with an approximately 29.7-kb genome. During the global epidemic, 8,098 probable SARS cases were identified across 29 countries (45). The mortality of SARS coronavirus ranged from 10 to 50% depending on age (46). Oropharyngeal specimens collected from a patient with SARS were used to inoculate cell cultures, following which, total nucleic acid extracted from the viral culture was hybridized to a virus microarray. The hybridization profile indicated that the virus was a novel member of the *Coronaviridae* family. The hybridized viral sequences were also directly recovered from the microarray and sequenced to validate the findings.

A significant advantage to the DNA microarray is the use of much longer oligonucleotide probes compared to primer lengths used in conventional PCR assays. This meant that a higher sequence divergence over the length of the probes could be tolerated during hybridization. For example, the Virochip DNA microarray identified a divergent human metapneumovirus subtype 1B that eluded clinical diagnosis in a reported case of a patient with respiratory illness (47). The microarray showed a hybridization pattern consistent with human metapneumovirus infection. However, results from four out of five clinically validated reverse transcription PCR (RT-PCR) assays for human metapneumovirus were negative. Subsequent sequencing of the virus genome revealed mutations in the regions targeted by the RT-PCR assay primers. Several pan-microbial microarrays have since been developed to detect a variety of microbial agents. These include a pathogen microarray for tropical and emerging infectious agents and biothreat agents called RPM-TEI (48) and broad-spectrum microarrays called the GreeneChip series designed to detect viruses, pathogenic bacteria, fungi, and protozoa (49, 50). A broad-spectrum microarray called the pan-microbial detection array was designed to detect viruses, bacteria, and plasmids (51).

VIDISCA

In 2004, virus discovery cDNA-AFLP (VIDISCA) was first described (52). VIDISCA was adapted from a technique called cDNA amplified restriction fragment polymorphism (cDNA-AFLP). The general rationale for this method is that an unknown pathogen should yield a unique banding profile after PCR amplification compared to a specimen where the pathogen is absent. This technique differs from RAP-PCR, which does not involve the use of restriction enzymes. Viral nucleic acid extracted from the specimen is converted to cDNA and digested with one or more restriction enzymes. The restriction fragments are ligated to specific adaptors corresponding to the overhang ends of the restriction sites. The ligated fragments are selectively amplified by primers designed to the adaptor-overhang region with a single specified nucleotide added. Varying the primers used at each end makes it possible for up to 16 primer combinations to be used in the selective amplification to reduce the complexity of the amplicons. This was done on a positive and negative specimen. The amplicons are then separated by gel electrophoresis and compared to the negative specimen to distinguish background amplicons.

VIDISCA was first applied in the discovery of human coronavirus NL63 (53). Virus from the clinical sample of an infant with acute respiratory disease was passaged in LLC-MK2 cell cultures. VIDISCA was performed on supernatant from infected LLC-MK2 cells and compared to uninfected LLC-MK2 cells. Several unique fragments were identified and found to be sequences of a divergent coronavirus. Using a combination of cDNA library, 5′ and 3′ rapid amplification of cDNA ends, the complete genome of human coronavirus NL63 was resolved. An advantage of VIDISCA is its reproducibility, because the method relies on digestion by specific restriction enzymes. VIDISCA has since been adapted to be used in conjunction with NGS. Adaptors containing the Roche-454 "universal tail" sequences are ligated to DNA fragments during VIDISCA and directly amplified in the emulsion PCR for 454 sequencing (54).

DIRECT HIGH-THROUGHPUT SEQUENCING

The previous sequencing-based methods all relied upon some form of enrichment or differential display to be able to select microbial sequences from the large background of host nucleic acid with a limited extent of sequencing. As the throughput of traditional Sanger sequencing increased due to the Human Genome Sequencing project, it became feasible to attempt direct sequencing of clinical specimens to identify the viruses present.

Meyerson and colleagues conducted the first proof of principle experiment to demonstrate that extensive sequencing of nucleic acids in a specimen in conjunction with computational subtraction could be used to detect pathogens (55). They examined a tissue specimen from a case of posttransplant lymphoproliferative disorder, knowing that most such cases are caused by Epstein-Barr virus. A cDNA library with 27,840 sequences was generated, and each sequence was compared to the human genome to identify sequences of human origin. These sequences were then eliminated from further consideration in a process termed "transcript filtering." The remaining sequences that did not match human genomic sequences were postulated to be derived from microbial organisms present in the sample. From this library, 10 sequences that did not match any human sequences but had similarity to Epstein-Barr virus were identified. Furthermore, these sequences could only be amplified from Epstein-Barr virus-infected tissues and not from controls, thus showing the potential power of mass sequencing strategies.

The discovery of human bocavirus (HBoV) marked the first discovery of a novel virus by direct high-throughput

sequencing of clinical specimens (40). In this study, pools of respiratory samples were ultracentrifuged, filtered, and DNase treated to produce samples for analysis that were highly enriched for intact viral particles (in which the genome is protected from DNase treatment) and to minimize the amount of other contaminating DNAs. In contrast to the discovery of human metapneumovirus, in which primers were arbitrarily chosen for amplification, a random PCR amplification strategy was used for amplification in the human bocavirus study. The amplified products were then cloned, and ~300 to 500 clones were sequenced without any effort to identify specific amplicons. In this analysis, 20% of the sequences showed similarity to viral sequences, the majority of which were derived from known viruses. However, some of the sequences had only limited similarity to viruses in the *Parvoviridae* family and, more specifically, the genus *Bocavirus*. The respiratory samples that were initially pooled together for sequencing analysis were independently screened by PCR for the presence of the novel bocavirus, and two tested positive. Further PCR screening of other pediatric respiratory specimens revealed that HBoV could be detected in 3.1% of the samples. The potential role of human bocavirus in respiratory disease and gastroenteritis has been investigated in numerous subsequent studies. These epidemiologic and seroepidemiologic analyses have determined incidence rates of bocavirus between 2.7 and 19% (in respiratory secretions) and seroprevalence rates of 94.7 to 98.3% in healthy adults (56). Other notable viruses identified using direct Sanger sequencing include KI polyomavirus and WU polyomavirus (57, 58).

NGS

While the principle of pathogen identification by direct sequencing had clearly been established by high-throughput Sanger sequencing, the advent of NGS, by lowering the cost of sequencing by multiple orders of magnitude, revolutionized the field of pathogen discovery. NGS provided the depth of sequencing to directly find the metaphoric needle in a haystack. The first NGS platform (454, Roche) was initially the primary platform used for pathogen discovery. As NGS technologies have evolved, most current efforts now primarily use the Illumina platform, although studies using other technologies such as Ion Torrent have also been described. Because of the significant impact of NGS on this field, we will briefly summarize key aspects of these platforms below (Table 2).

454 ROCHE

454 Roche sequencing is based on detecting pyrophosphate release, hence it is also known as pyrosequencing (59). Approximately 1 million sequencing reads are obtained from each 454 sequencing run, with an average read length of 450 bp. DNA library fragments are constructed with two different adaptors on each end. A single DNA fragment is immobilized onto a bead within a water-in-oil emulsion droplet and undergoes amplification across the surface of the bead (referred to as emulsion PCR). Each template-amplified bead is then deposited into individual wells of a PicoTiter plate in a manner in which each well holds only a single bead. Sequencing reagents are subsequently added across the plate in a predetermined order. As one or more nucleotides are incorporated into the complementary strand, a chemiluminescent signal is emitted during the pyrophosphate release. The chemiluminescent signal is recorded by a camera for base-calling. This allows sequences to be recorded at each bead location on the PicoTiter plate. The chemiluminescent signal intensity is proportional to the number of identical bases incorporated. A limitation to 454 pyrosequencing has been its inability to resolve long stretches of homopolymers, a stretch of a single base repeated consecutively. This occurs when the homopolymer length is incorrectly called from the signal intensity. Several algorithms have been developed to correct homopolymer-based errors in 454 sequencing data (60, 61).

The discovery of Merkel cell polyomavirus as the causative agent of Merkel cell carcinoma was one of the first successes of pathogen discovery by 454 sequencing (62). Merkel cell carcinoma is a rare, but aggressive, neuroendocrine cancer that is associated with immunosuppressed individuals (63). The cDNA from four Merkel cell carcinomas was pyrosequenced using a 454 platform. Human-derived sequencing reads were first removed, and then the remaining reads were aligned to known viral genomes. Out of approximately 400,000 sequencing reads, three sequencing reads were identified that had shared similarity to known polyomaviruses. This led to sequencing of the complete genome of Merkel cell polyomavirus by primer walking. Using a combination of PCR and Southern hybridization assays, Merkel cell polyomavirus infection was found to be associated with Merkel cell carcinoma. The authors also demonstrated that Merkel cell polyomavirus DNA was clonally integrated into the genome of carcinoma cells. Since then, Merkel cell polyomavirus has been shown to be in most Merkel cell carcinoma specimens (64, 65).

Subsequently, 454 sequencing has been used directly to identify viruses from clinical samples and from cultured samples. A few examples demonstrating the diversity of virus types and hosts include MERS-coronavirus, a virus associated with respiratory disease (2); Heartland virus, a novel phlebovirus identified in a patient with severe febrile illness (66); Lloviu virus, a filovirus from a pathogenic out-

TABLE 2 Comparison of NGS platforms

Instrument	GS FLX+	HiSeq 2500	MiSeq v2	Ion Proton I	Ion PGM 318	PacBio RS II
Platform	454 Roche	Illumina	Illumina	Ion Torrent	Ion Torrent	PacBio
Sequencing technology	Pyrosequencing	Illumina sequencing	Illumina sequencing	Ion semiconductor sequencing	Ion semiconductor sequencing	SMRT cell sequencing
Average sequencing reads per run	1 million	4 billion	22 million	60 million	4 million	47,000
Average read length	450 bp	125 bp	300 bp	200 bp	400 bp	10 kb
Run time	23 hours	6 days	55 hours	4 hours	8 hours	10 hours

break in bats in Europe (67); and Rosavirus 2, a picornavirus in fecal samples from children in The Gambia (68).

ILLUMINA

Illumina sequencing technology is also based on sequencing by synthesis. The DNA library is ligated with two unique adaptors at each end of the DNA fragment and attached to the surface of a flow cell. The DNA fragment undergoes solid-phase amplification in place on the flow cell through a process called bridge amplification, yielding a cluster of thousands of copies of a DNA fragment at each spot on the flow cell. Sequencing reagents are added to the flow cell that includes a sequencing primer that anneals to an adaptor at one end and nucleotides. Each nucleotide is labeled with a different fluorescent dye. During each sequencing cycle, a single nucleotide is incorporated into the complementary strand, and an image of the fluorescent signal is recorded indicating the corresponding nucleotide. However, DNA synthesis stops after incorporation of a single nucleotide because the fluorescent label on the nucleotide also acts as a reversible terminator. After imaging, the fluorescent label is enzymatically cleaved off to allow incorporation of a labeled nucleotide in the next cycle. This step-by-step sequencing proceeds from one end of the DNA strand for a specified number of cycles. In paired-end sequencing, both ends of the DNA fragment are sequenced.

Illumina libraries are often constructed with a slight overlap to facilitate downstream assembly analyses. For example, a 225-bp fragment will have a 25-bp overlap in the middle when 2 × 125-bp paired-end sequencing is performed. Thus, the insert fragment length can influence the number of useful base sequences. Illumina offers the HiSeq and MiSeq sequencing systems. The HiSeq platform offers considerably more reads than the MiSeq platform, but the HiSeq read length is shorter than MiSeq. Currently, the typical output of an Illumina HiSeq run is 4 billion pairs of reads, while a MiSeq run gives 22 million pairs of reads. Paired-end reads obtained from the Illumina HiSeq 2500 and MiSeq are 2 × 125 bp and 2 × 300 bp, respectively.

One of the earliest applications of Illumina sequencing technology in pathogen discovery was in the identification of a novel rhabdovirus associated with an acute hemorrhagic fever outbreak in the Democratic Republic of Congo (69). Three human cases presented with an abrupt disease onset, high fever, bloody vomiting, and diarrhea. Two of the patients died within 3 days. Initially, RNA extracted from a sera specimen from the lone survivor was sequenced on the 454 Roche platform. However, only a single candidate read out of 4,536 sequencing reads was identified, as it showed limited similarity to known rhabdoviruses. Thus, the RNA was subsequently sequenced to a greater depth on an Illumina HiSeq platform that yielded a total of 140 million sequencing reads. Ninety-eight percent of the novel rhabdovirus genome was assembled with at least 10-fold coverage across the genome. The viral load of Bas-Congo virus, the novel rhabdovirus, in the patient's serum was 1.09×10^6 RNA copies/ml. Additionally, serological assays indicated that an asymptomatic primary health care provider to the patient had developed neutralizing antibodies against Bas-Congo virus. Thus, Bas-Congo virus was identified as an emerging human pathogen associated with acute hemorrhagic fever. Other examples of novel pathogens discovered by Illumina sequencing include avian bornavirus, a virus associated with proventricular dilatation disease of parrots (70); and Theiler's disease-associated virus, a new pegivirus associated with equine hepatitis (71).

OTHER NGS PLATFORMS

Other recent NGS sequencing platforms on the market include Ion Torrent from Life Technologies and PacBio from Pacific Biosciences. Ion Torrent platforms use a semiconductor chip to detect hydrogen ions during DNA polymerization. Similar to 454 sequencing, nucleotides are added to the chip in a predetermined order. As each nucleotide is incorporated during strand synthesis, a hydrogen ion is released that alters the pH value. Changes in pH are then converted to and measured in voltage values. The changes in pH, and hence voltage, are proportional to the number of nucleotides that are incorporated. Currently, Ion Torrent offers sequencing reagent kits for 200-bp and 400-bp read length configurations. The number of sequencing reads obtained per run can be varied based on the type of chip used. Options range from half a million reads to more than 60 million reads per chip.

PacBio sequencing technology is based on a single-molecule, real-time (SMRT) DNA sequencing system. Individual molecules of DNA template are fixed to the bottom of a chip in an optical waveguide called a zero-mode waveguide, together with a single DNA polymerase enzyme. Each zero-mode waveguide is a nanophotonic structure that creates an individual illuminated observation area. The four nucleotide bases are labeled with different fluorescent dyes. During nucleotide incorporation, the fluorescent tag is cleaved and detected in the zero-mode waveguide, allowing the base to be called accordingly. Each SMRT cell is embedded with 150,000 zero-mode waveguides, and the average sequencing read length can be up to 10 kb.

PARAMETERS DRIVING THE CHOICE OF NGS PLATFORM

Amid the myriad sequencing platforms currently available, new NGS sequencing platforms will undoubtedly continue to emerge in the coming years with rapid advances in technology. However, certain instruments are ideally suited for specific applications such as detection of single nucleotide polymorphisms, *de novo* genome assembly, and metagenomic studies. Some of the key considerations in deciding on the NGS approach to use are sequencing cost, number of reads desired, and sequencing read length.

1. Sequencing costs have dropped tremendously in recent years, outpacing the exponential predictions of Moore's Law (72). Nonetheless, sequencing costs are nontrivial. While the cost per base is many orders of magnitude lower than with traditional Sanger sequencing, NGS platforms have a fixed cost per "run," so in many cases multiple samples are pooled to optimize cost-efficiency. For reference, 454 Roche sequencing costs approximately 100 times less than Sanger sequencing per megabase of DNA. However, Illumina sequencing costs approximately 100 times less than 454 Roche sequencing per megabase. This is partly because Illumina sequencing costs are averaged across a much higher number of sequencing reads.

2. The sequencing depth necessary to detect microbial sequences present in a specimen depends on the relative copy number of the microbe compared to the host nucleic acid. For samples that are relatively poor in host sequences (e.g., respiratory secretions), sequencing of as little as 10^2 to 10^3 reads may suffice for detection. By contrast, a tissue specimen, which contains the entire human genome and transcriptome, may require much greater sequencing depth (>10^7 reads).
3. Read length is an important parameter in the detection of divergent sequences from individual reads. For sequences that do not share nucleotide similarity, translation to amino acids is often necessary to identify the read as microbial in origin. Longer reads enable alignments of longer stretches of amino acids, thereby increasing the probability of detection of divergent microbes. Naturally short reads may be assembled into longer contigs, but the assembly process also poses its own challenges. The average read length from the 454 FLX Titanium is 450 bp, while the read length from Illumina is 2 × 125 bp (HiSeq) or 2 × 300 bp (MiSeq) through paired-end sequencing. However, the average read length of the PacBio RS II platform is 10 kb.

NGS BIOINFORMATICS WORKFLOWS

While increased sequencing capacity and decreased costs of sequencing have been a boon for the field of pathogen discovery, it has also raised significant computational challenges in the processing and analysis of the resulting sequencing data. A number of bioinformatics workflows have been developed to identify viruses from high-throughput sequencing datasets (73–79; www.pathseek.eu). The general approach can be summarized in three stages. First, sequencing reads are preprocessed for quality control such as trimming low-quality and adaptor sequences. Next, host sequences are subtracted from the dataset. For instance, for a human specimen, sequencing reads that map to a human reference genome are removed. This is done to reduce the amount of sequencing data because the subsequent downstream analyses are often computationally intensive. Finally, sequencing reads are aligned to a virus database to identify virus sequences using algorithms such as BLAST. Other workflows have additional features such as detecting viral integration sites in VirusFinder (76). Pathoscope uses Bayesian statistics to assign a posterior probability to the matching hits (77). Additionally, SURPI supports a cloud-compatible workflow (78), and MetaVir can be accessed through a web server (79). A bioinformatics workflow is also being developed by the PATHSEEK consortium with the goal to provide a diagnostic assessment within 48 hours from clinical samples sequenced by NGS (www.pathseek.eu).

METAGENOMICS

Beyond the identification of individual, novel microbes that may be pathogens, the advent of NGS has made it possible to define entire microbial communities that occupy a given biologic niche. Multi-institutional consortiums such as the European Metagenomics of the Human Intestinal Tract (MetaHIT) and the U.S. Human Microbiome Project (HMP) have made significant efforts to characterize the human microbial communities (80, 81). These metagenomics studies show that healthy individuals harbor a tremendous diversity of bacterial microbes that is highly variable over time and across populations (82, 83). However, while the specific bacterial lineages can vary between individuals, the profile of enzyme gene functions encoded by the bacterial community is highly similar between individuals (84). Thus, it has been proposed that the microbial community's functional gene composition might be associated with altered physiological states (85). In support of this model, the pathogenesis of some diseases has been linked to the imbalance of the commensal microbial community, rather than invasion by specific microbial agents. In Crohn's disease, a form of inflammatory bowel disease, microbial dysbiosis is consistently observed, where the proportion of *Proteobacteria* increases concomitant with a decrease in *Firmacutes* (86). Additionally, bacterial dysbiosis mediated by a high-fat diet has been shown to accelerate intestinal tumor progression (87). Taken together, the complex ecological community of commensal microbes can influence human health, and this concept presents a new model of microbial pathogenesis beyond the traditional "one microbe, one disease" paradigm. As another example of this concept, a recent intriguing observation emerged from a study of the enteric virome of simian immunodeficiency virus-infected macaques. In this study, significant expansion of the enteric virome (predominantly an increase in picornaviruses and adenoviruses) was observed specifically in pathogen simian immunodeficiency virus-infected monkeys as compared to controls (88). Whether similar changes in the enteric virome are seen in HIV-infected humans remains to be determined.

NGS has become an attractive tool for bacterial community profiling because short 16S gene amplicons (less than 500 bp) can be easily sequenced within the standard read lengths of most sequencing platforms. 16S sequencing was used to profile the microbial communities of different niches such as the mouth, skin, vagina, gut, and lung in the human microbiome project (81, 89). Metagenomic workflows such as QIIME perform high-throughput analyses on sequencing datasets to assign operational taxonomic units and estimate sample diversity (90). A complementary metagenomic profiling approach is to analyze the microbial community using clade-specific marker genes. Rather than sequencing 16S gene amplicons, shotgun sequences from the microbial community are mapped to a curated catalog of clade-specific marker sequences using classifiers such as MetaPhlAn (91). This approach is able to measure species-level relative abundance of bacterial and archaeal microbes. Likewise, NGS has been adapted to interrogate entire fungal communities using high-throughput ITS sequencing (25, 92).

CHALLENGES OF PATHOGEN DISCOVERY

The advent of NGS has dramatically increased the rate of detection of novel microbial sequences. With the tremendous sequencing depth achievable with current technology, it is possible to detect microbial nucleic acids that are present at a frequency of less than 1 per 10^8. However, this also poses significant challenges in interpretation of the biological significance of these discoveries. One key point to address is the criteria for establishing a causal link between the newly detected agent and disease. Traditionally, this has relied on Koch's postulates, which have been revised over the past decades (93–95). This issue is beyond the

scope of this review. However, the focus of those approaches is to distinguish pathogens from nonpathogenic infections. Additional challenges faced in this molecular era include the potential for contamination artifacts. Contamination may arise at any point from initial sample collection through the end of the computational analysis pipeline. We will provide several examples to illustrate the pitfalls that may arise from inadequate control of contamination.

LABORATORY CONTAMINATION

Xenotropic murine leukemia virus-related virus (XMRV) illustrates the confusion that can arise when the discovery of a *bona fide* novel virus is incorrectly associated with a disease. XMRV was initially linked to human prostate cancer and subsequently to chronic fatigue syndrome. Mutations in the ribonuclease L gene were previously linked to prostate cancer (96). Using a pan-viral DNA microarray approach, XMRV was identified in tissue samples from prostate cancer patients (97). Subsequently, XMRV was detected in the blood of patients with chronic fatigue syndrome (98, 99). However, nearly all follow-up studies failed to detect the association of XMRV with these diseases (100). The origin of XMRV was traced to a prostate cancer cell line that had been xenografted in a mouse, where XMRV emerged from a unique recombination event between two endogenous murine retroviruses (101). Thus, XMRV was shown to be a laboratory-derived contamination that had no association with human prostate cancer and chronic fatigue syndrome (100, 102). This highlights the importance of interpreting disease association with the newly identified novel pathogens and the need for controls that can detect contamination especially in highly sensitive discovery techniques.

REAGENT CONTAMINATION

During the course of the XMRV controversy, multiple labs identified similar murine retrovirus-derived sequences that were present in standard molecular biology reagent preparations such as commercial reverse transcriptase (103, 104). Another example of reagent contamination emerged following the discovery of a novel circovirus-parvovirus hybrid DNA virus called National Institutes of Health Chongqing virus (NIH-CQV), putatively identified in sera specimens from patients with non-A-E hepatitis (105). The discovery had significant implications because up to 20% of acute hepatitis cases have an unknown etiology (106). The cDNA from pooled sera of patients with non-A-E hepatitis was deep-sequenced. Bioinformatics analyses identified a 3.7-kb contig that shared limited identity with known parvoviruses. The complete genome of NIH-CQV was subsequently verified in eight overlapping PCR fragments. Additionally, genetic heterogeneity was detected in the NGS data and inferred to represent intrapatient quasispecies. Using quantitative PCR and serological assays, the authors concluded that NIH-CQV was highly prevalent in patients with non-A-E hepatitis. However, NIH-CQV has since been shown to be a reagent contaminant of nucleic acid extraction spin columns (107, 108). NIH-CQV could be detected by PCR in mock extractions of water eluted from the silica column. Importantly, samples that were previously positive for NIH-CQV subsequently tested negative when they were reextracted using other vendor kits and platforms. Further analyses indicated that NIH-CQV sequences could be detected in NGS metagenomic data from marine environmental samples. Therefore, the origin of NIH-CQV has been proposed to be an inadvertent contamination of silica during spin column manufacture (107).

METAGENOMICS LIMITATIONS

Microbial contamination during specimen sampling can also significantly influence metagenomic studies that survey taxonomic distributions and frequencies (109, 110). Samples with low microbial biomass, such as spinal fluid, blood, and lung specimens, are particularly prone to these effects (111). Serial 10-fold dilutions of a pure *Salmonella bongori* culture were sequenced at three research institutions and analyzed to compare the effect of limiting sample dilution on bacterial community composition analyses. While the bacterial taxonomic profiles were generally similar among the three sites, some bacterial genera were only detected in a site-specific manner, indicating that contaminants could vary between laboratories. Importantly, the proportion of bacterial contaminants was dominant in samples at increasing dilutions. These observations were consistent in bacterial 16S amplicon sequencing and shotgun sequencing. Thus, as NGS becomes more widely accessible, studies should include added precaution to minimize contamination.

CONCLUSIONS

Significant advances have been made in the methodologies for pathogen discovery. NGS has changed the face of pathogen discovery by providing a high-throughput cost-effective approach. As this technology becomes more widely accessible and new innovative methods are developed over the coming years, it is important to emphasize the value of good practices in microbiology sterile techniques. This can have significant implications when the disease causation is established. Thus, the field of molecular diagnostics is poised to offer the first clues to many unsolved clinical syndromes. Moreover, the improved technologies have enabled the field to develop more nuanced concepts of microbial pathogenesis.

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Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry for Microbial Identification in Clinical Microbiology

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8

After several decades of limited progress, the practice of clinical microbiology is beginning to evolve on a more rapid scale. The introduction of immunological methods in the 1970s was followed by the introduction of automated biochemistry-based identification systems, automated antimicrobial susceptibility testing systems, and automated blood culture instruments in the 1980s and 1990s, and thereafter by the gradual adoption of nucleic acid amplification tests over the past 25 years. Today, there is an accelerated pace of introduction of novel technologies into clinical microbiology laboratories (1). Easy-to-use panel-based molecular testing and automated workflow are in the process of being adopted. Workflow optimization is being facilitated by the introduction of automated equipment, with rapid identification of microbial pathogens being a key component (2). A revolutionary new entry, matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS), has been introduced into clinical microbiology laboratories over the past 5 years as a tool for microbial identification (3–5). The quality of MALDI-TOF MS with respect to bacterial identification already seems to surpass that of more classical growth-based technologies (6). The reason for the rapid adoption of this technology is that it is simple, high speed, cost-effective, and at least as good as, if not better than, conventional identification strategies. Sometimes the combination of classical technologies such as Gram staining with MS is advocated, but most people agree that MS will be able to function as a stand-alone technology for bacterial identification (7).

MS methods can be fairly easily standardized, and although in some cases culture conditions may affect the nature and overall quality of the mass spectra (8), this is the exception rather than the rule and has not hindered the development of spectral databases and dedicated identification assays. Results can be obtained in as few as 6 min, and identifications are very reproducible (5). The technology has been broadly adopted, and many laboratories around the world use MALDI-TOF MS as their main microbial identification strategy. Given this success and the availability of many good reviews on the topic, this chapter does not aim to provide a detailed description of MALDI-TOF MS as such. Instead, this chapter aims to summarize some of the major developments surrounding the use of MALDI-TOF MS in clinical microbiology with a focus on the recently performed comparative studies (MALDI-TOF MS versus other technologies) and multicenter evaluations.

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SAMPLE PREPARATION

A key component of MALDI-TOF MS is sample processing. DNA testing using MS has been proposed and commercially developed (9), but in most current clinical microbiology applications of MALDI-TOF MS, the focus is on the evaluation of protein rather than nucleic acid spectra. For this purpose, the only requirement is to have a relatively pure bacterial, mycobacterial, or fungal culture with a sufficient number of cells, typically at least 10^5 microbial cells per sample for most species, to generate useful spectra and microbial identification. When cultures have grown on a commercial agar medium, simply picking a colony and transferring the biomass to a MALDI-TOF MS sample plate suffices in most cases, although spectra may differ depending on culture media used and the age of the colony. Adding a small amount of a chemical matrix (α-cyano-4-hydroxycinnaminic acid or others) needed for ionization of the biomaterial is all that is required for processing most bacteria.

For some bacterial and fungal species, the addition of a simple preparatory on-plate extraction step with formic acid can improve the diagnostic outcome. This has been demonstrated particularly for the *Corynebacterium* species and Gram-positive cocci of the species *Streptococcus mitis* and *Streptococcus dysgalactiae* using a commercially available MALDI-TOF MS system (10). The on-plate formic acid preparation or a more formal formic acid–ethanol extraction approach is generally required to analyze fungal cells (11, 12). For yeasts, this is typically performed using the aforementioned on-plate formic acid strategy (13). Molds may require formal protein extraction prior to being placed on the sample plate. Likewise, bacteria grown in liquid media (e.g., in blood culture bottles) need preparatory treatment to purify the bacterial cells free from other contents

present in the liquid. For this purpose, a variety of methods have been used. Whereas the accuracy of bacterial species identification of MALDI-TOF MS is usually over 96%, in the case of positive blood culture bottles, this percentage may drop depending on the bacterial species in the sample. Some commercial systems are available, but in-house lysis-filtration and lysis-centrifugation methods have been proposed (14–16). These methods have been claimed to be economical and time-saving compared with the more classical methodologies, especially when coupled with rapid antimicrobial susceptibility testing (17). Some very impressive results have been reported for the mycobacteria, a particularly cumbersome group of organisms when the processing of bacteria grown in liquid or solid media is involved (18). When handling potentially hazardous organisms, such as *Brucella melitensis*, work with live organisms should be exclusively performed in biological safety cabinets.

Sample processing is more cumbersome if analyses are performed directly from clinical specimens. Even detection and identification of bacteria from positive blood culture media may require adaptation of the laboratory workflow to enhance reliability. Several studies have documented good results while using a variety of sample preparation methods where selective lysis of host cells seems to be a common experimental theme. For the postculture detection of blood-borne pathogens, PCR methods have also been developed, but the results of such molecular methods are similar to those of mostly cheaper MS analyses (19). The sensitivity of the MALDI-TOF MS methods ranges between 80 and 90%, so there still is room for improvement (20, 21), which will be primarily sought in the preanalytical steps.

Urine specimens are the only clinical specimens that regularly contain the requisite 10^5 or more cells compatible with direct microbial detection in clinical specimens, although these samples may contain mixed species (22, 23). A recent study reported a successful complementation of urine flow cytometry with MALDI-TOF MS where the combination of the results obtained with both methods provided actionable data (24). However, the method failed in some instances of mixed infection. To address this issue, proposed solutions that seem to work quite well involve the adaptation of interpretative software (25). In addition, focus has been on the development of sample preparation methods that improve the sensitivity by eliminating contaminating and potentially MS-confounding compounds (such as human defensin proteins, phosphate crystals, and leukocytes). Protocols involving differential centrifugation (26), short preincubation of urine sediments on agar-based growth media (27), and diafiltration (28) have all shown their analytical utility.

Interestingly, organisms from completely different phyla can be analyzed by MALDI-TOF MS: a recent paper clearly showed that ticks of a variety of species and origins can be differentiated (29). Work on larvae, cell lines, mosquitoes, and midges has been published as well (30, 31). We expect soon to see even more widely differing applications in all fields of medical, environmental, pathological, chemical, and toxicological diagnostics.

MICROBIAL IDENTIFICATION

Once samples have been processed and the sample plate has been put in the mass spectrometer, the process is highly automated, with the end result being a report indicating top matches and confidence in the proposed identifications. Generally speaking, MALDI-TOF MS yields identifications that are as good as, if not better than, currently available biochemical and nucleic acid–based identification systems for both bacteria and yeast. In some instances, such as the case of nontypeable *Haemophilus influenzae* versus *Haemophilus haemolyticus*, MALDI-TOF MS supplements classical taxonomy. Whereas classical microbiologic techniques may have difficulty distinguishing the two, MALDI-TOF MS has been shown to provide a means to identify both organisms (32). Similar data were obtained for identification of *Bacteroides* spp. and *Streptococcus* spp. including *Streptococcus pneumoniae* (using the VITEK MS system), with MALDI-TOF MS being as good as classical testing with regard to sensitivity and with enhanced specificity (33–35). In addition, applications were developed for microbial species as diverse as *Leptospira* spp. (36), *Mycobacteria* spp. (37), and *Propionibacterium* spp. (38) (see Fig. 1).

The current commercially available systems cover a broad array of species, and as databases and strategies expand, the spectrum continues to broaden. For example, although select agents have historically not been adequately addressed, using appropriately constructed databases, including Bruker's Security-Relevant Library, *Brucella* species, *Francisella* species, and *Burkholderia pseudomallei/mallei* may be identified (39, 40). It has also been shown that detection and identification of *Bacillus anthracis* may be reliably performed using 2.5×10^6 spores (41). For fungi, MALDI-TOF MS has added significantly to the reliable identification of these organisms (42–44). Increasingly, commercial databases are being expanded to include the majority of the clinically relevant fungal species (45, 46) and will be expanded to include additional species that are currently hard to identify by phenotypic methods as well (such as the *Nocardiae*; see Fig. 2 and Fig. 3). Obviously, MALDI-TOF MS is not a panacea. When taxonomy is not clear, MS may have issues with correct microbial identification. Examples include the differentiation between *Escherichia coli* and *Shigella* species and the correct identification of several streptococcal species.

ANTIMICROBIAL SUSCEPTIBILITY TESTING AND BACTERIAL TYPING

To date, commercial MALDI-TOF MS offerings in clinical microbiology have been solely focused on microbial identification. However, there are other applications that have the potential for impact on clinical patient management. Among these are strain-typing and antimicrobial susceptibility testing; the latter was recently reviewed by several authors (47, 48). Methods may be simple; in some cases the combination of MALDI-TOF MS and automated antimicrobial susceptibility testing facilitated a better workflow for the timely management of positive blood cultures (4, 49–51). Alternatively, four major types of assays can be envisioned: (i) detection of antibiotic modification or degradation based on changes in mass of the antibiotic, (ii) detection of proteomic changes in resistant versus susceptible bacterial species in the absence or presence of the antimicrobial compounds, (iii) semiquantitation of intracellular or extracellular antibiotics, and (iv) detection of modified antibiotic target sites (52). Practical examples have been recently published. For example, detection of specific β-lactamase activities (53–55), including carbapenemases (56), assessing the susceptibility of *Candida* species and *Aspergillus* species to caspofungin (57), and simple and straightforward testing for ampicillin resistance among *E.*

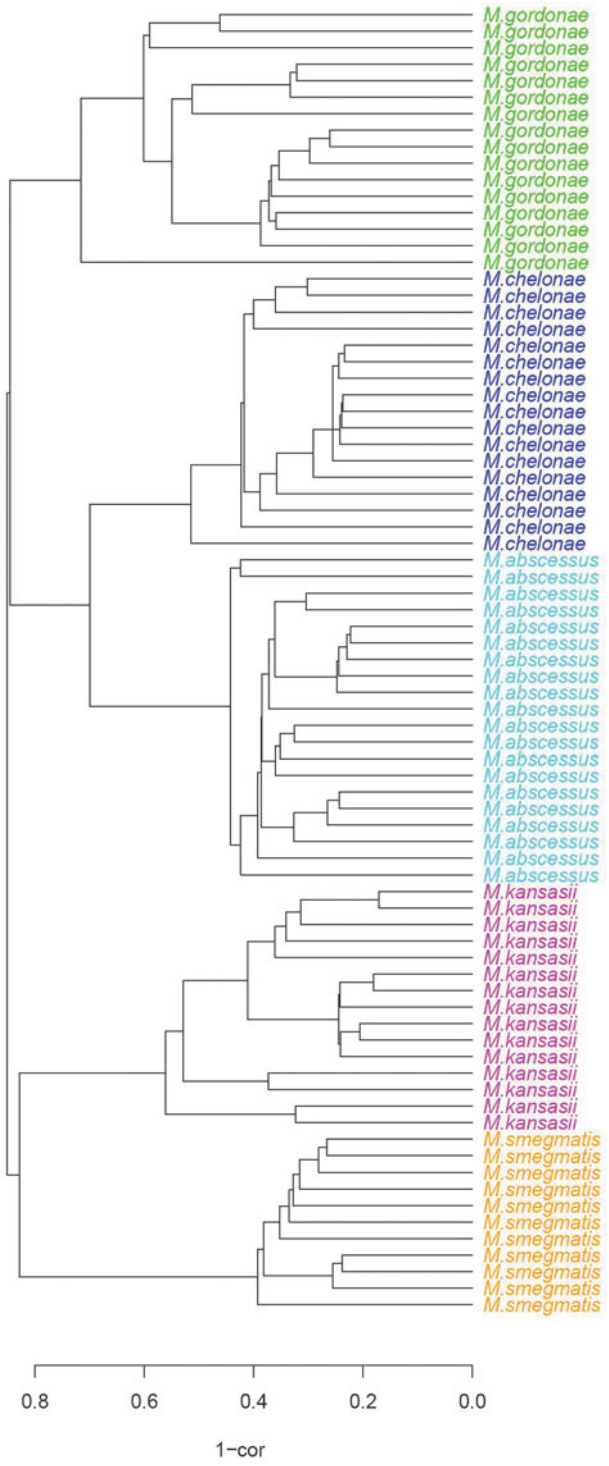

FIGURE 1 Dendrogram (Spearman correlation dissimilarity index, single linkage criteria) based on the MALDI-TOF MS spectra of some strains of *Mycobacterium* species used for the VITEK MS knowledge base creation. For each strain, replicate measurements were summarized into a prototype spectrum by retaining only the peaks found in 50% or more of the spectra. *Mycobacterium abscessus*, *Mycobacterium chelonae*, *Mycobacterium smegmatis*, *Mycobacterium gordonae*, and *Mycobacterium kansasii* are clearly separated by MALDI-TOF MS.

coli strains (58) have been convincingly demonstrated. Details can be found in several good reviews on the subject (59, 60).

Bacterial strain-typing can also be performed using MALDI-TOF MS (61). While identification assays usually focus on those peaks that are universally conserved among all isolates from within a microbial species, high-resolution typing within a species relies on comparison of peaks that are unique to an isolate. It has been suggested that MALDI-TOF MS–based typing may be quicker than molecular typing for investigation of *Acinetobacter baumannii* outbreaks (62). Also, clonal lineages of *Staphylococcus aureus* can be identified on the basis of MALDI-TOF MS analysis of point mutations in stress and ribosomal proteins, as can *E. coli* pathotypes (63–66). Interestingly, MALDI-TOF MS can also be used to track plasmids (67). This allows for the investigation of infectious outbreaks related to horizontal plasmid transfer rather than local spreading of bacterial strains. Additional studies into reproducibility and robustness of these analyses are eagerly awaited, and methods for data interpretation are yet to be described. Again, there are currently no commercial offerings for this type of analysis, but there is proof of principle with regard to the MALDI-TOF MS–based typing procedures. It remains to be shown whether this sort of typing procedure will be competitive with nucleic acid sequencing–based approaches.

MOLECULAR DIAGNOSTICS INCLUDING MS ANALYSES

To date, MALDI-TOF MS has been primarily used for proteomic analyses. The two main distributors of MALDI-TOF mass spectrometers, Bruker (Bremen, Germany) and bioMérieux (Marcy l'Etoile,France), have a significant installed customer base, and the method is increasingly popular among clinical microbiologists. However, MS procedures can also be used for the characterization of DNA molecules, especially PCR-amplified DNA fragments. Many papers have been published, primarily ones that use electrospray ionization (ESI) MS applications rather than the current MALDI-TOF strategies (68, 69). Such methods have proven useful to demonstrate microbial diversity that goes beyond the resolution of MALDI-TOF MS and have been shown to be particularly capable of detecting genetic variability after DNA amplification. However, only a single such commercial system, using ESI MS, has been demonstrated to have potential utility for routine clinical microbiology applications. The Ibis PLEX-ID system (currently renamed the IRIDICA system, marketed by Abbott) was demonstrated as a useful tool for bacterial detection and identification (70). Since the system first amplifies DNA and then characterizes the amplified material, the approach is compatible with direct analyses of clinical samples without the time-consuming cultivation step required for "classical" microbiological MALDI-TOF MS. So the sequence of events is DNA purification from a clinical sample, targeted amplification of this genetic material, ESI MS, and finally, using sophisticated mathematical algorithms, identification of pathogens. Although the method is costly and may be relatively difficult to adapt for routine use in all but high-throughput microbiology laboratories, the strategy holds promise as demonstrated in recent literature.

Detection of potential bioterrorism-relevant organisms using ESI MS has been studied in detail, and reports show good sensitivity and applicability in a variety of matrices (70). Among clinically relevant fungal species, *Aspergillus*

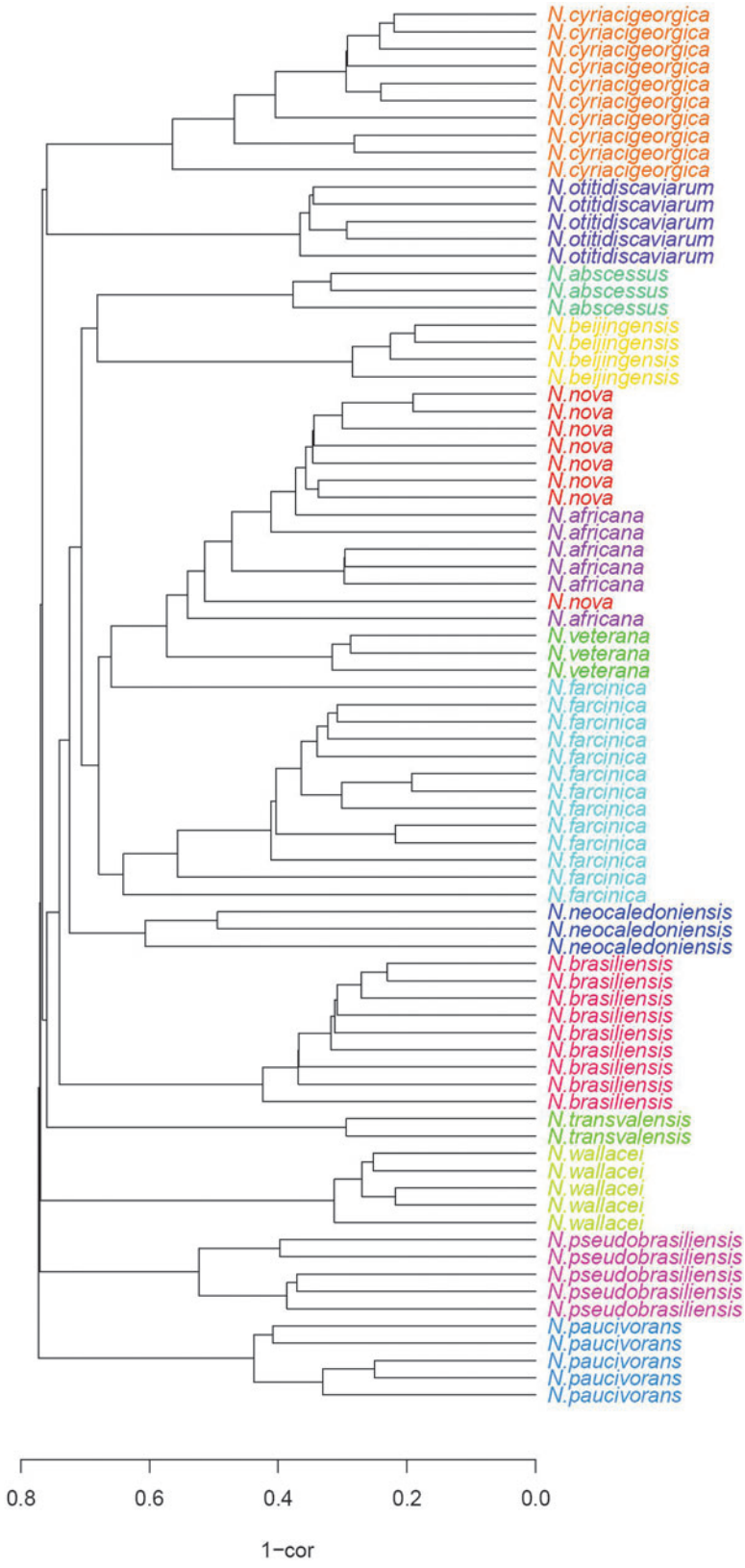

FIGURE 2 Dendrogram (Spearman correlation dissimilarity index, single linkage criteria) based on the MALDI-TOF MS spectra for strains of *Nocardia* spp. used for the VITEK MS knowledge base creation. For each strain, replicate measurements were summarized into a prototype spectrum by retaining only the peaks found in 50% or more of the spectra. Most *Nocardia* spp. are clearly separated by MALDI-TOF MS except *Nocardia nova* and *Nocardia africana*, at least with the VITEK MS system.

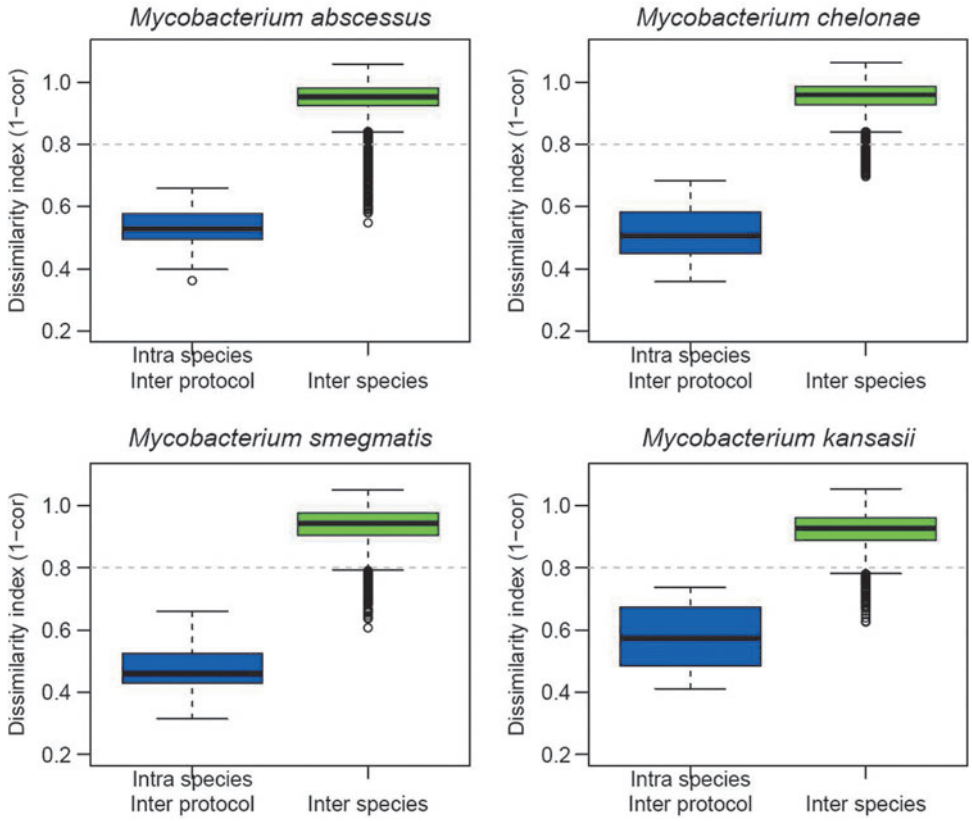

FIGURE 3 Distribution of intraspecies pairwise dissimilarity compared to interspecies pairwise dissimilarity based on the MALDI-TOF MS spectra of some species of *Mycobacterium* used for the VITEK MS knowledge base creation. For each strain, replicate measurements were summarized into a prototype spectrum by retaining only the peaks found in 50% or more of the spectra. For intraspecies pairwise dissimilarity, prototype spectra obtained from liquid cultures were compared to prototype spectra obtained from solid, agar-based cultures. For interspecies pairwise dissimilarity, prototypes from one species were compared to those from all other species. Despite the variability observed in spectral patterns in response to culture conditions, the intraspecific variability of spectra was found smaller than the interspecies variability.

fumigatus and *Candida albicans* showed sensitivities of 94.4 and 99.2%, respectively (71). Similar performance was demonstrated for the detection and characterization of other bacterial species (72), even when working directly from clinical specimens (73). Because of the initial PCR amplification step, the method is also compatible with the detection of viruses, a capability that is still quite cumbersome with the MALDI-TOF MS approach (74). Compared to alternative viral detection assays (including fluorescent antibody staining or other commercial PCR systems), the ESI MS assays performed very well (75). The method is also well suited for further typing of microbial pathogens including the detection of specific virulence or resistance genes (76). In conclusion, the use of ESI MS may very well complement the more classical MALDI-TOF MS assays, although it remains to be seen whether ESI MS–based sequence identification will be supplanted by (next-generation) enzymatic DNA sequencing.

CLINICAL IMPACT

Applications in the field of mass spectrometry need to be cost effective and provide added value in the clinical care of patients (77, 78). In this regard, clinical evaluations have recently appeared in the international literature. Preliminary studies in the United States have demonstrated the added value of MALDI-TOF MS–mediated identification of bacterial isolates from patients with recurrent bacteremia (79). Such individual analyses are important, but longitudinal studies on the overall effect of rapid diagnostics in changing patients' morbidity and mortality are required to more clearly document medical value. A Dutch study on the effect of faster identification of bacterial species in positive blood cultures demonstrated that identification via MALDI-TOF MS reduced the time to identification by more than a day (80); this was confirmed in a subsequent Swiss study (81). This resulted in a significant increase in the number of patients receiving appropriate antibiotic therapy: 64.0% in the control group versus 75.3% in the MALDI-TOF MS group ($P < 0.01$) (82). There was no demonstrable effect on morbidity and mortality, but the study was notably small. Also, results may differ in settings where the prevalence of multiresistant microorganisms is greater. A report from Belgium showed an 11% therapy change based on MALDI-TOF MS results and also proposed that MALDI-TOF MS was helpful in defining contamination rather than real infection in septic patients (83). When a Swiss study compared the impact of Gram staining versus

MALDI-TOF MS on the management of bacteremia caused by Gram-negative organisms, the outcome was optimistic (81). In 35% of cases of infection, MALDI-TOF MS affected the spectrum of prescribed antibiotics. MALDI-TOF MS was more frequently the basis of de-escalation of antibiotic treatment than Gram staining, although it has to be pointed out that all of the current studies were performed in settings where antimicrobial stewardship programs were already vigorously implemented before the study. As yet there are no data on precise assessment of the effect of rapid MALDI-TOF MS on morbidity and mortality.

COMPARATIVE AND TECHNICAL MULTICENTER STUDIES

When a new technology is introduced, its various iterations are compared to "gold standard" assays using side-by-side evaluations. In all comparisons performed thus far, MALDI-TOF MS has turned out to be at least as good as the reference standard method. In many cases, MALDI-TOF MS has outperformed classical testing (see, for instance, reference 84; there are many other papers showing similar results). Currently, the MALDI-TOF MS market is dominated by two commercial vendors: bioMérieux, offering the VITEK MS, VITEK MS RUO, and VITEK MS Plus systems (France), and Bruker Daltonics, marketing the MALDI Biotyper and MALDI Biotyper CA systems (Germany). Accordingly, most comparisons of performance have been done with these two brands. One of these comparative studies was published in early 2012 and evaluated identification of nonfermenting Gram-negative bacilli from cultures of patients with cystic fibrosis (85), a study that was recently complemented by an additional one analyzing Gram-negative rods from a similar patient cohort (86). In the first study, species-level identification was not significantly different between the two MS methods. However, the MALDI Biotyper system required more extractions in order to generate interpretable spectra. The second study demonstrated agreement with 16S rRNA sequencing of 96.6, 90.1, and 93.6% for the Bruker Biotyper, VITEK MS systems using Saramis version 4.09, and VITEK MS with the Knowledge Base version 2.0, respectively (86). A similar study on yeasts was recently published by a combined team from Rome and Milan, Italy. Again, using the clinical databases of the two systems, no significant difference was found for correct species identification (87). The studies showed that open database extension, as can be done with both systems, improved results.

A third study performed in Brussels, Belgium, was the first where routine application of the two systems was compared. More than 1,000 isolates were tested using the MALDI Biotyper and the premarket version of the VITEK MS Plus system; spectra were interpreted using three databases (88). Among 986 routine isolates identified to the species level, 93% were correctly identified by the Biotyper and Vitek MS databases. Neither system was able to discriminate between *E. coli* and *Shigella* species. The performance of the Saramis database, which is available as an open database for the VITEK MS system, was globally poorer. A multicenter study employing a single type of instrument confirmed the reproducibility of the technology for identification of *Brucella* species, as did a multicenter study on yeast in 10 Korean hospitals (89, 90).

Finally, a recent study from Seattle compared the diagnostic performance of the two systems in combination with two simplified protein extraction protocols including vortexing with silica beads in the presence of ethanol for identification of *Mycobacterium* species (91). The commercial Bruker database was augmented with an in-house database composed of clinical *Mycobacterium* strains. The 198 isolates tested were correctly identified to the species level in 94.9% of cases using the augmented database and in 94.4% of cases using the bioMérieux system. Notably, only 79.3% were identified to the species level using the nonaugmented Bruker database; if a lower identification score threshold of ≥1.700 was applied, the identification percentage increased to 93.9% using the nonaugmented database. For mycobacteria, as for any other pathogenic bacterial species, sample preparation is key, and inactivation protocols for processing such samples outside of a biosafety level 3 environment are important (92).

Quality verification of diagnostic equipment and assays is within the domain of the U.S. FDA. To be able to apply for FDA approval of a test system, external validation studies are important (93, 94). For the VITEK MS system the performance of such studies included over 6,000 clinical bacterial isolates, among which about 180 different species were identified. This led to FDA approval for the identification of these species in clinical laboratories. This leaves open the question of how to adequately validate databases covering thousands of different species. However, overall, the commercially available systems work well and provide clinically actionable data. There are differences between systems and databases, but on the whole, the choice between the systems will likely depend on specific workflow needs, laboratory information system connectivity requirements, and unique characteristics of the purchasing laboratories or, on the other hand, the qualities or shortcomings of the manufacturers.

CONCLUSIONS AND FUTURE PERSPECTIVE

Clearly, MALDI-TOF MS is here to stay, although, of course, many studies are also focusing on the potential usefulness of next-generation MS tools (95). For the coming decade, the MALDI-TOF technology will continue to prevail and dominate the microbial identification market while applications in the domain of viral detection and identification are currently being developed as well (96). DNA whole-genome sequencing is currently identified as a potentially invasive technology covering the same domain, and it holds the potential to, in the end, take away market share because of its ability to identify resistance mechanisms, epidemiologically important genetic polymorphisms, and virulence traits as well as provide identification, all in the same assay. However, the simplicity, speed, and low cost of MALDI-TOF MS, including its convenient sample handling, will not be easily challenged by next-generation sequencing technologies in the very near future. While it is possible that sequencing may ultimately replace certain MALDI-TOF MS applications, this will only occur when the process is significantly simplified from a processing and timing standpoint and when the cost is much reduced. Today, MALDI-TOF is state of the art, and it is important to further enrich the MALDI-TOF MS field with innovative diagnostic applications.

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Multiplex Technologies

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9

It is well appreciated that the adoption of molecular methods by clinical laboratories has revolutionized the identification of microbial isolates, detection of pathogens directly from clinical specimens, and methods for monitoring response to antiviral therapy. As discussed in chapter 1 of this volume, hybridization and amplification technologies continue to mature and enable new diagnostic applications, particularly as the ability to multiplex has become a reality. Multiplexing allows for the simultaneous detection of more than one target, whether it is a pathogen and internal control, multiple pathogens, pathogen and resistance determinants, or pathogen and host sequences. Multiplex detection performed on a single specimen allows laboratories to expand the breadth of their testing menu while improving efficiency and decreasing reagent and personnel costs. This chapter will review the multiplex technologies with potential diagnostic applications, including multiplexed hybridization and amplification and microarrays. Although the ultimate multiplexing technology could be considered next-generation sequencing, this technology and its applications are discussed in chapters 5 and 6 and section II.

MULTIPLEX HYBRIDIZATION

Nucleic acid probes have long been used for culture confirmation as well as direct detection of organisms from clinical material. Applying this technology in a multiplexed manner allows for the expansion of clinical applications. Probes are single-stranded oligonucleotides that can vary in size from 20 base pairs (bp) to a few kilobases but are generally less than 50 bp. Probe specificity is defined by the nucleic acid sequence of the probe. Typical probe targets include genomic DNA or RNA, mRNA, or rRNA. Probe detection occurs by hybridization, or annealing, of a labeled probe to a target sequence which has been released by lysis of the organism. Hybridization generally uses stringent conditions (i.e., high temperature, low salt) to promote the highest specificity, often allowing even a single base pair change to be detected. Following hybridization, the probe-target hybrids are generally either isolated and detected using a luminometer or viewed using fluorescent microscopy.

Hybridization can occur in liquid phase or in solid phase. In liquid-phase hybridization, both the probe and the target are free in solution to interact, allowing for more rapid annealing and thus shorter assay times. Disadvantages of liquid-phase hybridization include a requirement for relatively pure target nucleic acid, and interference from related but not identical target nucleic acid sequence can affect detection (1). In contrast, solid-phase hybridization uses solid media such as nitrocellulose, nylon membrane, or glass slide to immobilize the target nucleic acids. Labeled probes are then added to the solid support, allowing for detection of the nucleic acid sequence of interest. The format can also be reversed such that the probe is immobilized on a solid surface and target sequences are added in solution. This reversed format allows for the detection of multiple analytes from a single specimen preparation or the detection of multiple polymorphisms in a single amplicon generated using consensus primers for amplification (i.e., hepatitis C and human papillomavirus genotyping, resistance detection in *Mycobacterium tuberculosis*). Examples of multiplexed hybridization detection include *in situ* hybridization (ISH) and microarrays, the latter of which will be discussed under a separate heading.

Probes used for ISH can be either DNA or RNA and are typically short (15 to 30 bp), which allows for easier penetration and access to the target site. Lysis of cellular membranes and proteins must occur to allow permeation and hybridization of the probe. Both colorimetric and fluorescent ISH probes have been described; common fluorescent labels include fluorescein, rhodamine, their derivatives, and cyanine dyes (1). A powerful application of ISH is the use of multiple probes tagged with different fluorophores for the simultaneous detection of multiple organisms in a single specimen or the simultaneous detection of an etiologic agent and immunohistologic markers. Advantages of ISH applications in histopathology are that the host tissue response can be evaluated, and the exact cells displaying a specific morphotype can be probed for suspected etiologic agent(s). Disadvantages include autofluorescence exhibited by some microorganisms (including *Pseudomonas*, *Legionella*, many yeasts, and molds), highly probe-sequence-dependent specificity and reliability, insufficient penetration of sample material, secondary structure of target sequence, low target content, and photobleaching (2).

For direct identification of microbial organisms in patient samples or cultures, peptide nucleic acid fluorescent ISH (PNA-FISH) has been described. PNA probes have a neutral peptide-like backbone, as opposed to the negatively charged sugar phosphate backbone found in DNA

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probes (3). However, like DNA probes, PNA probes still hybridize to DNA and RNA in a sequence-specific manner and can be labeled fluorescently for ease of detection. Reported advantages of PNA probes include stronger and faster hybridization, ability to discriminate 1-bp differences, resistance to nucleases and proteases, survival under stringent conditions that allow for access to regions with secondary structure, and increased hydrophobicity that allows for penetration of cell membranes during ISH (3). PNA-FISH allows for multiplexed detection directly from positive blood culture bottles since different fluorophores can be used to identify different target sequences. Commercially available PNA-FISH tests for multiplexed detection and differentiation of microorganisms from positive blood cultures include *Staphylococcus aureus*/coagulase-negative staphylococci, *Enterococcus faecalis*/other enterococci, *Escherichia coli*/*Pseudomonas aeruginosa*/*Klebsiella pneumoniae*, and *Candida albicans* or *Candida parapsilosis*/*Candida tropicalis*/*Candida glabrata* or *Candida krusei* (AdvanDx, Inc., Woburn, MA) (4).

MULTPLEX SIGNAL AMPLIFICATION

Another methodology that is used for the multiplex detection of nucleic acid sequences in clinical specimens is signal amplification. This technique is unique in that the signal, as opposed to the target (as in PCR), is amplified. Due to the amplification of the signal generated from hybridization, signal amplification is more sensitive than nonamplified direct detection but is generally not as sensitive as target amplification methods. However, there seems to be less risk of contamination with signal amplification than with target amplification techniques.

Although signal amplification methods have been available for over a decade (see chapter 1) and have been used clinically for quantification of a variety of viruses, their use in multiplex detection is relatively new. The introduction of gold nanoparticle technology has allowed sensitive detection of nucleic acids and proteins without the need for target amplification (Nanosphere, Northbrook, IL). However, for the most sensitive detection, either culture amplification or target amplification should occur prior to signal amplification. The most common clinical application of multiplex signal amplification (in the absence of target amplification) is detection and differentiation of organisms and resistance determinants from positive blood cultures (Verigene System, Nanosphere). Since the organism is amplified by culture, target amplification is not needed prior to detection by signal amplification. Target sequences from lysed organisms are hybridized on a microarray by capture oligonucleotides (Fig. 1). A gold nanoparticle (13 to 20 nm) with sequence-specific probes is subsequently hybridized via specific mediator oligonucleotides. The end result is hybridization of the target sequence to a gold nanoparticle on a microarray spot. The nanoparticles are then coated with silver, which increases their size (0.5 to 1 um) and their reflectivity. Optical analysis of LED light scatter is used to detect signal from hybridized spots on the microarray and generate test results (5).

Commercially available tests using multiplex signal amplification without the need for target amplification include the Gram-positive blood culture test and Gram-negative blood culture test; a yeast blood culture test is in development. The Gram-positive blood culture test includes targets to detect staphylococcal, streptococcal, and enterococcal species, as well as *Listeria* spp. and resistance determinants *mecA*, *vanA*, and *vanB*. The Gram-negative blood culture test detects *E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *P. aeruginosa*, *Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp., *Proteus* spp., and select resistance genes for extended spectrum beta-lactamases (CTX-M) and carbapenemases (IMP, KPC, NDM, OXA and VIM).

MULTIPLEX AMPLIFICATION

With careful planning and design, it is possible to create sets of primers that can be run in the same amplification reaction together or multiplexed. By multiplexing, the user

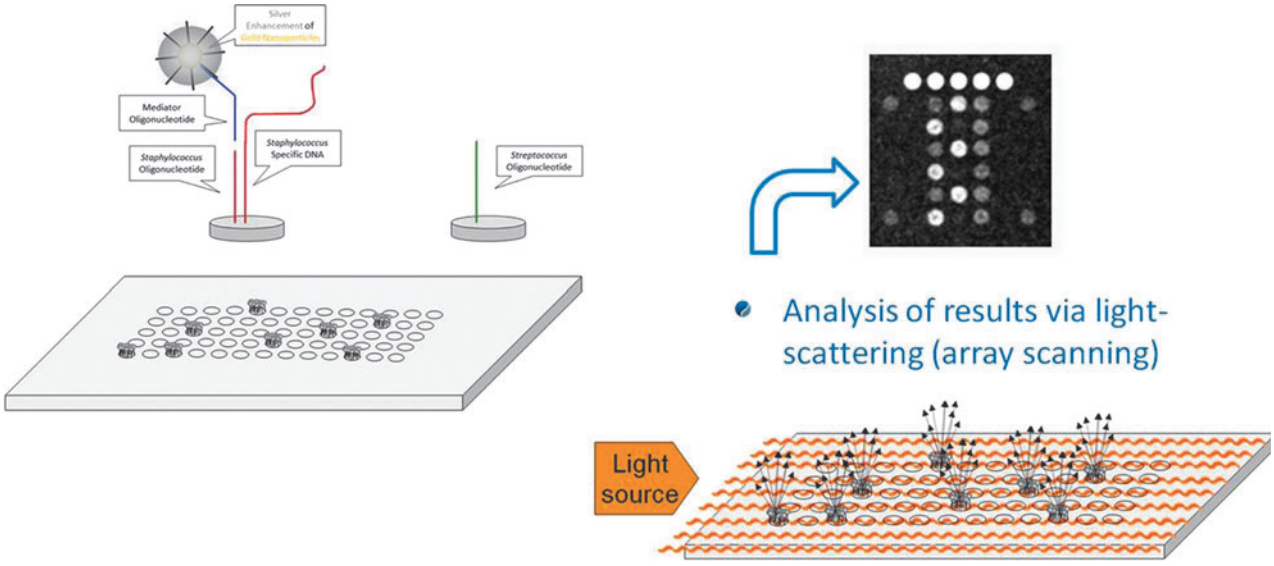

FIGURE 1 Signal amplification and hybridization. This diagram depicts the detection of *Staphylococcus*-specific DNA using gold nanoparticles followed by signal amplification. The targeted DNA is hybridized to a specific oligonucleotide on an array. A mediator oligonucleotide is bound to a gold nanoparticle and then hybridizes at the specific array site. Following silver-based signal amplification, light scattering is detected at each of the array locations. Image provided by courtesy of Nanosphere.

is able to amplify and detect multiple targets from one nucleic acid sample in one reaction vessel. As discussed in chapter 1, there are numerous amplification and detection methods that also apply to multiplex reactions. Multiplex detection methods essentially fall into two broad categories: endpoint detection, where only the final products are analyzed (either by composition or hybridization), and real-time detection, where the products are evaluated throughout the reaction.

Endpoint Detection by Composition

There are two predominant methods for endpoint detection of multiplex PCR: composition based and hybridization based, though within each category there are a variety of specific methods. The most basic of multiplex reactions uses different primer sets that generate amplicons of different, but predictable, sizes that can be resolved using standard gel electrophoresis. Gel electrophoresis utilizes the fact that DNA is negatively charged and will migrate away from a positive source within an electric field. In this case the DNA has to move through a matrix of gel, typically made of agarose. Larger molecules have a more difficult time moving through the gel matrix, and their movement in the assay is impeded. Smaller amplicons have an easier time moving through the gel, and this is demonstrated by increased migration away from the field source. In this way, the composition of the product directly influences the result. The PCR products are stained using dyes such as ethidium bromide or SYBR Green, often within the gel itself, and can be visualized using UV or blue light. This method is useful when looking for only a few targets and can be implemented without special thermocyclers or other equipment beyond what is typically used for PCR in a laboratory. This approach is limited by the number of amplicons that can be reliably resolved, and quantitative analysis may be difficult.

SYBR Green is also commonly used in a different type of endpoint analysis: melting curve analysis. Since SYBR Green is most fluorescent when intercalated within double-stranded DNA (6), seeing a reduction in fluorescence while increasing the temperature applied to the PCR product indicates the point at which the two strands of the product disassociate. By manipulating the plot that is generated, typically by applying a negative first derivative, a characteristic peak(s) can be derived, equating to the melting point of the product (6). Importantly, the melting point of the product is directly related to the G/C content (6). Therefore, even products of similar sizes can be resolved as long as their G/C content is sufficiently different.

Another more sophisticated method of composition-based endpoint detection is using mass spectrometry (MS) to analyze the end products of a PCR reaction. Under the names Ibis Biosensor, PLEX-ID, and IRIDICA, PCR is followed by electrospray ionization with mass spectrometry (ESI-MS) (Abbott Molecular, Des Plaines, IL). In ESI-MS, the molecule of interest, the PCR product in the case of PCR ESI-MS, is introduced to the mass spectrometer via ESI (Fig. 2). In this method the sample must first be nebulized, which is achieved by introducing the product to a high electric field at the tip of a capillary tube. Eventually, the electrostatic forces overwhelm the surface tension, and an electrically charged droplet is released from the tip into the surrounding chamber. This droplet contains multiple ionic species. As the droplet travels through the gas in the surrounding chamber, solvent evaporation

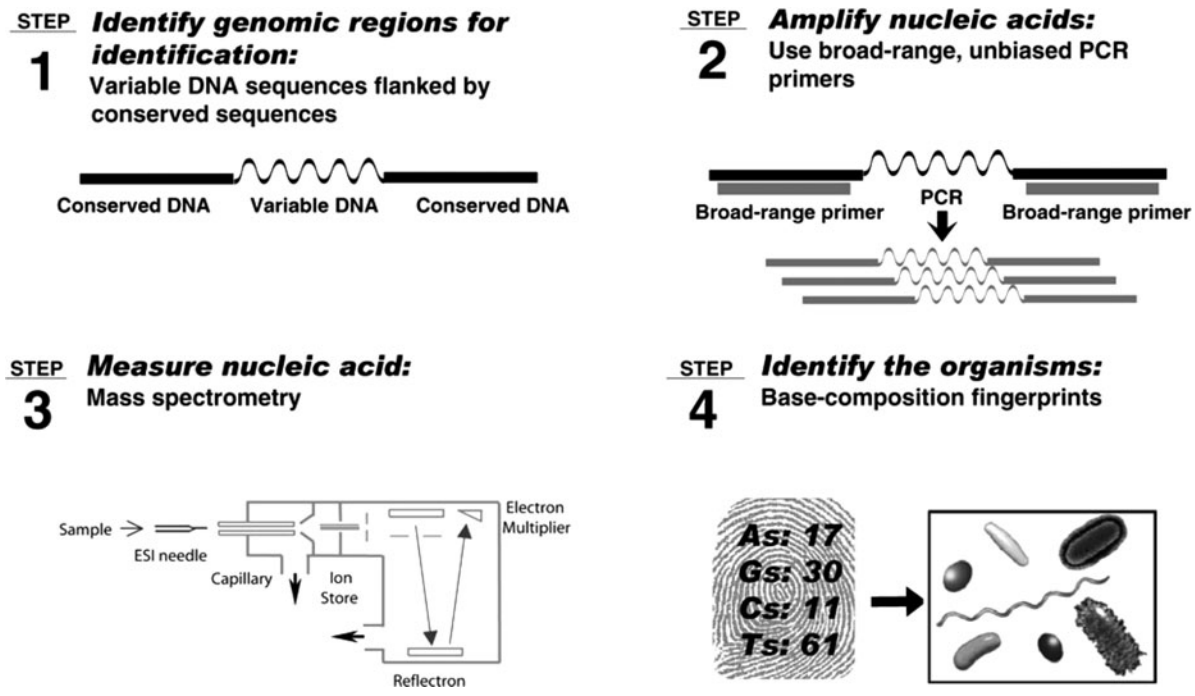

FIGURE 2 PCR electrospray ionization with mass spectrometry. Genetic regions of interest are first identified (step 1). Broad primers near the region of interest are utilized to amplify that region (step 2). The products of these reactions are measured using mass spectrometry (step 3). The sample is introduced via an ionization needle into a capillary, where the solvent is stripped away. Ions are then introduced into a chamber, where they are detected via an electron multiplier. The size of the ions is directly related to the base composition of the PCR product (step 4), which is utilized to determine the identity of the target.

and shear forces result in the release of individual ions (7). In the case of PCR ESI-MS, these ions are components of a single-stranded species. These ions are then introduced into a spectrometer and a mass is determined. Each mass is associated with a unique combination of nucleotides such that for any one mass, the base composition (but not sequence order) is known. By combining multiple primer sets, one can reliably determine the identity of an unknown target with a few different reactions (8).

The iPLEX MassArray system by Sequenom (San Diego, CA) is another system that utilizes MS as a method for detection of a PCR reaction. In the case of the iPLEX, a number of single-base extension PCRs are performed and then analyzed using matrix-assisted desorption ionization–time of flight MS. One of the keys for analysis on this instrument is the utilization of mass-modified terminating nucleotides that allow for the location of the incorporation of these specific nucleotides to be determined. Matrix-assisted desorption ionization–time of flight MS is becoming more common in the diagnostic microbiology laboratory for its ability to discriminate protein profiles of different microorganisms (see chapter 8). The technology used in the iPLEX is similar to that used for identification of organisms except that the starting product is nucleic acid as opposed to bacterial or fungal cellular proteins. After being mixed with an organic matrix to aid in energy distribution, laser energy is utilized to ionize the sample. These ions are then organized in an electric field before release into a drift tube. Ions with a lower mass/charge ratio move faster through the tube, while ions with larger ratios move more slowly. A detector at the end of the tube registers the frequency of ions at any one mass/charge ratio. This technology has been shown to be an effective method of phylogenetic investigation (9) and has potential applications for not only outbreak investigation but pathogen discovery as well.

Methods such as PCR ESI-MS and iPLEX MassArray have the potential to change molecular diagnostics by providing an approach that has limited bias. The question that remains is will these technologies remain restricted to those with the financial resources and expertise, much like next-generation sequencing currently is, or will these technologies become more useful for routine diagnostic laboratories? The ability to assess the composition of dozens of products simultaneously, however, makes these technologies attractive platforms for future development and ensures that they will be ones to monitor in the coming years.

Endpoint Detection by Hybridization

One common way of detecting PCR products while increasing the specificity is to utilize a nucleic acid probe. This probe binds to a complementary sequence internal to the PCR product and can be detected via a variety of methods. While this was initially done using radiolabeled probes, the most common way today is to use different fluorescent probes for each target. The use of fluorescently labeled probes has allowed for the development of real-time monitoring strategies (see below and chapter 1). Many commercial multiplex platforms use target amplification followed by array-based detection in which probes are fixed to a substrate and their spatial location determines their identity. The Nanosphere Verigene system currently has amplification assays for the detection of respiratory and enteric pathogens that use a printed array on a glass slide. The amplified products hybridize to complementary oligonucleotides at specific locations on a glass slide. Secondary mediator probes hybridize to the captured target and allow for further hybridization with the gold nanoparticle signal probes. The Genmark eSensor (Carlsbad, CA) has assays to detect respiratory viruses, distinguish hepatitis C virus genotypes, and identify cystic fibrosis mutations. This platform uses an electrochemical array to identify products. The products of a multiplex PCR reaction are labeled with iron-containing probes. The labeled products then pass through a flow cell over gold electrodes which contain complementary oligonucleotides. If the target of that oligonucleotide is present in the sample, then the ferric probe allows for the completion of an electronic circuit, the position of which determines the identity of the target. The FilmArray system (BioFire Diagnostics, Salt Lake City, UT) combines multiplex and nested PCR with melting curve analysis in their multiplex arrays for respiratory and gastrointestinal pathogens as well as blood culture identification. After nucleic acid purification, an initial large volume massively multiplexed reaction occurs. This reaction mixture is then diluted into wells, where a second-stage single-plex reaction takes place. Each well has a predetermined secondary reaction, such that the presence of a positive signal specifically identifies the target. Additionally, the products of this second, nested reaction are analyzed via melting curve analysis to further increase specificity. Suspension (or liquid)–based arrays where the probe is attached to a specific-sized bead and analyzed via flow cytometry are also available commercially (see "Microarray Technologies" below).

Array-based detection methods greatly expand the number of targets that can be discriminated in a single amplification reaction beyond what can be determined using other methods of endpoint detection such as gel electrophoresis. This has allowed for the development of syndromic panels for infectious disease for conditions such as respiratory infections and gastroenteritis (10–12). Using a syndromic-based approach greatly reduces the diagnostic bias compared to single-plex reactions, though because these methods are still looking for specific targets, they are more biased than methods such as next-generation sequencing or PCR ESI-MS. Because these panels may detect anywhere from 14 to 22 targets, additional measures must be taken to ensure the sensitivity and specificity of the reactions for all analytes. This can be achieved in a number of ways including using multiple reactions for the same target since the product discrimination step is no longer rate limiting. Additionally, since array-based detection of amplified products is an endpoint method, no information is available until the process is complete, which limits the ability to perform these assays rapidly without sacrificing the number of cycles performed in the amplification step. A drawback of some array-based detection technologies is that they require the handling of amplified material in the laboratory, increasing the likelihood of contamination. This typically translates to a need for staff with specialized training and expertise to run these assays while limiting contamination. More assays are being developed like the FilmArray and Verigene, which do not require pre-extraction or manipulation of postamplification material and are more applicable to implementation in laboratories with limited molecular experience.

Real-Time Detection

Hydrolysis probe– or Taqman-based chemistry is at the heart of many real-time detection methods. In hydrolysis probe–based real-time methods, a probe is designed that is

internal to the primers of the target of interest and contains a 5′ fluorophore and a 3′ quencher. See Tables 1 and 2 for common fluorophores and quenchers used in these reactions (13). The advantages of hydrolysis probe–based real-time PCR are its facile adaptation to multiplexing and utility in reporting styles. As long as nonoverlapping fluorophores are used, multiple targets can be queried in the same reaction. This can either be additional targets amplified using different primers or different targets of a variable region amplified by a set of common primers. The assay can provide a positive/negative readout based on whether or not the fluorescent signal crosses a predetermined threshold. This threshold is typically set using control or other known material, and it represents the amount of fluorescence that needs to be generated for a reaction to be considered positive. Different thresholds can be set for different targets in the same reaction to allow for more stringent determination of some analytes (e.g., process or internal control). Finally, results can be reported semiquantitatively using the cycle threshold value because, in general, specimens with lower cycle threshold values start with more template DNA than those with high cycle threshold values. This can be particularly useful in multiplex reactions to determine the relative abundance of the different targets compared to one another.

Many commercial and laboratory-developed multiplex assays use real-time hydrolysis probes, though they all have similar strengths and weaknesses. These assays are easy to design and relatively simple to implement since many molecular laboratories already have the necessary equipment. Since the detection step occurs during the reaction, there is no need to have additional equipment such as that needed for mass spectrometry or array-based detection of multiplex products. These assays can also be performed and resulted quickly since there are no additional steps after the reaction is set up. Finally, real-time detection allows the evolution of the reaction itself to be queried. This can play an important role in discerning falsely positive or negative results, which is not possible with endpoint detection methods. The main problem with hydrolysis probe–based real-time detection is that it is limited by the number of unique emission wavelengths that can be read by the spectrophotometer. In most cases this limits the number of members of the multiplex reaction to between 2 and 6 (13). This limits not only the number of targets that can be queried in any one reaction, but introduces challenges in assay design because there is typically no room for the redundancy seen with array-based detection methods. Thus, targets must be chosen carefully for each assay.

In addition to being a method of endpoint detection, melting curve analysis can also provide real-time results. The LightCycler instrument (Roche Diagnostics, Indianapolis, IN) has multiple assays, both laboratory- and commercially developed, that utilize this technology. The CE-marked SeptiFast test is one example that can detect eight different Gram-negative bacteria, six different Gram-positive bacteria, and six different fungi from a whole-blood sample in a few hours, with modest performance compared to blood culture (14).

TABLE 1 Common fluorophores used in multiplex detection

Fluorophore	Excitation (nm)	Emission (nm)
FAM	495	515
TET	525	540
JOE	535	555
TMR	555	575
ROX	575	605
Texas Red	585	605

TABLE 2 Common quenchers used in multiplex detection

Quencher	Maximum absorption (nm)
BHQ-1	534
BHQ-2	580
BHQ-3	670
Dabcyl	475
Iowa Black RQ	645

Scalable transcriptional analysis routine (STAR) combines PCR with real-time capillary electrophoresis. The Modaplex (also ICEplex) system by PrimeraDx (QIAGEN, Mansfield, MA) has several research-use-only kits available including assays for sexually transmitted infections and a viral panel designed for transplant recipients. For a STAR experiment, each target of interest has primers that generate a fluorescently labeled product. Each target must have a size variation of at least 5 nucleotides for the product to be distinctly resolved. After each round of PCR, a small aliquot of the reaction is removed and subjected to capillary electrophoresis. The area under each target on the electropherogram is used to reconstruct amplification curves. These reconstructed curves can then be treated similarly to real-time PCR curves for the determination of a cycle threshold or even quantification. Studies have shown that using STAR for the quantification of gene transcripts is equivalent to either TaqMan or SYBR Green–based methods with increased signal generation (15). STAR has a number of additional advantages over traditional real-time PCR including increased tolerance of amplicon size (up to 1,000 bp). Additionally, because STAR uses incorporation of a fluorescent primer, the system eliminates the need for a probe and allows for quantitation based on the presence of the product, not a byproduct of the reaction as with hydrolysis probes (16).

Multiplex detection of amplified products has a number of benefits. Multiplexing allows for more targets to be queried using the same amount of input specimen or nucleic acid, which is ideal for situations where volume is limited due to specimen type. Multiplexing also reduces a component of sample bias since all of the targets in the reaction query the same input nucleic acids. This can be especially valuable when looking for things such as polymorphisms or other small nucleotide changes. The expansion of methods of detection such as arrays and STAR has allowed for the development of massively multiplexed reactions with dozens of primer sets used in a single reaction capable of being discriminated from each other. One major drawback to multiplex reactions, however, is that oftentimes these methods are less specific and/or less sensitive than corresponding single-plex reactions. This primarily is due to sacrifices made in either primer design or reaction conditions to allow multiple sets of primers to work simultaneously, albeit at different efficiencies. Additionally, even though massively multiplexed platforms are playing a larger role in diagnostic testing, they still represent a biased approach. Whether an unbiased approach such as next-generation

sequencing is capable of matching the speed and value of multiplex amplification reactions remains to be seen. Until it does, however, multiplex amplification will remain a staple of diagnostic laboratories.

MICROARRAY TECHNOLOGIES

Similar to the PCR revolution, hybridization array technologies have transformed the complex analysis of transcriptomes, genomes, and proteomes in the research setting and have begun to modernize multiplex detection in the clinical diagnostic arena. Simply defined, an array is a collection of macroscopic or microscopic features that can be probed with target molecules to produce either qualitative or quantitative data. Though other arrays exist, such as protein arrays (17, 18), the focus of this section is DNA-based hybridization array technology.

Microarrays can be distinguished based on characteristics such as the nature of the probe, the type of solid surface support used, and the methodology of feature addressing or target detection. This section reviews the methodologies of cDNA, oligonucleotide, and suspension bead arrays. In each of these methodologies, the probe refers to the DNA sequence bound to the solid surface support in the microarray, whereas the target is the "unknown" sequence of interest. In general terms, the probes are synthesized and immobilized as discrete features, or spots. Each feature contains millions of identical probes. The target is labeled fluorescently and subsequently hybridized to the probe microarray. The fluorescence intensity of a successful hybridization event between the labeled target and the immobilized probe is measured by a fluorescent scanner, the data from which can then be analyzed by a variety of methods depending on the individual experiment. Experimental specifics such as probe length and synthesis, number of possible features (density of the microarray), and the solid surface used vary depending on the type of microarray employed and are discussed below.

Printed Microarrays

Printed arrays were the first type of microarray used in research settings and are so called because of the spotting, or "printing," of the probes onto the microarray surface, typically a glass microscope slide. There are a multitude of advantages to using glass slides, including that they are inexpensive, durable throughout high temperatures and stringent washes, and nonporous, allowing for effective kinetics of hybridization, and they have low background fluorescence (19). Printing occurs via either a contact or a noncontact printer. In contact printing, each print pin directly applies a solution of the probe to the slide, resulting in the application of a few nanoliters of probe solution to create a feature of ~100 to 150 μm in diameter. Noncontact printing uses the same technology as computer printers (i.e., bubble jet or ink jet) to expel small droplets of probe solution on the surface. Regardless of the method of printing, contamination control and printing consistency are crucial to preserving the quality of the printed microarray and subsequent hybridization data. Printed microarrays allow for ~10,000 to 30,000 features to be queried, which is a considerably lower density than synthesized oligonucleotide microarrays but a higher density than suspension bead arrays, discussed below. Printed arrays can be further characterized as double-stranded DNA (dsDNA) or oligonucleotide microarrays based on the nature of the probes. Figure 3 depicts a general workflow for the processing of printed microarrays.

In dsDNA microarrays, PCR amplicons are used as the probes. Probes can be amplified using primers designed from known genomic sequences, from shotgun library clones, or from cDNA (20–22). Ideally, each dsDNA probe (amplicon) is 200 to 800 bp and represents a different gene. Amplicon probes generally have a high sensitivity but may suffer in specificity. Hager et al. reported that 21 to 34% of probes either did not match the intended target and/or were contaminated (23). The decreased specificity of dsDNA probes may be beneficial if the user is interrogating regions rich in natural polymorphisms but detrimental if the user requires discrimination of highly similar target sequences (24). Redundancy in the inclusion of multiple gene segments increases the specificity of downstream hybridization experiments. While the production of a single PCR amplicon is straightforward, when this is scaled up to the thousands of amplicons needed for a pan-microbial array or bacterial genome, quality control and information management, as well as efficiency and accuracy, are challenging. PCR amplicons for microarrays must have maximum specificity, maximum yield, and no contamination (due to either nonspecific amplification or contaminants that affect attachment to the microarray surface or that autofluoresce) (25). The purity and quantity of the amplified product should be assessed by agarose gel electrophoresis, followed by sequencing if financially feasible. Attachment of amplicons to the glass slide surface typically occurs by the electrostatic interaction of the negative charge of the phosphate backbone of the dsDNA with a positively charged coating of the slide surface (26). In addition, UV cross-linking can be used to form covalent bonds between the thymidine bases in the DNA and amine groups on treated slides (19).

Oligonucleotide microarrays use short chemically synthesized sequences as the spotted probes. The length of probes typically used in printed microarrays varies from 25 to 80 bp, but Chou et al. report that 150-mer probes are optimal for gene expression microarrays (27). The shorter probe length of oligonucleotide microarrays may adversely affect sensitivity and specificity as compared to dsDNA probes, but they are also less prone to errors during production. Generally, sensitivity and specificity increase with the length of the probe, as does the strength of the hybridization signal. If shorter probes are needed experimentally, the hybridization signal strength can be improved by using spacers or a higher concentration of probe during printing (27). Very long probes may adversely affect specificity due to random matching to nontarget sequences. Thus, it is critical to carefully determine optimal probe length for each microarray design. The probe length also determines how many replicates of the probe or other gene target sequence are needed on the microarray to allow for optimal specificity; i.e., longer probes need fewer probes per gene. Oligonucleotide probes, though easier to manufacture, must be designed such that all probes have similar melting temperatures (within 5°C) and are void of palindromic sequences. Ideally, each probe should be tested experimentally to ensure nonbiased hybridization data (27). Oligonucleotide probes are attached to glass slides by covalent coupling of their modified 5′ or 3′ ends (usually a 5′ amino group) to coated slides that provide aldehyde or epoxy functional groups. Covalent linkage is necessary because, in contrast to dsDNA probes, a significant portion of oligonucleotide probes would be lost during subsequent

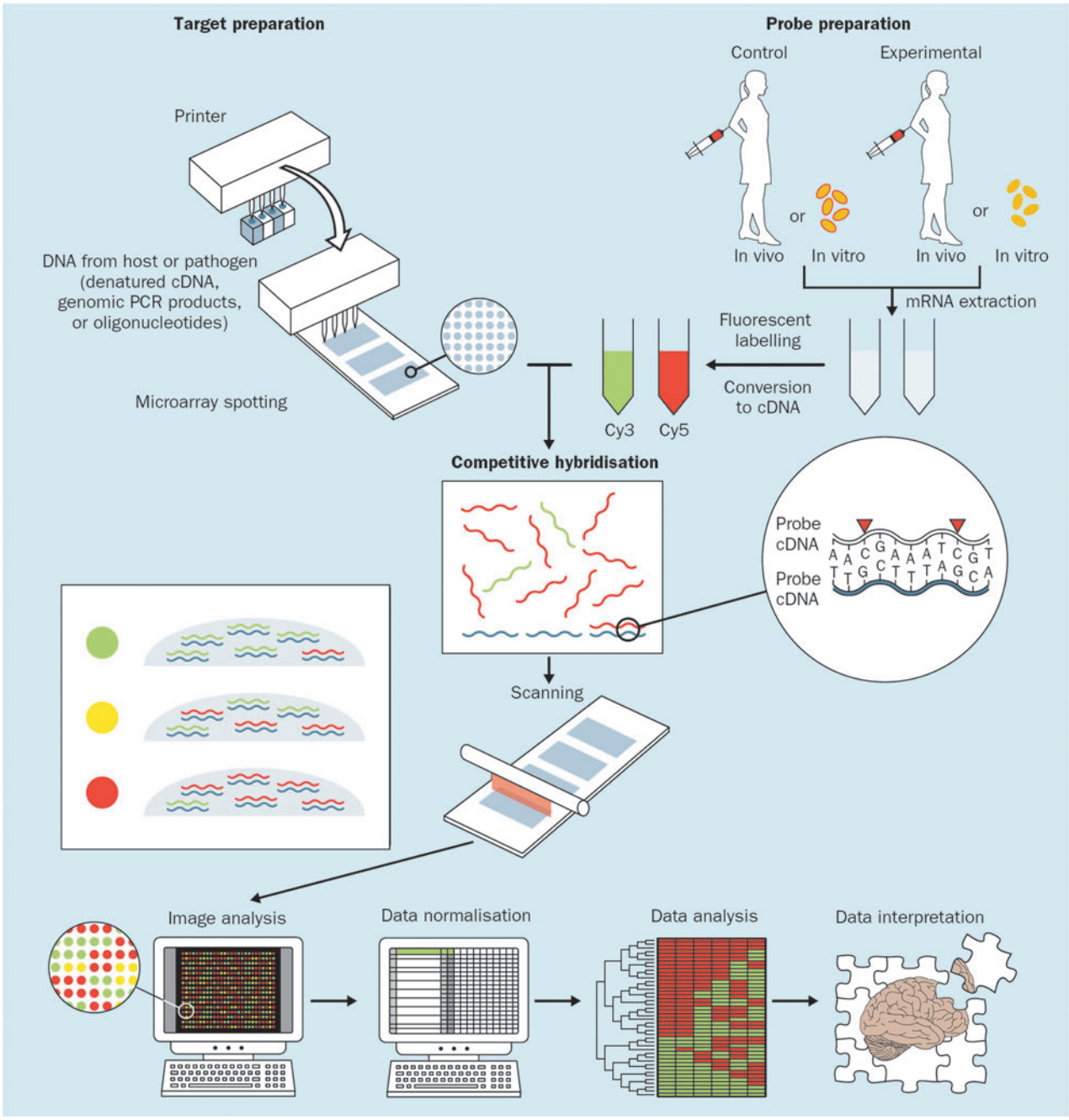

FIGURE 3 General workflow of printed microarrays. Probe preparation begins with the production of either denatured cDNA, genomic PCR products, or oligonucleotides that are subsequently spotted in an array format on a glass slide. In this example, two samples (one control and one experimental) are being compared by extracting mRNA and converting the RNA sets into differentially labeled cDNA sets for hybridization. The inclusion of multiple fluorescent labels allows for color differentiation based upon the quantity of target cDNA from each set that hybridizes to the microarray probes. The fluorescence signal is then scanned and analyzed. Reprinted from reference 55 with permission from Elsevier.

wash steps due to their small size. However, an advantage of the shorter probes is the ability to interrogate smaller genomic regions, such as polymorphisms.

Printed microarrays have the advantages of simplicity and relatively low cost compared to synthesized microarrays, discussed below. Nonetheless, the initial setup of microarray facilities is relatively expensive and requires a dedicated environmentally controlled space, with dust, humidity, and temperature well regulated. Many universities have dedicated microarray core facilities to overcome these challenges. However, these laboratories are generally not Clinical Laboratory Improvement Amendments certified, thereby limiting their diagnostic capability. Printed microarrays are most conducive to user-defined testing, which has largely been limited to microbiology researchers. However, the ability to quickly adjust spotted probes based on

updated annotations or the discovery of new, emerging pathogens makes this approach attractive for potential diagnostic applications. Drawbacks include the laborious production of PCR products or design of oligonucleotide probes and the errors introduced from probe synthesis. Though there are commercially available whole-genome microarrays for select organisms that are useful for research endeavors, there are no commercially available printed microarrays applicable for use in diagnostic microbiology.

In Situ Synthesized Microarrays

Synthesized arrays are high-density microarrays in which the synthesis of the oligonucleotide probes occurs directly on the surface of the array, i.e., *in situ* synthesis. The most widely known *in situ* synthesized microarrays are the GeneChips (Affymetrix, Santa Clara, CA). In contrast to printed oligonucleotide microarrays, *in situ* synthesized arrays are typically made of very short probes (20 to 25 bp), and 11 to 15 probes per target are included to improve analytical performance and statistical accuracy. Probes are manufactured in terms of probe sets: one perfect match probe and one mismatch probe that contains a 1-bp difference in the middle position of the probe (typically position 13 of a 25-bp probe). The use of probe sets increases the specificity of using a very short probe because the mismatch probe acts as a negative control to identify possible nonspecific cross-hybridization events. Generally 11 probe sets are used per 600 bases being interrogated (28). Each probe within a probe set is located in a separate feature.

Manufacturing of GeneChips occurs on a quartz wafer using semiconductor-based photochemical synthesis, which allows for $>10^6$ features on a typical 1.28-cm^2 array, depending on the interfeature space (28, 29). The quartz wafer is modified such that the surface offers hydroxyalkyl groups which can be covalently linked to photo-labile protecting groups. This creates a surface that prevents the addition of reactive nucleotides unless the surface is spatially activated by light. Photolithographic masks are used that have windows acting as filters to either transmit or block UV light from the chemically protected microarray surface. The pattern of windows in each mask represents the desired sequence content for each probe to be included on the microarray by instructing the order of nucleotide addition. UV light deprotects the exposed surface of the microarray, which allows for nucleotide coupling. Areas of the microarray surface in which UV light has been blocked remain protected from the addition of nucleotides. Each added nucleotide is modified with a light-sensitive protecting group such that the synthesis of the oligonucleotide probes occurs by cycling through masking, light exposure, and the addition of either A, C, T, or G bases to the growing oligonucleotide (Fig. 4) (26, 28).

Though GeneChips have been the most utilized to date in the literature, Agilent (Palo Alto, CA) also manufactures high-density oligonucleotide microarrays based on 60-mer probes and inkjet technology. The use of longer oligonucleotides reportedly increases sensitivity by 8-fold relative to GeneChips (30). Agilent microarrays synthesize 60-mer oligonucleotides on glass slides using five-ink (four bases plus catalyst) inkjet printing of nucleotide precursors combined with phosphoramidite chemistry (31, 32). Thus, lithographic masks are not needed.

Synthesized microarrays are not conducive to self-manufacturing, but rather rely on commercial manufacturing. There are limited numbers of different microorganism genome arrays currently available, and commercial customized microarrays are not easily updated and are very expensive. The advantages of these systems are the reproducibility of the manufacturing process and the standardization of reagents, instrumentation (fluidics system, hybridization oven, scanner) and data analysis (28). Additional advantages include controls such as reference probes for intensity normalization, internal standards of known concentrations, and probes arranged in a checkerboard pattern that are homologous to an internal control included in the hybridization mix. It should be noted that experiments performed with GeneChips are limited to one label, whereas Agilent allows for two-color hybridizations. Oligonucleotide microarrays (printed and synthesized) overall allow for much cleaner downstream data than amplicon-based microarrays. This is due to the ability to standardize probe concentrations and hybridization temperatures while avoiding significant nonspecific hybridization (24). Though synthesized oligonucleotide arrays are very robust systems, none are currently commercially available with direct diagnostic microbiology applications.

Suspension Bead Arrays

Expanding on the two-dimensional arrays discussed above, suspension bead arrays are essentially three-dimensional arrays based on the use of microscopic polystyrene spheres (microspheres) as the solid support. Suspension bead arrays build on the well-established technique of flow cytometry to provide multiplexing capability. Initial bead-based assays focused on the detection of antigens and antibodies and were first described in 1977 (33). Early multiplexing approaches used different-sized microsphere sets for the simultaneous detection of two to four antibodies (34, 35). To extend the multiplexing capability of microsphere-based assays, two different fluorochromes (red and infrared, 658-nm and 712-nm emission) are used at varying concentrations to fill 5.6-μm microspheres. Each microsphere then has a specific spectral address based on the red/infrared ratio within the microsphere such that the identity of each of 100 beads is distinct. Microspheres with specific spectral addresses can be coupled to a specific oligonucleotide (probe) that is unique for the target nucleic acids of interest. Once the individual microspheres have been coupled, a mixture of microspheres can be used to probe the sample type of choice. Thus, one can theoretically detect 100 features simultaneously using modern liquid bead array technology. Similar to planar arrays, the target is detected using a third fluorescent reporter.

After target hybridization, the microsphere suspension is analyzed using flow cytometry instrumentation. Luminex Corporation (Austin, TX) developed compact cytometers that specifically detect and process their proprietary microspheres with a high signal-to-noise ratio. The cytometers have two different lasers that the single-file microsphere suspension flows past. A 635-nm laser excites the fluorochromes contained within the microspheres, which allows for the identification of the bead and therefore the probe being analyzed. A 532-nm laser excites the reporter fluorochrome (most commonly, *R*-phycoerythrin or Alexa 532) to quantify the probe-target reaction on the microsphere surface (Fig. 5). The X-Y platform of these compact systems facilitates the reading of 96-well microtiter plates and therefore increases throughput.

Using the principles of the above technology, several chemistries have been developed for nucleic acid detection

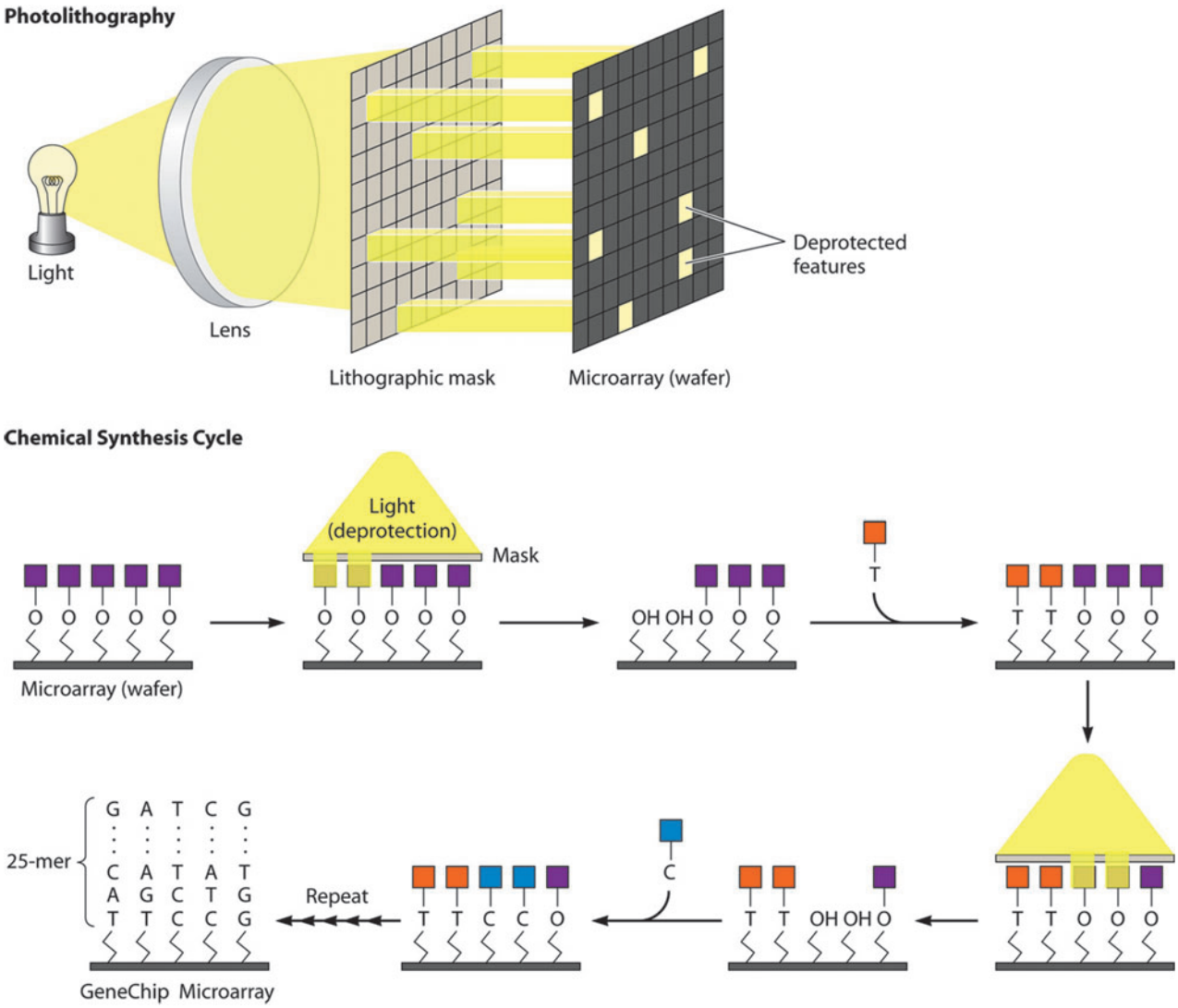

FIGURE 4 GeneChip oligonucleotide microarray. Photolithography: UV light is passed through a lithographic mask that acts as a filter to either transmit or block the light from the chemically protected quartz wafer. Multiple lithographic masks are applied sequentially to determine the sequence synthesis on the microarray surface. Chemical synthesis cycle: As the mask-filtered UV light removes the protecting groups (squares), a single nucleotide washed over the microarray surface is able to couple to the deprotected oligonucleotide chains. Sequential rounds of nucleotide addition combined with changes in the masks form a quartz wafer with 25-mers of predetermined sequence. Adapted and reprinted from reference 28 with permission from Elsevier and Affymetrix.

such as direct DNA hybridization, competitive DNA hybridization, and solution-based chemistries with microsphere capture (36). The earliest assays using liquid bead arrays used the direct hybridization format (37, 38). Direct DNA hybridization uses fluorescently labeled PCR amplicons hybridizing directly to probe capture sequences immobilized on the microspheres. Typically, one of the primers is biotinylated, and a positive hybridization event at the bead surface is measured using streptavidin-*R*-phycoerythrin (Fig. 5, upper inset). In competitive DNA hybridization, unlabeled PCR amplicons compete with labeled oligonucleotides for hybridization to the microsphere-bound capture probes. In this instance, the competitor DNA is biotinylated. The absence of target DNA in the sample results in a positive hybridization event between the labeled competitor DNA and the capture probe on the microsphere and therefore increased fluorescent intensity. The presence of target DNA in the sample binds the labeled competitor DNA and results in low fluorescent intensity.

Solution-based chemistries, including allele-specific primer extension (ASPE), oligonucleotide ligation assay, and single base chain extension, take advantage of the inherent properties of DNA polymerases and ligases and employ unlabeled primers (Fig. 5, lower inset) (36). These chemistries incorporate a capture sequence during the solution-based extension or ligation reaction. During ASPE, the capture primer is only extended if the 3′ nucleotide is homologous to target DNA. In ASPE, one of the deoxynucleoside triphosphates (dNTPs) is biotinylated. Like ASPE, oligonucleotide ligation assay uses a capture primer but is ligase-dependent as opposed to polymerase-dependent. In oligonucleotide ligation assay, a reporter probe homologous to target DNA is biotinylated; ligation only occurs if the complementary target DNA is present in the sample. Single

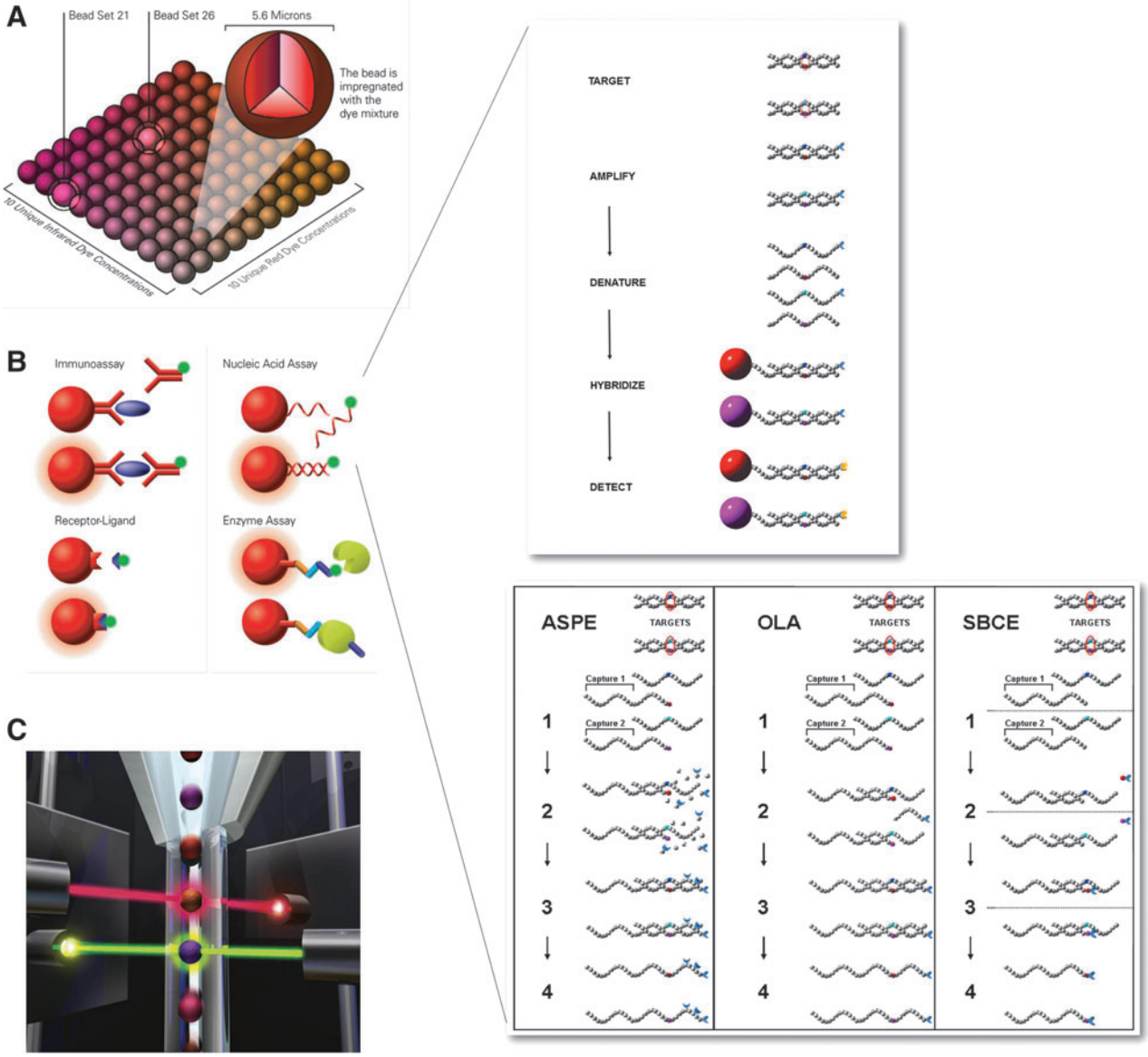

FIGURE 5 Suspension bead microarray. (A) Microspheres 5.6 μm in diameter are filled with varying concentrations of an infrared dye and a red dye to create 100 spectrally distinct beads. (B) Microspheres can then be used in a variety of assays depending on the ligand bound to the bead surface. (Upper inset) Suspension bead direct hybridization. The target is amplified using a biotinylated primer and subsequently denatured and hybridized to microspheres tagged with target-specific sequence probes. A positive hybridization reaction at the microsphere surface is detected using streptavidin-*R*-phycoerythrin. (Lower inset) Solution-based chemistries for microsphere capture. ASPE (allele-specific primer extension): 1, denaturation of target DNA in the presence of specific capture sequence-tagged primers; 2, annealing of target DNA and primers; 3, primer extension and incorporation of biotinylated dNTP; 4, capture sequence-tagged ASPE products. OLA (oligonucleotide ligation assay): 1, denaturation of target DNA in the presence of capture sequence–tagged allele-specific probes; 2, annealing of target DNA and probes in a reaction containing a DNA ligase and biotinylated reporter probe; 3, oligonucleotide ligation; 4, capture sequence–tagged OLA products. SBCE (single base chain extension): 1, denaturation of target DNA in the presence of a capture sequence–tagged primer (in separate reactions for each allele); 2, annealing of target DNA and primers; 3, single base primer extension with incorporation of biotinylated ddNTP; 4, capture sequence-tagged SBCE products that can be multiplexed for detection. (C) After hybridization with the target of interest, the microsphere suspension is analyzed using a flow cytometer. A red laser (635 nm) excites the impregnated dyes of the microspheres to determine the spectral identity of the bead and therefore the probe being analyzed. A green (532 nm) laser excites the reporter fluorochrome to quantify the probe-target reaction on the microsphere surface. Insets reprinted from reference 36 with permission from Elsevier. Other images courtesy of Luminex.

base chain extension requires separate reactions for each nucleotide query and is used specifically for single nucleotide polymorphism (SNP) detection. A biotin-labeled dideoxynucleoside triphosphate (ddNTP) serves as the chain terminator when the target sequence is homologous to the capture sequence. For every SNP, one oligonucleotide probe with a unique capture sequence is used to assay the two alleles in each of two separate wells with a different labeled ddNTP per well (39). This technique allows for multiplex SNP analysis.

Arguably the most powerful and expansive application of these chemistries is the use of universal capture sequences. The first microsphere-based universal sequences were the ZipCode/cZipCode capture sequences originally used with single base chain extension in SNP genotyping assays (39–42). These 25-bp capture sequences were based on random sequences from the *M. tuberculosis* genome (39). A DNA sequence (ZipCode) is included in the 5′ end of the capture probe, while the complementary sequence (cZipCode) is used to tag specific microspheres. Subsequently, Tm Biosciences (Luminex Molecular Diagnostics, Inc., Toronto, Canada) developed the xTAG (formerly Tag-It and FlexMAP) sequences for use in their assays marketed for clinical use and user-defined assays. The specificity of the xTAG capture sequences resides in their use of only three of the four nucleotides; thus, these sequences are unlikely to hybridize to naturally occurring sequences. In addition, all of the xTAG sequences are matched such that the thermodynamics and hybridization efficiency are not variables in the target hybridization reaction. While the first described use of this technology used competitive hybridization in an HLA assay (43), the applications of the xTAG technology have expanded such that it is used in all commercial assays available through Luminex.

Diagnostic Challenges and Applications of Microarrays

Since its inception in research laboratories in the 1990s, microarray technology has continued to improve due to advances in fabrication, robotics, and bioinformatics as well as the rapid increase in available genomic sequences. Though microarrays have become the preferred method for gene expression analyses, they have yet to routinely replace traditional and other molecular methods (such as real-time PCR) in diagnostic microbiology. For microarray analysis to become a key diagnostic technology in pathogen detection, antimicrobial resistance detection, and genotyping, many obstacles still need to be addressed (see below). It is unlikely that "conventional" planar microarrays will transition into diagnostic laboratories, due to the costs associated with producing and analyzing them, as well as the limited sample throughput they offer. A low-density microarray that offers limited, reliable, and straightforward results without sophisticated equipment and data processing is most appropriate for routine clinical diagnostics and will better comply with regulatory requirements (44). Alternative array platforms, such as suspension bead arrays, have begun to meet these criteria.

With the potential power of microarray analysis come abundant challenges, particularly in relation to the diagnostic laboratory. Clinical implementation of microarrays requires careful consideration of verification and validation methods, quality control monitoring, and reimbursement strategies, to name a few. Additionally, one must account for assay reproducibility in production and analysis, cost of implementation, acquiring the training and skill level needed for execution, as well as information management and intellectual property obstacles. One major drawback to many microarray platforms is the limitation of one patient per chip. Multicolor analysis does allow for the comparison of two samples, but this is not applicable to patient specimens. A major preanalytic challenge not discussed in this chapter is efficient and reproducible sample preparation. Because microarrays can potentially detect nucleic acids from a wide variety of taxa (bacteria, viruses, fungi, protozoa, human), different strategies may be required for sufficient recovery of target nucleic acids. In addition, controlling the coextraction of inhibitors needs to be accounted for in the analytical process. Compared to real-time PCR, microarray analysis requires additional steps such as hybridization and washing, which increases the risk of contamination and the amount of hands-on time needed.

Even with these challenges, microarrays are being used for diagnosing infectious diseases, which can be categorized into two main applications: detection of known pathogens and discovery of novel or uncharacterized microorganisms (45). As mentioned above, low-density microarrays are best suited for detection of known pathogens, in part due to their lower complexity and costs. For targeted detection, fewer features (spots or beads) are needed to detect the most common infectious agents. High-density arrays, including resequencing arrays, are best suited for large surveillance studies and/or pathogen discovery, but their use clinically is limited by their increased complexity and costs. Like other multiplex platforms, microarrays allow for syndromic testing or the detection of numerous pathogens that cause similar clinical presentations.

Diagnostic tests that are both FDA-cleared and CE-marked rely on the suspension bead array technology by Luminex. A respiratory viral panel for the detection of influenza A, influenza B, respiratory syncytial virus, parainfluenza viruses 1 through 3, metapneumovirus, adenovirus, and rhinovirus/enterovirus is commercially available in the United States. The Canadian and European versions of the test also include parainfluenza virus 4 and coronaviruses. More recently, a gastrointestinal pathogen panel has become available which detects *Campylobacter*, toxigenic *Clostridium difficile*, *E. coli* O157, enterotoxigenic *E. coli*, Shiga-like toxin–producing *E. coli*, *Salmonella*, *Shigella*, *Vibrio cholerae*, adenovirus 40/41, norovirus GI/GII, rotavirus A, *Cryptosporidium*, *Entamoeba histolytica*, and *Giardia*. In addition, *Yersinia enterocolitica* is included in markets outside of the United States.

Genomica (Coslada, Spain) offers numerous CE-marked low-density microarrays for *in vitro* diagnostic testing outside of the United States, including detection and typing of human papillomavirus and respiratory viruses; detection of bacterial respiratory pathogens, enteroviruses and herpes viruses, gastrointestinal pathogens, and sexually transmitted infectious agents; and pan-microbial identification from positive blood cultures. The Genomica microarrays use Clinical Array Technology (CLART), which comprises multiplex PCR with subsequent analysis by low-density microarray. The CLART Entherplex microarray (enteroviruses and herpes viruses) has been used to detect infectious causes of encephalitis and meningitis with high accuracy; notably, it detected several previously undetected co-infections (46). The CLART PneumoVir (respiratory viruses) and FluAVir (influenza detection and typing) microarrays have been used to diagnose infections in patients with influenza-like illness and in infants hospitalized with bronchiolitis (47, 48). Depending on the level of service a diagnostic laboratory provides, these microarrays could serve as front-line testing (i.e., national centers, epidemiologic surveillance) or could be implemented as part of a testing algorithm. For example, a more rapid diagnostic test could be used first with only negative specimens or specimens from critically ill patients receiving additional testing by microarray analysis (49).

Low-density arrays, such as the ViroChip developed by DeRisi and colleagues (Howard Hughes Medical Institute

and University of California, San Francisco), have been used to identify emerging viruses such as severe acute respiratory syndrome coronavirus and parainfluenza virus 4 and known viruses causing unusually severe clinical presentations (50–52). High-density microarrays have been developed by Lipkin and colleagues (Columbia University, NY) that detect a large number of known pathogens and also allow for pathogen discovery (53, 54) (see chapter 7). While these microarrays are not used in routine diagnostic laboratories, they have been very beneficial in identifying new and emerging infectious agents for which more targeted assays can later be developed and used diagnostically.

SUMMARY

The evolution of molecular technology to allow for multiplex amplification and detection has provided a number of commercially available diagnostic products that allow for syndromic-based testing. These tests range from rapid identification of organisms from positive blood cultures to detecting common pathogens associated with acute respiratory tract infections and gastrointestinal infections. The ability to multiplex has allowed some of these platforms to detect not only the potential pathogen but also resistance genes. Since many infectious agents clinically present very similarly, it stands to reason that a syndromic-based diagnostic approach has the potential to greatly benefit the patient, the clinical provider, and the laboratory. In addition, many academic medical centers and large health care systems have developed their own user-defined multiplex assays for targeted or syndromic-based testing. This type of testing aims to reduce the number of tests ordered on any single patient, ultimately providing better patient care. Although these tests can be very expensive when compared to more traditional diagnostic tests, the overall financial and clinical impact must be considered. In general, the less expensive options of hybridization and PCR are more widely used in the clinical laboratory than the more expensive, though more robust, options of microarrays, mass spectrometry, and next-generation sequencing. It remains to be seen whether the cost of next-generation sequencing will continue to decrease to allow its use in diagnostic microbiology laboratories and therefore replace some of the technologies discussed here. It is likely that, for the foreseeable future, due to the wide variance in molecular expertise among diagnostic laboratories, next-generation sequencing will not replace multiplex hybridization, signal and target amplification methods, and suspension bead arrays because they provide a cost-effective approach to syndromic-based testing and require only limited to moderate molecular expertise and analysis.

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section II

Metagenomics: Implications for Diagnostics

Molecular Microbiology: Diagnostic Principles and Practice, 3rd Edition
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The Skin Microbiome: Insights into Potential Impact on Diagnostic Practice

ELIZABETH A. GRICE

10

THE SKIN HABITAT

No type of culture medium can exactly replicate the complex environment of the skin surface. Slightly cooler than body temperature, exposed to the external environment, and generally arid compared to other body habitats, the skin presents a formidable challenge for the colonization and growth of most microorganisms. To fully appreciate the microbial complexity of the skin, one must first be familiar with the unique features of the skin as a habitat. The outer layer of the skin, the epidermis, is composed of keratinocytes in various stages of differentiation. The most superficial layer of the epidermis, the stratum corneum, forms a semi-impenetrable barrier consisting of several layers of dead, flattened, enucleated, polyhedral, keratin-filled cells, termed corneocytes. Through the process of desquamation, corneocytes are continuously shed from the skin surface through terminal differentiation, providing a continuous supply of nutrients (i.e., keratins) to support the growth of microorganisms. Beneath the epidermis lies the dermis, a connective tissue layer rich in blood and lymphatic vessels. Subcutaneous connective tissue and fat separate the skin from the muscles and organs of the body.

Within the skin, several types of appendages are present, which present unique microenvironments for the skin's inhabitants. Eccrine sweat glands function in temperature regulation and are most densely concentrated on the palms and the soles of the feet. Eccrine secretions consist of primarily water and sodium chloride and, notably, small amounts of antimicrobial peptides. Apocrine sweat glands are present in much fewer numbers than eccrine sweat glands and are most densely concentrated in the axilla and the anogenital area. Apocrine glands have no known useful function but are responsible for the characteristic body odor. Apocrine secretions consist of proteins and lipids that are odorless until acted upon by skin bacteria.

The pilosebaceous unit consists of the hair follicle and the associated sebaceous gland. Sebaceous glands secrete sebum, an oily substance consisting primarily of triglycerides. These glands are most dense on the face and scalp but are found on all of the skin except the palms of the hands and the soles of the feet. Sebum exits from the hair follicle and functions in emolliating and waterproofing the skin. Sebaceous gland size and secretions are under androgenic control and thus become active and attain full size around puberty.

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OVERVIEW OF THE SKIN MICROBIOTA

Bacteria

Culture-based isolation of the healthy human skin microbiota most frequently recovers coagulase-negative staphylococci, with *Staphylococcus epidermidis* and *Staphylococcus hominis* being the most abundant isolates (1). These coagulase-negative staphylococci mainly live a commensal lifestyle but can cause nosocomial infection in patients with indwelling or implanted devices (2). Once classified as a *Corynebacterium* species, *Propionibacterium acnes* is also frequently recovered from the skin by cultures. *P. acnes* is a facultatively anaerobic, Gram-positive inhabitant of the hair follicles and sebaceous glands but is also implicated in acne (3). *P. acnes* hydrolyzes triglycerides found in sebum, thus releasing free fatty acids on the skin that then function to acidify and emolliate the skin (4). Particularly favoring the intertriginous regions, the *Corynebacterium* species are somewhat lipophilic, Gram-positive, slow-growing organisms. Species commonly isolated from the skin are *Corynebacterium jeikeium*, *Corynebacterium minutissimum*, *Corynebacterium tenuis*, and *Corynebacterium xerosis*. *Brevibacterium* is also considered a coryneform bacterium, is frequently isolated from the feet, and is implicated in foot odor (1). Gram-negative bacteria are less commonly cultured from the skin, but *Acinetobacter* isolates may sometimes be isolated from intertriginous, moist areas.

Molecular approaches, based on sequencing of the prokaryotic 16S rRNA gene, have revealed an even greater diversity of bacteria present on the human skin. These approaches require broad-range amplification of the 16S rRNA gene, encompassing at least one of the nine hypervariable regions. The hypervariable regions contain sequences that allow taxonomic classification of the bacteria. While Sanger sequencing was initially utilized for bacterial community characterization based on the 16S rRNA gene, high-throughput sequencing technologies that have emerged in recent years such as 454 GS FLX (Roche,

Branford, CT) and MiSeq/HiSeq (Illumina, San Diego, CA) are now most commonly employed for these studies. The studies described below will be primarily those that employ 16S rRNA sequencing-based approaches unless otherwise specified.

Fungi

Culture-independent studies analyzing fungal skin communities have lagged behind bacterial community studies, in part due to slower development of the appropriate databases and tools. Similar to bacterial 16S rRNA studies, fungal diversity may be characterized by sequence analysis of its ribosomal RNA genes, but which gene and/or amplicon to choose is not always clear. The three most commonly used ribosomal amplicon targets are the 18S rRNA gene, the 5.8S rRNA gene, and the internal transcribed spacer region. Generally, the internal transcribed spacer is the most effective locus for providing species-level identification. In fact, this locus is now commonly used as the fungal "species barcode" region because it nearly always contains a sufficient level of variation for species differentiation (5, 6). The 18S and 5.8S loci are more conserved and are therefore most effective for phylogenetically based analyses (7).

By far the most common fungal inhabitants of the skin are the *Malassezia* species (formerly *Pityrosporum* species), as demonstrated by both culture-dependent and -independent techniques (8–10). *Malassezia* is less frequent on the skin of prepubescent individuals (10), indicative of its lipid dependency. Just as the bacterial microbiota of the skin varies topographically, so too does the fungal microbiota. A culture-independent, topographical analysis of the skin of 10 healthy adults at 14 skin sites demonstrated that *Malassezia* dominated core body and arm sites, but sites on the foot were characterized by much greater diversity including *Trichophytum*, *Penicillium*, and *Aspergillus* (8). Further, *Malassezia* species differed in relative abundance by topographical location, with *Malassezia restricta* predominating on the head and face and *Malassezia globosa* predominating on the trunk.

Demodex Mites

A unique feature of the skin microbiota, as compared to other body sites harboring microbial communities, is the presence of the arachnid skin mite, *Demodex*. *Demodex*, particularly *Demodex folliculorum* and *Demodex brevis*, colonizes the pilosebaceous units and increases in prevalence with age (11). *Demodex* produces lipases and utilizes sebum as a food source (12). Other nutritional sources may be cellular debris or bacteria such as *P. acnes* that reside in close proximity within the pilosebaceous unit (9).

While there are currently no reported molecular typing schemes to identify and speciate *Demodex* mites, their discussion is worthwhile because they may both directly and indirectly (through their gut microbiota) contribute to rosacea, a chronic inflammatory disorder affecting the face. Increased density of *Demodex* has been associated with rosacea, particularly the subtypes erythematotelangiectatic rosacea and papulopustular rosacea (13–15). These associations are somewhat controversial, though, because systemic tetracycline treatments improve rosacea without a concurrent decrease in mite populations (16). *Bacillus oleronius*, a Gram-negative bacterium that is a part of the *Demodex*-associated microbiota, has been suggested to play a role in rosacea and triggers inflammatory pathways in a manner reflective of rosacea (17). Serum reactivity to *B. oleronius* proteins was found in 82.6% of rosacea patients, compared to only 26.9% of healthy controls (18). Increased production and aberrant processing of cathelicidin, a cutaneous antimicrobial peptide, are also associated with rosacea (19), which in part may contribute to dysbiotic cutaneous states.

Viruses

Unlike bacteria and fungi, characterization of skin-associated viruses and bacteriophage presents a unique difficulty since they lack a consensus gene that can be amplified and sequenced for widespread taxonomic identification. The phylogeny of closely related groups may be analyzed using specific conserved genes, such as the human papillomavirus (HPV) L1 gene. Comparative genome analysis is further complicated by the high frequency of gene transfer between virus and host genomes and the lack of comprehensive, annotated reference databases and assigned taxonomy. Whole-genome shotgun metagenomics, which does not rely on amplification and sequencing of marker genes, may in part allow a more thorough characterization of skin viral communities. This strategy does not rely on a conserved gene, bypasses PCR, and may provide insight into what microbial communities are doing on the skin surface by analyzing gene content and function. Whole-genome shotgun metagenomics is particularly difficult to perform on skin-derived samples, because high amounts of host DNA and low amounts of microbial DNA present technical limitations for these approaches.

Eukaryotic Viruses

HPV is one of the most extensively studied human skin viruses. PCR amplification and quantification of HPV marker genes have revealed that healthy skin is a habitat for a broad spectrum of HPV strains (20, 21). Sequence analysis of the conserved L1 open reading frame similarly revealed a diverse community of HPV types on healthy skin while identifying novel HPV strains (22). Multiple studies have confirmed the ubiquity and diversity of HPV types throughout human populations (23–25).

Human polyoma viruses (HPyVs) are another major group of commensal human skin viruses. Originally studied in the context of cancer, HPyVs have been found on healthy human skin (26). Molecular techniques have enabled the discovery of multiple types of HPyVs; the most common to human skin are HPyV6, HPyV7, and Merkel cell polyoma virus (26, 27). Whole metagenomic shotgun sequencing of the facial skin virome of five healthy individuals and one individual with Merkel cell carcinoma confirmed a cutaneous viral microbiota dominated by HPVs, HPyVs, and circoviruses (28).

Bacteriophage

Limited knowledge currently exists regarding the bacteriophage of the skin, though these microorganisms likely have complex roles in bacterial community dynamics, at the population level and the genomic level. In particular, bacteriophage may mediate antibiotic-resistance-gene transfer between bacteria, as demonstrated by *Staphylococcus aureus* strains (29, 30), a major cause of skin and soft tissue infections. Culture-dependent techniques paired with genomic analyses as well as prophage analyses have been employed to understand and characterize the

genomic diversity of subsets of skin bacteriophage communities. For example, *P. acnes* bacteriophage seem to be quite limited in their genomic diversity but have a broad host range among clinical isolates (25). This is in contrast to *S. aureus* bacteriophage, which appear to be quite diverse at the genomic level (31–33). Whole metagenomic shotgun sequence analysis of facial skin swabs from five healthy individuals and one individual with a previous Merkel cell carcinoma lesion indicates that two families dominate cutaneous bacteriophage communities, the *Microviridae* and *Siphoviridae* (28).

Acquisition and Early Colonization

At 34 weeks of gestation, the skin has formed a competent barrier able to withstand the terrestrial environment. Immediately following birth, the neonate skin acquires microbiota, and delivery mode may in part contribute to colonization. A study analyzing neonate skin microbiota indicated that babies delivered via vaginal birth ($n = 4$) had skin microbiota similar to their mother's vaginal microbiota (i.e., *Lactobacillus*, *Prevotella*, and *Sneathia* spp.), and babies delivered via Caesarean section ($n = 6$) were colonized with skin microbiota similar to their mother's skin microbiota (i.e., *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* spp.) (34). However, it is still unclear how long this effect lasts and how these potential differences in skin microbiota colonization contribute to health or disease, if at all. Following birth and adjustment to the extrauterine environment, the skin barrier significantly changes, including pH, transepidermal water loss, water content, and sebaceous gland activity (35). Thus, it is expected that the skin microbiota would significantly shift as well, adjusting to the changing habitat that selects for some microbiota and prohibits colonization of others. In analyzing the shifting skin microbiota during the first year of life ($n = 31$), it was found that evenness of the relative abundance of microbiota increased with time (36). In infants, the dominant phylum detected was *Firmicutes*. And though infant skin produces a limited amount of sebum in contrast to adult skin, *Propionibacterium* was detected in the top five abundant genera during 4 to 6 months of age.

Topographical Diversity of Skin Microbiota

Numerous studies employing 16S rRNA gene sequencing have characterized bacterial communities colonizing healthy human adult skin. Generally, the four dominant phyla of bacteria found via 16S rRNA sequencing to reside on adult skin are the *Actinobacteria*, *Proteobacteria*, *Firmicutes*, and *Bacteroidetes* (37). A unique feature of the skin habitat, as highlighted above, is the variability across the surface and the ability to sample that variability in a minimally invasive manner. Differentially dense and numerous hair follicles, eccrine and apocrine sweat glands, and sebaceous glands contribute to the variable cutaneous microenvironments and select for microorganisms that thrive in those specialized environments. Intrapersonal variability is high, similar to other sites of the human body colonized by microbiota (38). However, the dominant types of bacteria recovered from the skin appear to be relatively constant, with the rarer, less abundant types of bacteria accounting for the variability seen within and between individuals. The dominant types of bacteria identified by molecular techniques are the same as those recovered by culture-based techniques, primarily *Staphylococcus*, *Propionibacterium*, and *Corynebacterium* (38–40). Laser capture microdissection followed by 16S rRNA sequencing suggests that bacteria may also be present in subepidermal compartments of the skin (41), though it is unclear if these or any microorganisms identified by sequencing-based approaches are alive. One general limitation of molecular techniques is that they are not able to distinguish between viable and dead microorganisms.

Areas dense in sebaceous glands, such as the face, scalp, and back, are colonized by higher proportions of the lipophilic bacterium *Propionibacterium* (38–40). Heavily sebaceous areas also tend to be low in microbial diversity and are relatively stable temporally, compared to moist and dry sites of the skin (42), likely a reflection of the unique microenvironment afforded by sebaceous secretions. The role of sebum in defining the skin microbiota is apparent in age-associated changes in the composition and diversity of the skin microbiota. Puberty and its associated androgenic-controlled release of sebum from the sebaceous glands coincide with a shift in skin microbiota toward greater relative abundance of *Actinobacteria*, including *Corynebacterium* and *Propionibacterium* (43).

Staphylococcus and *Corynebacterium* dominate moist and/or occluded areas of the skin. For example, in the axilla, abundant amounts of *Corynebacterium* process odorless eccrine, apocrine, and sebaceous gland secretions, thus producing volatile organic fatty acids and thioalcohols that are responsible for the characteristic odor associated with axillary sweat (44–47). 16S rRNA gene sequencing and analysis has confirmed that *Corynebacterium* and *Staphylococcus* are the bacteria present in the highest relative abundance, and interestingly, people using deodorants harbored greater bacterial diversity in their axillae (48).

Dry and exposed areas of the skin harbor more bacterial diversity and less bacterial load than sebaceous, moist, occluded, and haired areas and seem to have less of a selective force, because those bacterial populations are not dominated by individual taxa (40). Instead, drier areas contain greater amounts of *Proteobacteria* and *Bacteroidetes*, in comparison to moist and sebaceous sites (39, 40, 49, 50).

Whole Metagenomic Shotgun Sequencing of Skin Microbiota

Metagenomic techniques to analyze skin microbiota, which employ whole-genome shotgun sequencing to identify and characterize microbiota without relying on amplification of a marker gene such as 16S rRNA, have not yet been widely used. The advantages of such techniques include gaining functional and metabolic insight into the coding potential of the microbiota, strain-level resolution of microbial taxa, and elimination of PCR-associated biases. Such studies have to this point been limited by the low burden of microbes present on the skin (in comparison to other sites, such as the gastrointestinal tract) and high amounts of human genomic contamination derived from skin cells. The Human Microbiome Project Consortium performed a metagenomic analysis of the retroauricular crease skin (the area behind the ear) in 242 individuals. Their findings indicate that the metabolic and functional pathways encoded by the skin microbiota were more constant and diverse than the taxonomic composition, suggesting low metabolic diversity among a taxonomically diverse population (38). An additional finding was that age was associated with differential metagenomically encoded pathways on the skin, including the glyoxylate cycle (38).

DISORDERS CAUSED BY OR ASSOCIATED WITH SKIN MICROBIOTA

Skin Disorders Caused by or Associated with Bacterial Microbiota

A variety of skin disorders are associated with bacterial colonization and/or infection. These disorders may include overt infection, such as folliculitis, carbuncles, furuncles, and other skin and soft tissue manifestations of infection by organisms such as *S. aureus* or *Pseudomonas aeruginosa*. Bacterial colonization and/or infection may also contribute to multifactorial disease, interacting with host and other environmental factors. This is likely the case for disorders such as acne, atopic dermatitis, and dandruff. Finally, commensal bacteria may become pathogenic and cause nosocomial infections, especially on indwelling devices, as exemplified by *S. epidermidis* and *Corynebacterium jeikeium*. Here we will discuss two disorders, acne and atopic dermatitis, in which microbiome science has provided important clues to the pathogenesis of disease. Other common cutaneous bacterial infections and disorders are listed in Table 1 but will not be further discussed.

Acne

Acne vulgaris is the most common disease seen by dermatologists and is generally associated with puberty and androgen exposure and coincides with the time when the sebaceous glands increase in size and commence sebum secretion. It is distinguished by open and closed comedones ("blackheads" and "whiteheads," respectively), pustules, papules, and nodules. The association between *P. acnes* and acne vulgaris has been well established (3), though it is generally regarded as a multifactorial disease in which host factors, environment, and genetics likely play a role in the pathogenesis. Antibiotics are relatively effective in acne treatment, further supporting a microbial role in disease pathogenesis. However, both culture-based and culture-independent 16S rRNA gene sequencing studies have established *P. acnes* as a member of the commensal microbiota in those without acne. Therefore, the question arises of when and how does a commensal become a pathogen? Recent insights into this question suggest that strain-level analysis of *P. acnes* may be required to differentiate commensal from pathogen; a recent study showed that even though the relative abundance of *P. acnes* did not differ between individuals with acne compared to healthy individuals, certain *P. acnes* ribotypes (types of 16S rRNA sequences) were highly associated with acne (51). Whole-genome sequence comparison of the different ribotypes showed that acne-associated strains carry unique genetic elements not present in the strains associated with healthy individuals, which may contribute to virulence and pathogenicity.

Probiotic applications may offer some benefit for treatment of conditions such as acne. Succinic acid, a fatty acid fermentation product of *S. epidermidis*, has been shown to inhibit the growth of *P. acnes* (52). Similarly, *P. acnes* itself may function in some regard as a probiotic and has been shown to suppress growth of USA300 methicillin-resistant *S. aureus* through fermentation of glycerol (53). Bacteriophage therapy has also been suggested as a novel therapeutic for acne. *P. acnes* bacteriophage are limited in genetic diversity, have a broad host range, and are unable to form stable lysogens within their hosts (31), rendering them

TABLE 1 Skin disorders caused by or associated with bacterial colonization and/or infection

Bacteria	Characteristics	Associated disorders
Staphylococcus aureus	Gram-positive, coagulase-positive cocci, often antibiotic resistant	Impetigo
		Folliculitis
		Furuncles
		Carbuncles
		Wound infections
		Atopic dermatitis
		Staphylococcal scalded skin syndrome
Staphylococcus epidermidis	Gram-positive, coagulase-negative cocci	Indwelling catheter infections
		Prosthetic and device infections
Propionibacterium acnes	Gram-positive, lipophilic, aerotolerant anaerobe; resides in hair follicle and sebaceous glands	Acne vulgaris
		Device infection
		Surgical site infection
Corynebacterium minutissimum	Gram-positive aerobic or facultative aerobic coryneform; produces porphyrins that fluoresce coral-red upon Wood's light exam	Erythrasma
Corynebacterium jeikeium	Gram-positive, aerobic, lipophilic coryneform; often antibiotic-resistant; formerly known as group JK coryneform	Indwelling device infections
		Other nosocomial infections
Corynebacterium tenuis	Gram-positive coryneform	*Trichomycosis axillaris*
		Trichomycosis pubis
Pseudomonas aeruginosa	Gram-negative, aerobic, motile	Toe web infections
		Green nail syndrome
		Folliculitis
		Wound infections

ideal candidates for a topical phage therapeutic. Novel probiotic, prebiotic, or bacteriophage-based approaches to treat acne would be desirable, because long-term antibiotic treatment for acne has been associated with acquisition of antibiotic resistance (54, 55). Gram-negative folliculitis (56) and pharyngitis (57) are also associated with antibiotic treatment of acne; these are opportunistic infections that likely result from disturbed microbial community ecology on the skin and in the airways, respectively.

Atopic Dermatitis

Atopic dermatitis (AD) is a chronic relapsing disorder affecting ~15% of children in the United States (58). Features include eczema with papules, vesicles, oozing, and crusting. Typically affected skin sites are the antecubital and popliteal fossae. The pathogenesis is likely multifactorial, with contribution of genetics, environment, and immune response. The incidence of AD has increased significantly over the past 3 decades, highlighting what may be an environmental component of the disease. Commonly associated with AD are colonization and/or infection with *S. aureus*. Profiling of the bacterial microbiota via 16S rRNA gene sequencing confirmed increases in the relative abundance of *S. aureus* during AD flares, with a corresponding decrease in overall bacterial community diversity (59). In the same study, treatments consisting of topical corticosteroids, systemic antibiotics, and/or dilute bleach baths increased skin microbiota diversity and decreased colonization with *S. aureus*, in parallel with improving symptoms of AD. In addition to bacteria, it appears that there may be an overgrowth of the fungi *Malassezia* in AD, with one study finding three times the amount of *Malassezia* in lesional compared to nonlesional skin of AD patients (60). Gastrointestinal probiotics, both during pregnancy and in neonates, may offer some protection against the infant developing AD, though the data thus far appear to be conflicting (61).

Skin Disorders Caused by or Associated with Fungal Microbiota

Fungal infections of the skin commonly present as scaling, erythematous papules, plaques, and patches. Here we will discuss both infections generally accepted as fungal in nature and those with a proposed association with fungal colonization and growth. Infections that we will not discuss in detail, but that are of clinical importance (Table 2), include tinea versicolor (also known as pityriasis versicolor; usually caused by *M. globosa* or *Malassezia sympodialis*), tinea pedis ("athlete's foot"; usually caused by *Trichophyton rubrum*), tinea cruris ("jock itch"; usually caused by *T. rubrum*), intrigo and diaper rash (both usually caused by *Candida albicans*), *Malassezia* folliculitis, and dermatophytoses generally caused by *Trichophyton* or *Microsporum* species.

Dandruff and Seborrheic Dermatitis

Though separate clinical entities, dandruff and seborrheic dermatitis (SD) have many similar features. In both conditions, itching, inflammation, and flaking of the skin are apparent. Dandruff occurs on the scalp, while SD additionally affects facial areas and hairy regions of the body. In both disorders, shampoos that contain antifungal agents (e.g., zinc salts, selenium salts, ketoconazole) are treatment mainstays, suggesting the involvement of fungi in the disease process. Further, a decrease in the amount of *Malassezia* found on the scalp generally correlates with improved scalp condition. Culture-based studies have recognized an overgrowth of *Malassezia* fungi in dandruff and SD (62). Fungal community profiling employing the 26S rRNA gene similarly demonstrated that an increased relative abundance of *Malassezia* was on the dandruff-afflicted-scalp skin (63). The same study reported that dandruff-afflicted skin was colonized with increased relative abundances of *Penicillium* and *Filablasidium floriforme* that correlated with increased severity of dandruff. Nested PCR assays to distinguish *Malassezia* species identified *M. globosa* and *M. restricta* as the major species associated with SD, and the SD strains isolated were significantly different than those from healthy subjects (60). Further studies indicate that oleic acid, a by-product of *Malassezia* metabolism of sebum, administered to human scalps can induce flaking in subjects susceptible to dandruff (64). The picture that is emerging of dandruff and SD is that of multifactorial disease, with a significant role for *Malassezia*, which is also a targetable factor for effective treatment.

Onychomycosis

Onychomycosis is a fungal infection of the nails and is generally thought to be caused by dermatophytes (in particular, *Trichophyton* spp.), which are slow-growing and difficult to culture, resulting in high rates of false-negative

TABLE 2 Skin disorders caused by or associated with fungal agents

Fungal genus	Characteristics	Associated disorders	Associated species
Malassezia	Dimorphic, lipophilic, high lipid content in cell wall; formerly known as *Pityrosporum;* resides in infundibulum of the sebaceous gland	Tinea versicolor	*Malassezia globosa*, *Malassezia sympodialis*
		Dandruff	*M. globosa*, *Malassezia restricta*
		Seborrheic dermatitis	*M. globosa*, *M. restricta*
		Folliculitis	*M. globosa*, *M. restricta*, *M. sympodialis*, *Malassezia furfur*
Candida	Dimorphic, characteristic yeasty odor	Intertrigo	*Candida albicans*
		Diaper rash	*C. albicans*
Trichophyton	Dermatophyte, develops macro- and microconidia	Tinea pedis	*Trichophyton rubrum*
		Tinea cruris	*T. rubrum*
		Onychomycosis	*T. rubrum*, *Trichophyton interdigitale*

culture results. Molecular techniques to detect the pathogenic agent for onychomycosis are becoming more common for its diagnosis. One study of 346 patients with onychomycosis used 18S rRNA PCR and sequencing to detect fungi, in parallel with routine cultures, and found that 18S rRNA sequencing was able to detect about double the positive results as cultures (65). The fungi identified, primarily *T. rubrum* and *Trichophyton mentagrophytes*, were consistent when comparing cultures and sequencing methods. However, a limitation was the inability to resolve taxonomy to the species level in eight of the cases. Other direct DNA detection techniques have been employed, including primers specific to known nail pathogens (66, 67) and PCR followed by restriction fragment length polymorphism (68, 69). Generally, the molecular techniques are more sensitive than cultures for detection of fungi. However, in most studies, a significant portion of cases are found to be both culture- and PCR-negative, suggesting that techniques such as metagenomics that target a broader range of organisms may be of utility for resolving the causative agent in these cases.

Skin Disorders Caused by or Associated with Viruses

Molecular techniques have been particularly useful in identifying known viruses in association with skin disorders. We discuss two viral skin disorders here: Merkel cell carcinoma and cutaneous warts. The identification of the virus associated with Merkel cell carcinoma was possible using a type of metagenomic shotgun sequencing. Greater precision has been attained in identifying HPV strains associated with cutaneous warts using molecular techniques. There are many other cutaneous manifestations of viral infection, and some of these are described in Table 3.

Merkel Cell Carcinoma

Merkel cell carcinoma is a rare but aggressive malignant, neuroendocrine tumor generally presenting as a solitary nodule on the face or neck of elderly or immunosuppressed patients, suggestive of a pathogenic cause. In 2008, Feng et al. employed a method called digital transcriptome subtraction to link a novel virus (Merkel cell polyomavirus [MCPyV]) to 80% of Merkel cell carcinoma cases in their study (70). The development of improved detection methods for MCPyV, specifically an expanded repertoire of quantitative PCR assays and monoclonal antibodies to detect integration and expression of the large T antigen, suggests that most Merkel cell carcinomas are associated with MCPyV (71). Molecular features of the MCPyV, such as genome copy number and mutation analysis, detected in tumors and peripheral blood mononuclear cells may be predictive of disease course (72). As mentioned previously, MCPyVs are commonly found on the skin of healthy individuals, are often asymptomatically carried, and can be shed from the skin as assembled virus particles (73–83). Therefore, there are likely other factors that interact with MCPyV to cause disease, including host immune function.

Cutaneous Warts

Warts are benign flesh-colored papules or nodules, commonly occurring on the hands and fingers, and particularly in children and immune-compromised patient populations. They are caused by HPV, most frequently of the alpha, gamma, and mu genera. Multiplex genotyping PCR assays have been successfully employed to identify HPV types, the most prevalent appearing to be HPV types 1, 2, 27, and 57 (84–86). An assay comprising PCR followed by restriction fragment length polymorphism analysis and Luminex xMAP Technology (Luminex, Austin, TX), revealed that HPV types 2, 3, 27, and 57 were most common in a cohort of Greek children (87). Data obtained from swabs taken from the skin overlying the wart are similar to data obtained from more invasive tissue biopsies (88). Plantar warts, in particular, have been associated with HPV types 1, 2, 27, and 57 (86, 89).

POTENTIAL CLINICAL DIAGNOSTIC APPLICATIONS OF THE SKIN MICROBIOME

While diagnostics based on the skin microbiome are not yet standard practice, the potential is great. It is foreseeable that variants of disease may be identified based on the colonizing microbiota, and as a result, a personalized treatment regimen may be administered. For example, perhaps variants of atopic dermatitis, based on their colonizing microbiota, may respond differently to various treatments (i.e., corticosteroids, antibiotics, dilute bleach baths). Identifying the ideal treatment for the disease variant would eliminate time-consuming troubleshooting and potentially unnecessary use of antibiotics. The microbiome may also be used to predict which cases of disease are at risk for downstream complications. If identified as "at risk," a patient could be more closely monitored, and measures could be taken to prevent the complication from arising. Though not discussed here, this may be a potential approach for

TABLE 3 Skin disorders caused by or associated with viruses

Virus	Characteristics	Associated disorders	Specific strain
Papillomavirus	Double-stranded DNA, circular genome, nonenveloped	Common wart	HPV1, 2, 27, 57 most common
		Plantar wart	HPV1, 2, 27, 57 most common
Polyomavirus	Double-stranded DNA, circular genome, nonenveloped	Merkel cell carcinoma	Merkel cell polyomavirus
Herpesvirus	Double-stranded DNA, linear genome, enveloped	Kaposi's sarcoma	HHV8
		Roseola	HHV6, 7
		Chickenpox, shingles	HHV3
Molluscum contagiosum virus	Double-stranded DNA, linear genome, enveloped, unculturable	Molluscum contagiosum	MCV1 most common

wound care, where the microbiota is thought to influence progression to complication (i.e., osteomyelitis, amputation), but it is unclear which cases are at risk for complication (90, 91). Finally, once a more complete understanding is obtained regarding what is "good" and "bad" microbiota, clinical diagnostic approaches may be used to quickly assess a patient's microbiome.

For any of these potential applications to be feasible, significant advances need to be made to overcome bottlenecks in the pipeline to obtain, analyze, and interpret the large datasets generated by microbiomic and metagenomic approaches. Sequencing technologies are continually evolving and becoming more cost-effective and rapid. However, a major bottleneck is the analysis required to gain meaningful information from the sequence data, which often requires specialized computational and bioinformatics skills that are in short supply. Additionally, standardized approaches are needed, such as sample collection, sequencing approach, and analysis parameters.

CONCLUSIONS

Molecular techniques have been employed for the diagnosis of some skin disorders, but especially those with a straightforward infectious etiology and a known pathogen (i.e., onychomycosis, cutaneous warts). The greater challenge will be applying molecular techniques for the diagnosis of complex, multifactorial diseases, where an alteration in microbial communities may be one of several factors in disease pathology. It is first necessary to gain a more complete understanding of the role of altered microbial communities in disease states. In the future, microbial community analysis may also be used to identify variants of disease, predict best treatment modality, and guide personalized management and care of the patient.

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The Gastrointestinal Microbiome

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11

Through the years, the individual microbes that reside in the human gastrointestinal (GI) tract have been labeled as pathogens, commensals, uncultivable, or unidentifiable. While exploration of particular species in the discovery and diagnosis of disease remains paramount, it is the landscape of the microbial community that continues to offer greater clues to the role of microbes in human health and quality of life. In contrast to other body systems, the human GI microbiome is ecologically diverse and complex and plays an active role in digestion, metabolism, behavior, heart size, and the development of the mucosal immune system, among other associations (1, 2). The composition of the gut microbiota is influenced by diet, age, host genetics, antibiotic treatment, and the environment (e.g., psychological stress, hygiene, climate, and allergies) (3). The microbial communities found in the gut have also been shown to contribute, both negatively and positively, not only to health issues rooted in the GI tract, but also to those of the respiratory and central nervous systems. An imbalance or shift of the gut microbiome has been linked to the development of a variety of disorders including inflammatory bowel disease (4–6), gastric ulcers and cancer (7–10), autism spectrum disorder (11–15), and obesity and diabetes (16–19). Because of the implications related to these changes and the development of "unhealthy" microbiomes, research is ongoing to continue to refine the definition and composition of a "healthy" gut microbiome.

MICROBIOME CHARACTERIZATION

The most rudimentary explorations of the composition of the gut microbiome employed basic microscopy and axenic culture of stool specimens. Today, most gut microbiome studies still rely on stool specimens for characterization. This has allowed for comparison of microbiome data to results obtained from conventional diagnostics. Routine diagnostics for a patient presenting with GI symptoms include Gram stain analysis, stool antigen screens, bacterial culture, and targeted molecular detection of enteric pathogens. The introduction of molecular detection of GI pathogens in clinical laboratories has allowed for the identification of known GI pathogens from stool or stool cultures, thus increasing sensitivity (20). While these testing modalities have improved identification of known pathogens, few techniques provided the ability to identify unknown microbes. However, in the case of a pure cultured isolate unidentifiable via conventional microbiology methods, alternative identification methods were quickly developed, including pyrosequencing of the variable regions of the 16S rRNA gene (21). Bacterial sequencing further expanded the capabilities for the identification of cultured organisms but offered no benefit in the detection and identification of the uncultivable microbes.

Current culture-based diagnostic techniques examine only a small portion of the entire microbial population (e.g., cultivable organisms), neglecting the ecological complexity of the community as a whole. Approximately 80% of the bacterial species in the gut are uncultivable, and many of these fastidious species have complex nutrient and oxygen requirements as well as dependence on one another (22). The microbial community structure of the gut varies among individuals, and its composition is not clearly defined. Greater understanding of the gut microbiome and microbial interactions within the community is essential to the development of future clinical diagnostics and therapies, and the potential utility of community manipulation, via introduction or reduction of a select bacterial population, remains promising.

The introduction of next-generation sequencing technology provided the opportunity to characterize the complete microbial community, exclusive of culture conditions, with massively parallel sequencing of the 16S rRNA gene (23). With a known commensal community, the GI tract was a frequent target for the initial studies to characterize the human microbiome. The employment of 16S ribosomal DNA sequencing methods led to the detection of more than 7,000 phylotypes in the human gut (24). It is the innovation in both high-throughput sequencing technology and bioinformatics applications that enabled the Human Microbiome Project and future 16S rRNA–based microbiome and shotgun sequencing–based metagenomic studies (25, 26).

LONGITUDINAL DEVELOPMENT OF THE GUT MICROBIOME

A healthy gut microbiome has the potential to limit infection and promote general well-being. Native gut populations can directly inhibit the colonization of pathogenic bacteria by blocking binding sites for pathogenic organisms at the mucosa surface, competing for nutrients, and producing bactericidal compounds (27). More than 100

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trillion microorganisms form the mutualistic community of the healthy gut. The gut microbiome is composed mainly of bacteria, which constitute 60% of total fecal mass (28). The total number of bacteria is 10-fold the number of human cells, and the microbial genome contains 100-fold more genes (24). Studies have consistently shown that healthy intestinal microbiomes exhibit more bacterial diversity compared to those associated with disease. The gut microbiota is characterized by approximately 1,100 dominant bacterial species, with approximately 90% of the bacterial population assigned to two phyla (*Firmicutes* and *Bacteroidetes*) (29, 30). *Actinobacteria*, *Cyanobacteria*, and *Proteobacteria* are also minor constituents in the gut microbiota. Looking deeper taxonomically, the genera most closely associated with a healthy gut microbiome include *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Eubacterium*, *Faecalibacterium*, *Lactobacillus*, and *Roseburia*.

The human GI tract is thought to be relatively sterile at birth, undergoing a dynamic and rapid expansion following the initial colonization during the birth process. A variety of factors influence the microbial profile at each stage of life, as depicted in Fig. 1, and these profound changes can be seen at phylum-level characterization (31). Mode of delivery (e.g., vaginal or caesarean) plays a role in the development of community structure and continues throughout infancy. A previous study reported that the pioneer species found in the infant gut were facultative anaerobes and thus species that then altered the gut to a more anaerobic environment (32). Those pioneer species found in the infant gut include but are not limited to *Escherichia coli* and *Streptococcus* spp. While this study identified key differences between the gut microbiomes of infants born vaginally and those of infants born via caesarean section, a more recent study suggested that mode of delivery affected the pace of colonization but ultimately did not affect the sequence (33). With the neonates in this study in the controlled environment of a neonatal intensive care unit, gestational age at birth appeared to affect the colonization pattern most strongly. In a study of the placental microbiome, this finding was upheld, as gestational age at time of delivery was associated with a specific microbial profile but mode of delivery had no such association (34). As infants grow older, the gut microbiota continues to increase in diversity, due to environmental factors as well as diet.

The diet of infants directly influences microbiota composition by supplying substrates important in bacterial function and growth (35). For instance, breast-fed infants have a more stable and fixed bacterial population than formula-fed infants (36). Another study comparing formula-fed infants to breast-fed infants found that the breast-fed infants had a more heterogeneous community than formula-fed infants (37). A major shift is seen in the gut microbial community during the transition from liquids (e.g., milk) to solids. The introduction of solid foods to breast-fed infants results in an increase in enterococci and enterobacteria as well as *Bacteroides*, *Clostridia*, and anaerobic *Streptococcus* spp. (38). After the second year of life, the gut microbiota of both breast-fed and formula-fed individuals begins to more closely resemble the typical microbiome identified in adults.

The infant gut microbiome exhibits greater variability in functional gene content between individuals than is seen in adults (39). These variations can be due to random

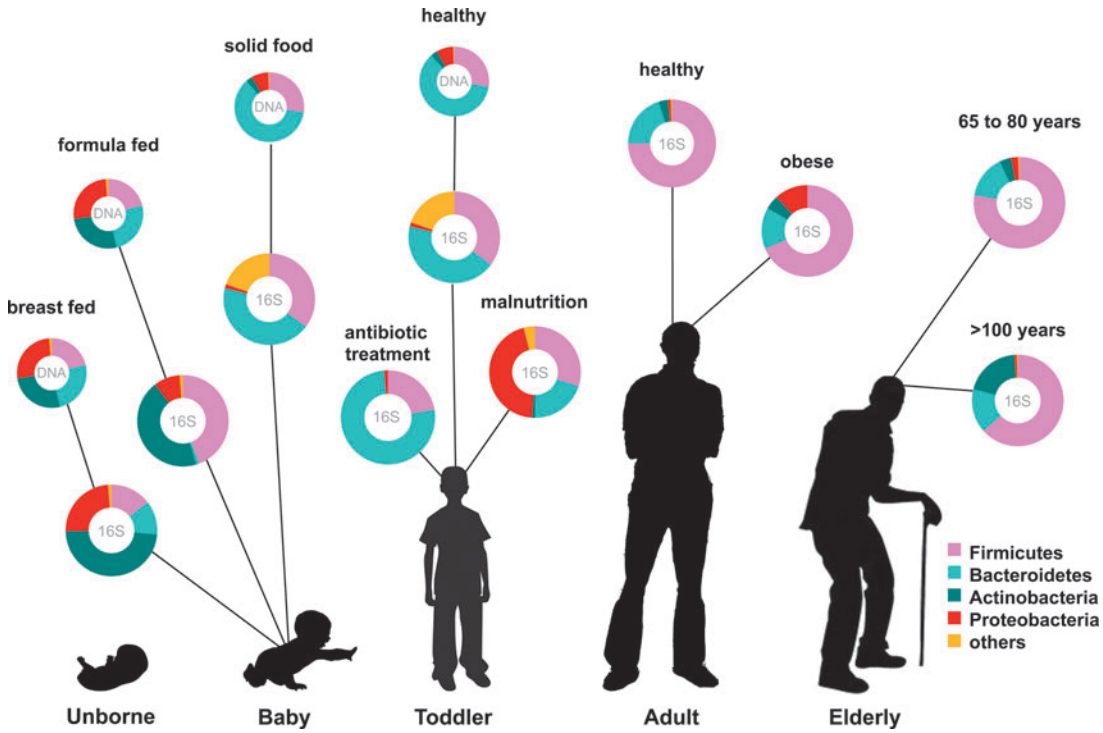

FIGURE 1 The dynamic nature of the gut microbiome over the span of a lifetime. With initial seeding of the microbiome at birth, the bacterial community of the gut continues to evolve through the course of a lifetime. Various factors affect the specific composition of an individual's gut community, and in return, the gut microbiome contributes to health and disease. (Reproduced with permission from reference 31.)

colonization events, differing immune responses, changes in host behavior, and lifestyle factors (40). In a case-study-based exploration of microbiome development, Koenig et al. monitored the gut microbiome of a full-term vaginally delivered infant over a period of 2.5 years (41). This male infant was fed breast milk and formula for the first 134 days of life, with the introduction of solid food to his diet at 4 months of age. Breastfeeding stopped at 9 months of age, and cow's milk was introduced at 1 year. The infant was typically healthy but was treated with antibiotics several times for an ear infection. He was immunized according to the Centers for Disease Control and Prevention-recommended schedule. A gradual increase in bacterial diversity was detected longitudinally, and these changes correlated with changes in diet and antibiotic treatments. Specifically, the introduction of peas and formula was related to a shift in functional genes associated with the adult gut and an increased abundance of specialized microbial groups, such as *Bacteroidetes*, that are known to break down plant polysaccharides. After weaning, a stable bacterial population was observed in regard to function, and this stable state more closely resembled the adult gut microbiome.

Palmer et al. describes the range of microbial profiles in 14 healthy full-term babies born to 13 healthy mothers (40). These authors observed diverse community profiles in the first 6 months of life that progressively became more similar to one another and moved to a more stable adult-like microbiome. This cohort of infants exhibited similar major contributing phyla and overall similar and stable populations over time. In this study, bifidobacteria did not appear to be major constituents of the gut and, if present, appeared after several months of life.

Throughout childhood and adulthood, changes in the relative abundance of bacterial species cause shifts in the overall bacterial composition of the gut. In general, *Verrucomicrobia* and *Actinobacteria* are in greater abundance in the stool of children compared to adults, where there is a predominance of *Bacteroidetes* and *Firmicutes* (42).

Yatsunenko et al. (4) examined how gut microbiomes differ with age and geography. The study included fecal specimens from 531 individuals, with gene content from 110. This study group included healthy children and adults from the Amazonas of Venezuela, rural Malawi, and large cities within the United States. Also included in the study were monozygotic and dizygotic twins. When examining individuals from all three populations, aged 0–17 years, the gut microbiome was shown to evolve toward an adult-like state within 3 years after birth. This group also found that the interpersonal variation was greater among children than adults, and lastly, they observed differences in the phylogenetic composition of fecal microbiota between individuals living in different countries. The latter was particularly distinct between those individuals from the United States and the Malawians and Amerindians, with U.S. individuals more closely related to each other than the non-U.S. individuals. Separation was also seen between the Malawian and Amerindian populations.

Analysis also revealed a dominance of bifidobacteria in the infant microbiome and distinct microbial profiles in the U.S. population and the non-U.S. populations. In the cohort including 110 individuals, Yatsunenko et al. observed an enrichment of genes involved in the *de novo* biosynthesis of folate, while adults in the cohort had a higher representation of genes that metabolize dietary folate. Also overrepresented in the infant population were fermentation pathways found in infant microbiomes. Pathways involved in cobalamin, vitamin B7, and vitamin B1 biosynthesis increased in relative abundance with increasing age of the subjects. Regarding geographical and age-specific differences, non-U.S. breast-fed infants had higher representations of genes related to vitamin B2 biosynthetic pathways and urease (both relevant to human milk) than found in U.S. breast-fed infants. Differences among U.S. adults and non-U.S. adult populations were related to diet, with U.S. adults consuming more rich protein compared to more corn and cassava in the Malawian and Amerindian populations. Genes related to vitamin biosynthesis, biotin and lipoic acid biosynthesis, the metabolism of aromatic compounds, and bile salt metabolism were found more frequently in the U.S. adult population.

Gradual changes in the microbiota occur during early childhood. The adolescent microbiota shifts from an aerobe- and facultative anaerobe–dominated community to one that is dominated by obligate anaerobes (43). From 1 to 2 years of age, the gut microbiome begins to resemble the profile of an adult, with 90% of the population identified as obligate anaerobes (28). The fecal samples of children aged 1 to 7 years are less diverse than those of adults and harbor increased proportions of *Enterobacteria* as well as higher abundances of bifidobacteria and *Clostridia* (44).

Agans et al. (45) employed array technology targeting the microbial community to quantitatively compare the gut microbiome of 22 healthy children of preadolescent/adolescent (11 to 18 years) age with that of 10 healthy adults (22 to 61 years of age). Similar microbiome profiles were observed in the preadolescent/adolescent group compared to the adult group. A 2-fold increase in *Bifidobacterium* was noted in children, but at the class level, there was greater similarity across both groups. The gut microbiomes of healthy adults and children were dominated by *Clostridia*, specifically *Ruminococcus* spp., which are considered the primary carbohydrate degraders in the human gut. *Clostridia* made up over 70% of the total sample abundance in both groups. Major classes such as *Actinobacteria*, *Bacteroidetes*, and *Bacilli* were also present in both groups; however, members of the class *Actinobacteria* appeared to be more abundant among adolescent samples.

The gut microbiome of six relatively healthy children (8 to 14 years of age) living in an urban slum in Bangladesh was compared with that of four healthy children living in an upper-middle-class suburban community of the U.S. over a 5- to 6-month time period (46). As expected, this study revealed distinct differences between the gut microbiota of Bangladeshi children and U.S. children, differences that were attributed to diet and ethnogeography. Overall, a greater microbial diversity in the gut of Bangladeshi children was observed, and a prevalence of *Firmicutes* was seen in the Bangladeshi cohort compared to a prevalence of *Bacteroidetes* in the U.S. children. In the phylum *Bacteroidetes*, *Prevotella* spp. were more abundant in the Bangladeshi population, whereas *Bacteroides* spp. predominated in the U.S. cohort.

The microbiome undergoes changes as individuals get older, and as individuals age, bacterial diversity decreases, a finding consistent with the development of additional health problems with advanced age. The gut microbiome remains relatively stable throughout adulthood but begins to vary more greatly between individuals in advanced age (>65 years), with diversity also differing based on age (47). Claesson et al. described the fecal microbiota of 178 elderly individuals and stratified them based on diet and

residence (e.g., residence location in the community, day-hospital, rehabilitation, long-term residential care), with antibiotic treatment as exclusion criteria (48). Distinct profiles were observed between community-dwelling and long-term residential care subjects. A control group of younger adults was also included and proved more similar to the community-dwelling group. Diversity decreased in the long-term residential care subjects when compared to the community-dwelling subjects as well as the rehabilitation subjects. The long-term residential care cohort had a higher proportion of *Bacteroidetes* compared to the community-dwelling group, which had higher abundances of *Firmicutes* in their gut microbiome. At the genus level, the community-dwelling individuals had higher proportions of *Coprococcus* and *Roseburia*. Metabolomics characterization was consistent with the microbiome findings, with markers of inflammation in higher levels in the long-term residential care individuals and rehabilitation individuals than in the community-dwelling cohort.

Biagi et al. surveyed the gut microbial composition of young adults (20 to 40 years of age), elderly (60 to 80 years of age), and subjects living for over 100 years (i.e., centenarians) in northern Italy (47). The microbiomes of young adults and elderly individuals were found to be similar, but significant differences were identified in the >100 years cohort. The two major phyla, *Bacteroidetes* and *Firmicutes*, dominated the gut microbiome of centenarians, and an increase in facultative anaerobes was observed including *Fusobacterium*, *Bacillus*, *Staphylococcus*, *Corynebacterium*, and certain *Proteobacteria* groups. Select *Proteobacteria* spp. are also considered pathobionts, and under specific circumstances (e.g., inflammation in the elderly), these bacteria can induce disease. As expected, the centenarians had higher levels of biomarkers associated with inflammation. A significant decrease in proportions of *Clostridium* cluster XIVa, which is a major component of the GI mucosal microbiota, was also observed in the centenarian cohort compared to an increase in younger adults and the elderly.

ENTERIC PATHOGENS

Understanding the progression of the gut microbiome from birth to advanced age allows us to identify detrimental perturbations in the healthy gut. The major organisms involved in GI infections and dysbiosis of the gut microbiome are *Vibrio* spp., *Campylobacter* spp., and those species belonging to the family *Enterobacteriaceae* (i.e., *Escherichia*, *Shigella*, *Yersinia*, and *Salmonella*). *Vibrio* spp. are the causal agent of cholera. Symptoms of this disease include watery stools and dehydration. *Campylobacter* is considered one of the leading causes of GI issues in humans (49). Symptoms of a *Campylobacter* infection include diarrhea, abdominal pain, and fever. There are multiple *E. coli* strains that can inhabit the environment as well as the human intestinal tract. This species is easily cultured and has both non-pathogenic and pathogenic strains, with six pathotypes associated with pathogenicity (e.g., enteropathogenic and enterotoxigenic) (50). Enterotoxigenic *E. coli* (ETEC) is the most common cause of diarrhea, which can be mild to life-threatening depending on the host. ETEC strains colonize and adhere to the intestinal tract and are characterized by production of toxins in the host. Enteropathogenic *E. coli* is the causal agent of acute and persistent diarrhea in infants and children in developing regions. Enteropathogenic *E. coli* strains adhere to the gut of the host and cause a decrease in the mucosal surface area as well as produce attaching and effacing lesions in the host. *Shigella* spp. cause invasive inflammatory colitis leading to bloody stool, fever, and severe cramps. These strains invade the host gut cells, replicate, and spread to nearby cells. *Salmonella* spp. cause gastroenteritis by colonization and attachment to the host tissue and release of proteins that are damaging to the host tissue. *Yersinia pseudotuberculosis* and *Yersinia enterocolitica* are the two enteropathogenic species of *Yersinia* and cause fever and gastroenteritis.

Clostridium difficile is a common species in the native gut microbiota but is also capable of causing diarrhea and severe colitis, generally reaching pathogenic population levels following antibiotic therapy. *C. difficile*-associated diarrhea begins with the release of enterotoxins from the pathogen that destroy the intestinal tissue and lead to an extreme inflammatory response. Another important gastric pathogen is *Helicobacter pylori*, a Gram-negative species that colonizes the upper GI tract, is a member of the phylum *Proteobacteria*, and infects 50% of the world's population (51). *H. pylori* uses urease to produce ammonia and bicarbonate, increasing the pH of the gastric fluid and mucosal layer and allowing the pathogen to colonize the mucosa. The colonization triggers an inflammatory response from the host, and *H. pylori* infection has been associated with the development of peptic ulcers, chronic atrophic gastritis, gastric cancers, and gut lymphoma. During infection, *H. pylori* has been reported to drastically alter the normal gut microbiota (7, 52). In addition, antibiotic resistance has also been documented in *H. pylori* (53).

DIET, OBESITY, AND THE GUT MICROBIOME

Considerable changes to the gut community have been attributed to diet alterations, specifically those involving plant dietary fiber (54). Geographic differences generally include dietary differences, and research in this area suggested the classification of the human gut microbiota into three enterotypes: those having predominantly *Prevotella*, *Bacteroides*, or *Ruminococcus* spp. (55, 56). *Prevotella*-dominated gut microbiomes are more closely associated with a rural society, while a *Bacteroides*-dominated gut microbiome is associated with a developed society, and little information is available on the *Ruminococcus*-dominated gut microbiome (56, 57). Microbial diversity in the gut is also greatly increased in individuals with a more plant- and fiber-based diet (e.g., vegetarian).

Diet is also associated with other factors influencing gut colonization such as ethnicity, sanitation, hygiene, and climate (58). Differences in the gut community can be introduced by self-selected diets (e.g., high-fat/high-sugar), and the gut microbiome responds quickly to changes. These diet-induced changes can also contribute to the development of GI disease (e.g., inflammatory bowel disease) (59). Diet has also been shown to affect the gut microbiome and its susceptibility to enteric pathogens. Gupta et al. (60) utilized pyrosequencing in stool specimens to examine the metagenome of the gut of a healthy child and a malnourished child. The family *Campylobacteraceae* was 35-fold higher in the malnourished gut microbiome, and *Helicobacteraceae* was 12-fold higher. The malnourished gut microbiome had a higher abundance of the families *Bacteroidaceae* and *Porphyomonadaceae*, and *Porphyomonadaceae* has been associated with Crohn's disease and inflammatory bowel disease (61).

While the role of diet in the development of obesity is well established, the microbiome also plays a critical role in the interaction between diet and weight. A reduction in microbial diversity, commonly found in disease states, has been observed in obese individuals compared to individuals of a healthy weight (16). Obesity and obesity-associated metabolic disorders have been associated with changes in the gut microbiome, with an increase in *Firmicutes* and a decrease in *Bacteroidetes* in obese individuals (62), but causation has not been definitively established.

In general, obesity is attributed to excess caloric intake compared with expenditures, but mounting evidence suggests that some individuals may be more susceptible to obesity due to their gut microbiome. In mouse model studies, Ley et al. examined the effects of the microbial community on increases in adiposity in mice homozygous for an obesity phenotype and their lean siblings (63). The two major phyla present in mice cecal communities were *Firmicutes* and *Bacteroidetes*, with more than 75% of the *Firmicutes* belonging to the *Clostridium* cluster XIVa (i.e., a mucosal-associated species) and *Eubacterium eligens*, and greater than 88% of the *Bacteroidetes* belonging to *Bacteroidetes* 4b, which is an uncultured group. *Protoebacteria*, *Cyanobacteria*, and *Actinobacteria* made up less than 1% of the mouse cecal microbiota. The cecal microbial communities of the obese mice had a 50% reduction in *Bacteroidetes* when compared to the lean mouse cohort. The obese cohort also had a greater proportion of *Firmicutes* than the lean group.

Via metagenomics and biochemical analyses, changes in *Firmicutes* and *Bacteroidetes* in the gut microbiota of obese mice were shown to affect the metabolic potential of the mouse gut microbiome (64). More environmental gene tags that matched *Archaea* were identified in the obese cecal microbiome than in the lean mouse cohort, and cocolonization of methanogenic *Archaea* spp. found in the gut change the specificity of bacterial polysaccharide fermentation, resulting in an increase in adiposity. The obese mice's gut microbiome was also enriched in genes related to starch and sugar metabolism, galactose metabolism, and butanoate metabolism. These mice also had increased concentrations of butyrate and acetate in their cecum as well as less energy remaining in the feces.

To further investigate the role of the microbiome in the onset of obesity, fecal transplantation using material from four adult twin pairs discordant for obesity was performed in germ-free mice (65). The mice were fed low-fat chow as well as a typical U.S. diet. The mouse transplanted with the obese twin's microbiota had a significant increase in adipose mass and epididymal fat pad weights in comparison to the mouse receiving the lean twin's microbiota. When gene expression was examined, the mice that received the obese twin's microbiota showed a higher expression of microbial genes involved in detoxification and stress responses, as well as in genes involved in the biosynthesis of cobalamin, the metabolism of essential and nonessential amino acids, and the pentose phosphate pathway. There was also an increase in branched-chain amino acids, which have been reported to be linked to obese and insulin-resistant individuals. The mice receiving a fecal transplant from the lean twin exhibited higher expression of genes related to plant-derived polysaccharide digestion, fermentation to butyrate, and fermentation to propionate. Overall, the transplanted mice exhibited distinct taxonomic, metabolic, and transcriptional profiles based on the donor microbiota (e.g., from the lean twin or the obese twin) and body mass index.

THE IMPACT OF ANTIBIOTIC TREATMENT ON GASTROINTESTINAL BACTERIA

Antibiotic treatments, by definition, alter the microbial population of the body. However, the effects of antibiotics on the gut microbiome reach beyond pathogen elimination and persist well beyond the last dose of treatment (see Fig. 2A) (66). Administration of antibiotics immediately reduces microbial diversity (67). Antibiotic treatment in newborns appears to decrease *Bifidobacterium* spp., and the effects can also be passed down, because newborns from antibiotic-treated mothers have shown lower *Bacteroides*

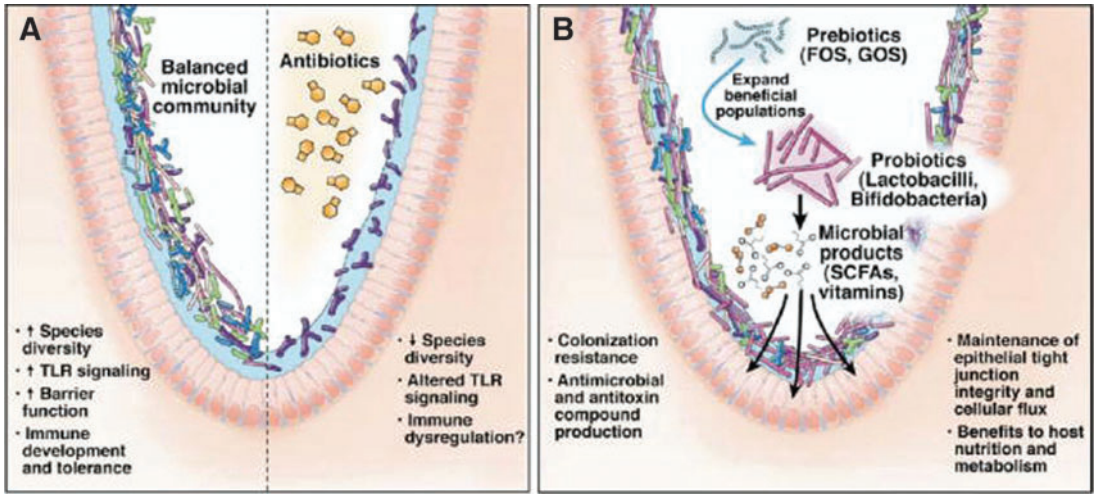

FIGURE 2 Antibiotics, probiotics, and prebiotics in the gut. (A) While antibiotics successfully eliminate pathogenic bacteria, the diversity of the overall bacterial community is diminished and can leave the host susceptible to further disease. (B) Probiotics and prebiotics are effective at manipulating the microbial community and promoting a healthier microbiome profile in the gut. (Reproduced with permission from reference 66.)

populations (68). These newly formed postantibiotic communities can remain in the host for days to several years. Inadvertently, antibiotic treatments can target beneficial populations rather than pathogenic populations. Antibiotic-resistant populations can also increase in the microbiome and cause additional structural shifts in the community.

In a study of three healthy adults before and after treatment with the fluoroquinolone ciprofloxacin, treatment was found to alter the abundance of approximately 33% of the bacterial taxa in the gut (69). The two major phyla present across all specimens were *Bacteroidetes* and *Firmicutes*, with most genera being found within the latter. The three other phyla present but less abundant were *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobia*. Antibiotic treatment also decreased the richness, diversity, and evenness of the community. Four weeks after treatment, the gut community appeared to recover to its pretreatment state.

A gut-based metabolomics study in mice before and after treatment with the fluoroquinolone enrofloxacin showed several differences in profiles. Of note, urea concentrations were increased in the antibiotic-treated mice, and because many bacteria in the gut produce ureases, a decrease in those specific taxa could lead to an accumulation of urea (70). A similar study using vancomycin-treated mice also detected significant differences in the metabolites of the gut following antibiotic treatment (71).

A thorough case study involving a 68-year-old male with no known intestinal disorders and an infected cardiac pacemaker used a multi-omics approach to investigate antibiotic-treatment-related changes in the fecal microbiome (72). Early during treatment, the microbiota was dominated by *Firmicutes*. During the first week of treatment, the families *Lachnospiraceae* and *Ruminococcaceae* were the most abundant taxa. Eleven days after treatment, the total bacterial community decreased. Also, there was a shift in the microbiota to a *Bacteroidetes*-dominated community, along with a significant increase in *Betaproteobacteria*. This group also examined the metabolically active bacteria present in the specimens, and *Firmicutes* was found to be the most active phylum in most samples. At day 14 of treatment, the metabolically active bacteria were *Bacteroidetes*, with *Parabacteroides* as the dominant genus. Also at day 14, there was a decrease in active bacterial taxa and richness. Forty days after the end of treatment, the total and active microbiota began to shift back to its original pretreatment state. However, there remained a decrease in diversity and an absence of *Slackia*, *Bifidobacterium*, *Gemmiger*, *Streptococcus*, *Roseburia*, *Barnesiella*, *Eubacterium*, and *Subdoligranulum*.

GASTROINTESTINAL DISEASE

Extensive research efforts have been dedicated to understanding the etiology and pathogenesis involved with irritable bowel diseases (e.g., Crohn's disease and ulcerative colitis), but these components have not yet been elucidated. These diseases have been linked with both human gene- and microbiome-associated factors (73). Patients with irritable bowel diseases have distinct intestinal microbiomes characterized by decreased bacterial diversity. An overall reduction in *Firmicutes* was observed in the microbiome of Crohn's disease patients (74). Another analysis examining the microbiota of ulcerative colitis patients in relapse and remission reveals the reduced bacterial diversity in ulcerative colitis patients, specifically within the *Firmicutes* (5).

Several microbiome-based studies have focused on irritable bowel syndrome (IBS). Next-generation sequencing technologies and DNA microarrays were utilized to identify specific microbial signatures in the gut microbiome of healthy children and children (7 to 12 years of age) with IBS (42). Two IBS subtypes were analyzed in this study, one described as having hard stool more than 25% of the time and loose stool less than 25% of the time and the other classified as unsubtyped IBS. These two subtypes had distinct microbiome profiles, with at least 70 to 75 different taxa. Analysis also revealed specific taxa to be associated with increased and frequent abdominal pain. Children with IBS had an increased abundance of *Proteobacteria*, specifically *Gammaproteobacteria*, compared to healthy children. At the genus level, these children had greater proportions of *Dorea* and *Haemophilus*. *Veillonella* and *Ruminococcus* spp. were also found to be associated with pediatric IBS, with an unclassified *Ruminococcus* sp. being linked with greater pain severity. Multiple species belonging to the genus *Alistipes* were associated with the children who had recurrent abdominal pain. An increased abundance of *Eubacterium* and *Bacteroides vulgatus* was seen in the gut microbiome of healthy children.

Jalanka-Tuovinen et al. used a phylogenetic microarray technology and a novel computational approach to explore the association between the intestinal microbiota and intestinal symptoms in healthy adults (6). *Firmicutes*, *Bacteroidetes*, *Actinobacteria*, and *Verrucomicrobia* were most abundant in the 15 Finnish subjects included in this study. The microbiota of the individuals who reported the most severe intestinal symptoms remained stable over the course of the study, and statistically significant bacterial taxa associated with symptomology were also identified. Bifidobacteria abundance was decreased in subjects experiencing abdominal pain, a finding consistent with the fact that a known probiotic, *Bifidobacterium infantis*, has been shown to reduce intestinal pain in patients with IBS (75). Taxa from the phylum *Firmicutes* (e.g., *Clostridiaceae*, *Ruminococcaceae*, and *Lachospiraceae*) also appeared to play a role in the manifestation of intestinal symptoms.

A combination approach of quantitative real-time PCR, high-performance liquid chromatography, and conventional culture-dependent techniques was employed to examine the GI microbiota and organic acid profiles of 26 patients with IBS (76). The IBS cohort had higher proportions of *Lactobacillus* and *Veillonella* than the control group (healthy subjects). In the organic acid analysis, the authors revealed that the IBS group had higher concentrations of acetic acid, propionic acid, and total organic acids. The higher acetic and propionic acid concentrations were also associated with GI symptoms, and these effects correlate with an altered IBS microbiome and could be an exacerbating factor in IBS.

Dietary interventions are often suggested as a treatment option for IBS. A low fermentable substrate diet in children with IBS appeared to decrease abdominal pain frequency in a subgroup identifiable by their microbiome profile (77). Diet responders exhibited increased abundance of *Sporobacter* and *Subdoligranulum* spp. and decreased abundance of *Bacteroides* spp. compared to nonresponders. These findings suggest that microbiome characterization may be a useful tool in the selection of treatment for IBS.

THE GUT-BRAIN-MICROBIOME AXIS

Advances in microbiome science have further elucidated the interconnectivity of the gut-brain axis. Specific microbiome profiles have been described in depression, and it is suggested that inflammation caused by the bacterial community in the gut interferes with neurotransmitter signaling. A recent study identified specific bacterial sequences, at the bacterial species/strain level, correlating with depression (78). Increased gut permeability in depressed individuals may also be a contributing factor in the inflammatory process, and increased IgA and IgM responses against commensal bacteria in the gut have been reported (79, 80).

Brain development and behavior are also impacted by the initial bacterial colonization of the gut (81), and the absence of a normal gut microbiome has been shown to alter central nervous system neurotransmission (82, 83). Perturbations in the brain-gut-microbiome axis are also considered as a cause of functional GI disorders (84–86). The correlation between the gut-brain axis, GI symptoms, and the gut microbiome has become highly relevant in the study of autism spectrum disorders (ASD). Gastrointestinal symptoms, ranging from constipation to diarrhea, are frequently reported in individuals with ASD, and several studies have identified altered gut microbiomes (11, 87–90). As in depression, increased intestinal permeability has been reported in patients with ASD, further supporting the "leaky gut" theory (91). Emerging research has also highlighted the potential utility of probiotics in the treatment of symptoms associated with ASD (92), but human clinical trials have yet to be reported.

Several studies have characterized the gut microbiome in children with ASD (11–15). Bacterial sequencing of intestinal biopsies in children with ASD and GI disease as well as neurotypical children with GI disease demonstrated decreased relative abundances of *Bacteroidetes* and increased relative abundances of *Firmicutes* and *Proteobacteria* in ASD compared to the neurotypical group (12). Additional studies have utilized stool specimens and performed comparisons with unaffected siblings and unrelated healthy controls but did not compare to neurotypical children with GI disease. In 2010, Finegold et al. recruited 33 children with ASD, 7 unaffected siblings, and 8 healthy controls (11). Results suggested increased bacterial diversity in ASD, with increased *Bacteroidetes* and decreased *Firmicutes* composition, findings that are contradictory to the study by Williams et al., where a larger sample size of 51 children with ASD was compared with 53 unaffected siblings (12). In further contradiction to previous studies, no significant differences were noted between the groups, but critics have suggested that an unrelated healthy control group is needed to strengthen the study (93). Microbiome comparisons between children with ASD (n = 20) and unrelated healthy controls (n = 20) revealed *Firmicutes* and *Bacteroidetes* as the dominant phyla in the ASD group, with no significant differences noted when compared to healthy controls (14). The most recent pediatric study (ASD [n = 10], pervasive developmental disorder–not otherwise specified [n = 10], and healthy controls [n = 10]) (15) demonstrated separation of groups based on microbiome profiles, but unfortunately the study excluded subjects with GI symptoms.

THE EFFECT OF PROBIOTICS ON THE GUT MICROBIOME

Probiotics have been extensively tested in various animal models, with particular attention given to the impact on inflammation of the GI tract and on the gut microbiome. While there is much anecdotal evidence regarding improved outcomes with probiotic intervention, published human clinical trials related to GI disease remain scarce. The use of probiotics and prebiotics has significant potential in the inhibition of pathogens, restoration of key microbial components, and supplying key nutrients such as short-chain fatty acids as described in Fig. 2B (66).

In a study of 18 healthy adults, administration of probiotics containing either *Bifidobacterium* or *Lactobacillus* strains was not shown to significantly alter the overall microbiome profile of each individual (94). This finding suggests that probiotic intervention will not elicit major changes in a healthy and robust gut community. Another study involving a *Lactobacillus paracasei* intervention found that the efficacy of the probiotic relied heavily on butyrate concentration prior to administration (95). Overall, probiotic treatment was found to increase the *Blautia:Coprococcus* ratio, a finding consistent with better health, and fecal butyrate concentrations represented a potential biomarker for determination of individuals who could benefit from this specific probiotic.

In contrast, a study investigating the effects of a probiotic (*Lactobacillus acidophilus*) and prebiotic (cellobiose) combination on the gut microbiome found that individuals taking the supplement displayed increased abundance of *Lactobacillus*, *Bifidobacterium*, *Collinsella*, and *Eubacterium* spp. partnered with a decrease in *Dialister* spp. (96). The results of these studies suggest that probiotic and prebiotic combinations may be most effective in gut microbiome alteration.

To assess the impact of probiotics on the prevention of infectious disease in the GI tract, a probiotic combination, consisting of *Lactobacillus helveticus*, *Lactobacillus rhamnosus*, *Bifidobacterium longum*, and *Saccharomyces cerevisiae* var. *boulardii*, was given prior to administration of live attenuated ETEC (97). While changes were seen in fecal output and GI complaints in both the probiotic and placebo groups, the probiotic did not increase resistance to orally delivered ETEC. Similarly, oral supplementation with *B. infantis* was assessed in the treatment of IBS, but while *B. infantis* was detected within the GI tract and subtle changes were seen in other taxa, no impact was observed on symptoms in patients with IBS (98).

MANIPULATING THE MICROBIOME VIA FECAL MICROBIOTA TRANSPLANTATION

C. difficile infection (CDI) is most often associated with individuals undergoing antibiotic treatment, causing diarrhea and severe colitis. De La Cochetière et al. examined the association between the most abundant resident fecal microbiota and the later development of CDI (99). The cohort included 260 adult subjects from a former clinical study that were residing in the Paris area. Specimens were collected before antibiotic treatment and 14 days after the beginning of treatment. Comparisons of the gut microbiome showed specific microbial profiles that represented permissive microbial communities and predicted *C. difficile* development. CDI is associated with the release of enterotoxins from the pathogen, which destroys the intestinal tissue and leads to an extreme inflammatory response. CDI is the leading cause of death and hospitalization from gastroenteritis in the United States (100). Occasionally, patients with CDI do not respond well to antibiotic treatments and have recurrent infections with an indefinite number of

relapses (101). A shift in the microbiome is the ultimate cause of CDI infection, and it is for that reason that a purposeful shift in the microbiome, via fecal transplantation, was first suggested as a potential treatment option for the eradication of C. *difficile* in the gut.

Fecal microbiota transplantation (FMT) is currently considered an effective therapy for the treatment of robust and recurrent C. *difficile* infections. FMT employs a bacterial community or bacterial strains from a healthy human gut to alter the composition of an unhealthy gut microbiome. The process as it relates to CDI is depicted in Fig. 3 (102). FMT is becoming a more common clinical practice due to the increased incidence and severity of CDI (103) and has proven to be an effective therapy in curing these patients and reestablishing healthy bacterial populations in the gut. Bacterial diversity in recurrent CDI patients is significantly reduced, with an increase in members of the family *Enterobacteriaceae* and a decrease in *Bacteroidetes* and *Firmicutes* populations.

A dramatic shift in the gut microbial communities of a cohort (n = 3) of patients with CDI was reported following fecal microbiota transfers (104). Prior to transplantation, the fecal specimens of the patients had a high abundance of *Proteobacteria*. Three days following transplantation, the patient gut microbiota began to mimic the donor gut, evidenced by an increase in the abundance of *Firmicutes* and *Bacteroidetes*, an increase in microbial diversity, and an overall shift toward a more healthy gut microbiome profile. After 1 month, a lack of symptoms and negative clinical diagnostic tests for C. *difficile* were noted in all patients. After approximately 4 months, two out of the three patients maintained a microbial profile similar to that of the donor. The third patient's profile began to resemble the pre-FMT composition with an increase in *Proteobacteria*, but this is likely a result of a short-term course of sulfamethoxazole-trimethoprim for the treatment of a urinary tract infection.

Similar findings were reported by Weingarden et al. when a multi-omics approach was employed to examine the effects of FMT on the fecal microbiota composition and bile acid concentrations in 12 patients with recurrent CDI (105). After FMT, the microbiome of each patient closely resembled that of the donor and resulted in an increase

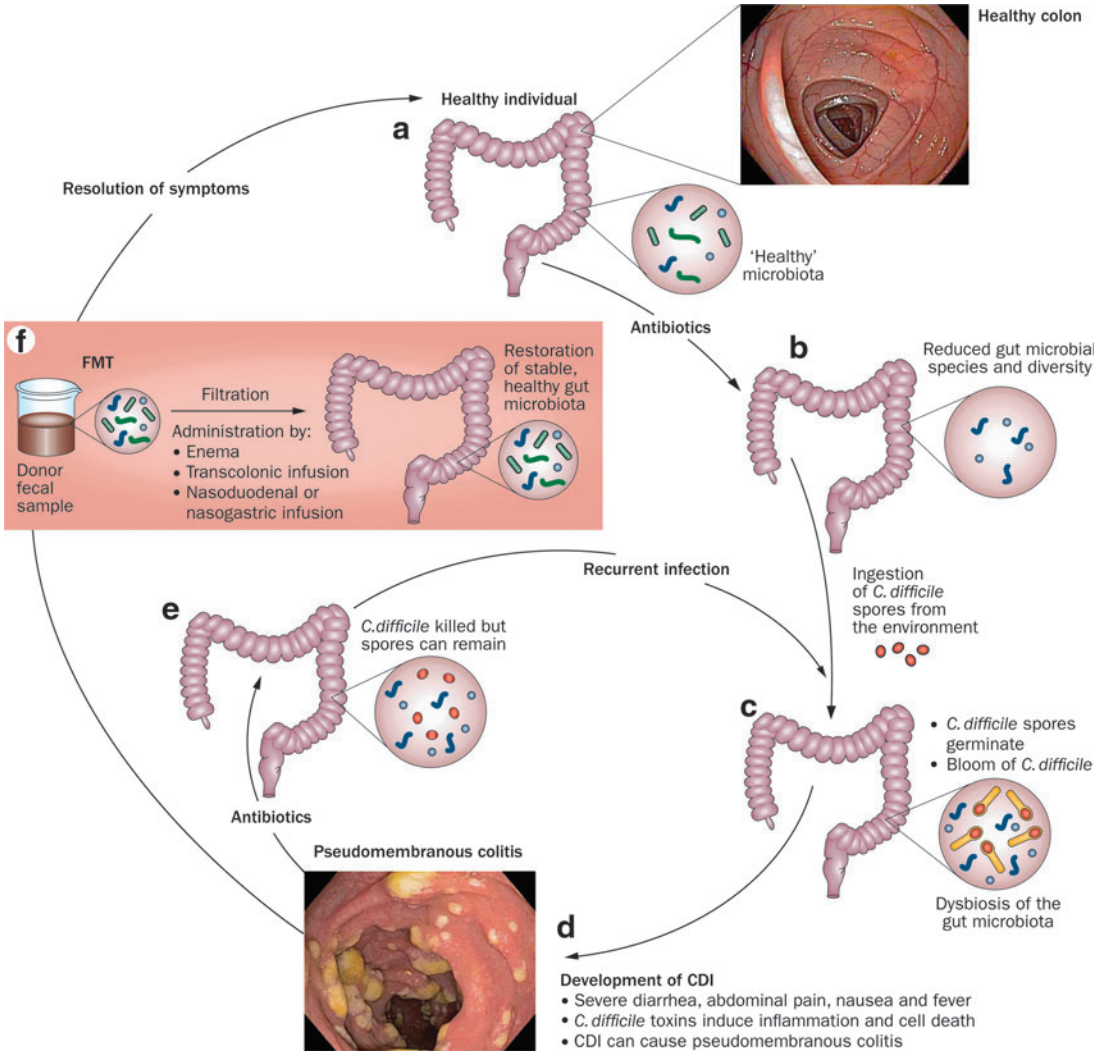

FIGURE 3 Fecal microbiome transplantation for the treatment of *Clostridium difficile* infection. Using material from a healthy donor, fecal transplants have successfully treated C. *difficile* infection and restored a healthy gut microbiome. (Reproduced with permission from reference 102.)

in abundance of *Bacteroidetes* and *Firmicutes* along with a decrease in abundance of *Proteobacteria*. Metabolomics further supported these findings, with the post-FMT metabolite profile resembling the donor profiles, and bile acids and primary bile salts, the predominant metabolites in the post-FMT and donor specimens, were restored to normal.

Beyond treatment of GI pathogens, FMT has also been evaluated in metabolic syndrome. Vrieze et al. transferred the intestinal microbiota from a lean donor to a subject with metabolic syndrome to examine the donor's effects on energy metabolism and energy sensitivity (106). Subjects with metabolic syndrome received small-intestine biopsies and bowel lavages through a duodenal tube. Subjects were assigned to a lean donor or assigned to the control group, which received an autologous gut microbiota infusion. Before and at 6 weeks after the microbiota transfer, insulin sensitivity, large and small intestine gut microbial compositions, and fecal short-chain fatty acids were measured. Improvement in peripheral insulin was seen in metabolic syndrome subjects at 6 weeks following transplant from a lean donor, and short-chain fatty acid concentrations also decreased. The microbiome profiles of obese subjects revealed a less diverse bacterial community characterized by increased abundances of *Bacteroidetes* and *Clostridium* cluster XIVa, which is a mucosal-associated group. Following transplant, microbial diversity increased in the lean donor transplant group, with no change in diversity seen in the control group. Specifically, there was an increase in *Roseburia intestinalis* and *Eubacterium halli* in the lean donor–assigned group.

A current trend in FMT therapy is the use of a more defined microbial population, derived from donor fecal specimens. The composition of these defined communities is known and can be controlled and replicated (107). More research is needed to define the most beneficial microbes from the donor community and determine which combinations would provide the most effective treatment.

THE GASTROINTESTINAL MICROBIOME AS A DIAGNOSTIC BIOMARKER

Significant shifts in the gut microbiome correlate with disease. Specifically, certain taxa appear to be associated with established markers of disease activity. These alterations may prove to be useful as diagnostic biomarkers. In 2010, Willing et al. (108) determined the composition of the gut microbiota in a cohort of 40 twin pairs who were concordant or discordant for Crohn's disease or ulcerative colitis. The authors noticed that several bacterial populations increased or decreased with disease type. Patients with ileal Crohn's disease had a decrease in *Faecalibacterium* and *Roseburia* and increased proportions of *Enterobacteriaceae* and *Ruminococcus*. Similar evidence of microbiome-based biomarker potential was found in a study by Swidsinski et al. (109), in which the mucosal flora in biopsy specimens of patients with inflammatory bowel disease (i.e., Crohn's disease or ulcerative colitis), self-limiting colitis, and IBS were characterized. They found that *Bacteroides fragilis* was responsible for >60% of the biofilm mass in inflammatory bowel disease but for only 30% of the biofilm mass in self-limiting colitis and <15% of the biofilm mass in IBS. Potential biomarkers have previously been proposed in pediatric Crohn's disease, where evaluation of *Campylobacter* spp. other than *Campylobacter jejuni* revealed a significantly greater abundance of *Campylobacter concisus* in children with Crohn's disease (110). Examination of the presence of *Helicobacter* in adults with and without ulcerative colitis revealed significantly higher abundance of the genus *Helicobacter* in patients with ulcerative colitis, as expected (111). These data suggest that robustly observed alterations in the abundance of certain microbes in the intestinal microbiome may prove to be useful biomarkers of disease as well as inflammation.

CONCLUSIONS

The human GI microbiome is dynamic in nature throughout the lifespan of an individual. With changes caused by genetics, diet, and the environment, the microbial community continues to adapt. During the evolution of the gut microbiome, each individual is uniquely predisposed to health and disease. Culture-independent methods could allow us to circumvent possible treatment failures by identifying antibiotic resistance in the metagenome of individual gut microbiomes. Defining the gut microbiome of healthy individuals and those patients affected by gastric diseases and disorders allows for the identification of disease-associated microbes and novel therapeutic targets. Microbiome manipulation via antibiotics, probiotics/prebiotics, and fecal transplantation can successfully alter the bacterial community of the gut and ultimately promote health and quality of life.

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The Vaginal Microbiome

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12

The collection of microbes (microbiota) in the human vagina with their associated genes (microbiome) and gene products helps to shape the physiology of the vagina (1). Different vaginal microbial communities are associated with different metabolic states, immunological responses, and risks of adverse health outcomes including preterm birth and acquisition of sexually transmitted infections. Tools in molecular microbiology have cataloged the diversity of vaginal microbes, leading to a more complete census, and have identified species that may be useful indicators of health or markers of disease risk.

The microbiota of the human vagina is distinct from that found at other human body sites and is characterized by more constrained bacterial species diversity, reflecting the unique properties of the vaginal environment (2). Vaginal microbes are dependent on host-derived nutrients for sustenance, unlike in the mouth or gastrointestinal tract, where nutrients from the diet can be easily co-opted by microbes. Sources of microbial nutrients in the vaginal environment may include glycogen in the vaginal epithelium, vaginal tissue fluid coating the surface of the vagina, mucus from the cervix, menstrual fluid (blood, protein, and endometrial cells) from the uterus, shed vaginal and cervical epithelial cells, and semen with sexual activity. The vaginal environment changes over the lifespan of an individual and also over the course of a menstrual cycle, with the potential for associated changes in the vaginal microbiota (3, 4).

The human vaginal microbiota is important to understand because it may impact the health of women and their sexual partners, the success of pregnancy and conception, and the health of newborns. The data showing a link between the vaginal microbiota and conception are sparse, though the presence of *Lactobacillus crispatus* vaginally and on a transfer catheter tip was associated with an increased rate of successful *in vitro* fertilization (5). Vaginal colonization with group B streptococci is associated with neonatal infection including sepsis and meningitis—hence the mandate for group B streptococci screening of pregnant women, coupled with antibiotic treatment if positive. Newborns delivered vaginally are initially colonized with maternal vaginal bacteria, and this initial exposure may shape the assembly of the indigenous neonatal gut microbiota. For example, there are interesting differences in neonatal microbiota when delivered vaginally versus by cesarean section (6). The vaginal microbiota has been associated with risk of preterm birth in numerous studies (7), with several vaginal bacteria found in amniotic fluid of women suffering from preterm labor (8). Unfortunately, efforts to reduce the rate of preterm birth through antibiotic therapy for bacterial vaginosis (BV) have not met with consistent success, with some studies showing benefit and other studies showing harm (9–11).

The vaginal microbiota (BV) has been linked to risk of sexually transmitted infections including gonorrhea, chlamydia, trichomoniasis, herpes simplex virus, and human papillomavirus infection (12, 13). In addition, BV has been linked to risk of HIV acquisition in women and transmission of HIV to men (14). The vaginal microbiota has also been associated with pelvic inflammatory disease, infertility, and risk of gynecologic infections such as posthysterectomy vaginal cuff cellulitis and postpartum endometritis in numerous studies. Clearly there are many known connections between the vaginal microbiota and states of health and disease, and new connections are coming to light with the development of advanced molecular diagnostic approaches for detecting vaginal microbes. More mechanistic studies are needed to dissect how the vaginal microbiota may mediate these risks, if indeed the microbiota is in the causal pathway to adverse health outcomes.

This chapter provides perspectives on the composition of the healthy vaginal microbiota, the changes in microbiota that ensue with BV, and the links between the vaginal microbiota and disease states. In addition, I will discuss the development of molecular diagnostic tools for detecting vaginal microbes.

THE HEALTHY VAGINAL MICROBIOTA

Before describing the microbiota associated with health, one needs to define "healthy" and distinguish between the terms "asymptomatic," "normal," and "healthy."

I will use the term "healthy vaginal microbiota" to refer to vaginal microbial populations that are common (present in a large fraction of women) and are associated with protection from adverse events such as preterm birth and acquisition of sexually transmitted infections. Women with vaginal microbiotas dominated by lactobacilli are considered healthy per this definition, though not all lactobacilli may impart the same degree of health benefit. Many studies have documented that women without BV have vaginal colonization with a few key *Lactobacillus* species, including *L. crispatus*, *Lactobacillus iners*, *Lactobacillus gasseri*, or *Lactobacillus jensenii*, though other lactobacilli can also be present including *Lactobacillus vaginalis* (15, 16). In

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some women a particular *Lactobacillus* species dominates the vaginal community, such as *L. crispatus* or *L. iners*. In other women there is more equal representation of two or more *Lactobacillus* species such that any single species cannot be considered truly dominant. It is common for a few other bacterial species to coexist with lactobacilli in the vagina of healthy women, including *Gardnerella vaginalis*, some *Prevotella* species, and other bacterial taxa that are usually present in low abundance.

Some investigators have proposed that vaginal bacterial communities in humans can be categorized into a handful of community types based on hierarchical clustering of 16S rRNA gene amplicon sequences. For example, Ravel et al. suggested that there are five bacterial community types in the vagina of asymptomatic women, with four of the five clusters notable for abundance of a particular *Lactobacillus* species, and the cluster designated "IV" having diverse microbiotas notable for the presence of species previously linked to BV (15). Indeed, women in cluster IV tended to have BV as documented by Gram stain, but clinical criteria for BV (Amsel criteria) were not assessed in that study. Other investigators have found different vaginal bacterial community profiles, and this may reflect differences in DNA extraction methods, broad-range PCR primers, study populations, and clustering algorithms used to generate community types. For example, Smith et al. found six bacterial community types using cervical swabs, including types dominated by *G. vaginalis* (17). The *G. vaginalis* community type may have been missed in the work by Ravel and colleagues due to use of a broad-range 16S rRNA gene PCR primer (27F) with mismatches to the *G. vaginalis* sequence (18).

Lactobacilli have been considered markers of vaginal health for several reasons. First, multiple studies have associated a lactobacillus-dominated vaginal microbiota with protection from adverse health outcomes, including preterm birth, pelvic inflammatory disease, risk of HIV acquisition and transmission, and risk of other sexually transmitted infections (19–21). It is possible that lactobacilli serve as markers of disease risk but do not have an active role in mediating risk, though this seems unlikely. What direct role could lactobacilli play in promoting health and modulating risks of adverse health events? Glycogen is present in the vaginal epithelium (22). Lactobacilli can convert glucose from vaginal glycogen to lactic acid. The lactic acid helps to decrease the vaginal pH, which may be microbicidal by creating an environment inhospitable for growth of other microbes at this acidic pH of 3.5 to 4.5. In addition, lactic acid has direct antimicrobial properties beyond that mediated by the change in vaginal pH (23). In support of this connection, higher levels of free glycogen in the vagina are associated with lower vaginal pH and higher abundance of lactobacilli (22).

Bacteriocins and other antimicrobial compounds secreted by vaginal lactobacilli may also be toxic for other bacterial species (24). Vaginal lactobacilli thus create an environment that is antagonistic to the growth of many non-*Lactobacillus* species, including some pathogens. In addition, lactobacilli may compete with introduced non-*Lactobacillus* species for existing resources, thereby providing colonization resistance. In summary, vaginal lactobacilli are likely markers of health because they have relatively benign interactions with the human host while tending to inhibit the growth of pathogens, though the relative importance of different inhibitory forces is debated.

THE NORMAL VAGINAL MICROBIOTA

Although having a vaginal bacterial biota dominated by lactobacilli tends to be associated with states of health, are there other vaginal bacterial communities that can be considered normal? The answer to this question depends on your definition of normal. Normal can mean several things, including being within a normal distribution of vaginal bacterial community types, occurring naturally, or found in normal women such as those women without a diagnosed disease. Using these definitions of normal, it is possible to argue that non-*Lactobacillus* vaginal bacterial communities can be normal. For example, vaginal bacterial communities dominated by *G. vaginalis* and some anaerobic bacteria can be found in women who appear to be currently healthy and not symptomatic. To wit, we used quantitative PCR assays targeting common vaginal bacteria in a longitudinal study of women to show that some apparently healthy women have a decrease in lactobacilli and a surge in concentrations of *G. vaginalis* during menses (3). The dynamic nature of the vaginal microbiota during menses was confirmed by other investigators using broad-range 16S rRNA PCR with sequencing, though *G. vaginalis* was likely underrepresented in this analysis due to primer bias, and therefore conclusions about relative abundances of *Gardnerella* during menses cannot be made from this study (25).

Some investigators have insisted that having a vaginal microbiota characterized by diverse anaerobic bacteria that includes BV-associated bacteria should be considered normal (15). It is true that about >50% of women with BV diagnosed by Gram stain of vaginal fluid lack symptoms (26, 27). But are these asymptomatic women with BV-associated vaginal bacteria normal? Since up to 29% of women in the United States have BV (27), which is mostly asymptomatic, one could make the argument that BV is within the normal distribution of vaginal microbiotas. But is a woman with BV as healthy as a woman without BV? Regardless of symptoms, women with BV have elevated risks of preterm birth, HIV acquisition, pelvic inflammatory disease, and other adverse events including postsurgical complications. An appropriate analogy is the middle-age adult who has severe hypertension and hypercholesterolemia; the person may be asymptomatic now, and even have blood pressure and cholesterol values that fall within an elevated but normal distribution. Yet this person has an elevated risk of myocardial infarction and stroke and therefore cannot be considered optimally healthy despite his or her outward appearance and lack of symptoms. Similarly, women with BV may be considered normal (based on some definitions of normal listed above) if asymptomatic, but this categorization ignores the health risks that are consistently associated with BV. Asymptomatic and normal do not equate with healthy.

Fungi can be found in the vagina of asymptomatic women, including *Candida albicans* and *Candida glabrata* (28) and, more rarely, *Saccharomyces cerevisiae* (29), but these organisms are also associated with symptomatic vaginitis. It is not clear why some women colonized with *Candida* species develop inflammation and symptoms while others do not, but differences in host factors are suspected (30).

Detailed surveys assessing for the prevalence of archaea in vaginal samples have not been reported.

Several viruses and numerous bacteriophage have been detected in the vagina of asymptomatic women, including herpes simplex virus, cytomegalovirus, and human papillomavirus. These viruses are also associated with pathology in the reproductive tract of some women, highlighting the

observation that microbes with pathogenic potential can also be found in normal or asymptomatic women. *Lactobacillus* phages have been detected in the human vagina, and some investigators have hypothesized that these viruses may shape the vaginal bacterial biota by depleting lactobacilli (31). However, there are no clear studies showing a temporal relationship between bacteriophage acquisition and loss of lactobacilli or development of BV.

BV

BV is a very common condition in women of reproductive age. The National Health and Nutrition Examination Survey (NHANES) assessed for the prevalence of BV using Gram stain of vaginal fluid in a nationally representative sample of women in the United States, revealing that 29% of women had BV and 84% of women did not report symptoms, although these women were not examined for the presence of vaginal discharge or amine odor (27). BV is the most common cause of vaginal discharge, though ~40% of women with BV by Gram stain of vaginal fluid lack symptoms in most studies (32). It is not known why some women with BV are symptomatic while others are less affected or completely asymptomatic, though differences in microbial populations and their metabolites may help drive differences in symptoms (16). BV is considered by many to be a dysbiotic condition and is characterized by a decrease in abundance of most lactobacilli coupled with an increase in abundance of anaerobic bacteria in complex communities. Although many risk factors for BV are known, the proximate factors that lead to this shift in vaginal microbiota are not clear.

The diagnosis of BV was originally based on assessing for the presence of four clinical criteria, commonly known as Amsel criteria based on the original publication by Amsel et al. (33). A diagnosis of BV requires the presence of three of the following four criteria:

1. A homogeneous, thin, gray-white discharge that smoothly coats the vaginal walls.
2. A vaginal pH of >4.5.
3. A positive "whiff test" for amines, which is the presence of a fishy odor when 10% potassium hydroxide (KOH) is added to a sample of vaginal fluid, usually on a microscope slide.
4. The presence of clue cells on saline wet mount visualized by light microscopy. Clue cells are shed vaginal epithelial cells that are studded with bacteria, usually coccobacilli, and obscure the edges of the epithelial cells. At least 20% of epithelial cells should be clue cells to yield a positive result.

The advantage of using Amsel criteria to diagnose BV is that the approach is relatively cheap and employs little technology except pH paper, KOH, and a light microscope. There is some subjectivity to the Amsel diagnostic approach since it relies on the sense of smell for the whiff test and the recognition and quantification of clue cells, which requires some experience.

A more objective approach for the diagnosis of BV is based on interpretation of vaginal fluid smear Gram stains, where multiple observers can assess and validate the results. The microscopist notes the abundances of several bacterial morphotypes (bacterial shapes and Gram stain characteristics) such as bacterial forms resembling lactobacilli, *Gardnerella/Bacteroides*, and *Mobiluncus* when using Nugent criteria to define BV (34). A low Nugent score of 0 to 3 is characterized by large numbers of lactobacillus morphotypes and normal vaginal microbiota, a score of 7 to 10 indicates decreased lactobacillus morphotypes and increased abundance of coccobacilli, indicative of BV, and a score of 4 to 6 is considered intermediate between these two poles. The scoring system of Ison and Hay is another approach for using Gram stain to diagnose BV (35). The problem with Gram stain as a diagnostic test is that interpretation of the smear results requires an expert microscopist skilled at this specific task, and the resulting turnaround time is long, so it is not a feasible point-of-care test. Gram stain remains the "gold standard" for diagnosis of BV and thus is commonly employed in research studies where turnaround time of results is not paramount.

Numerous reviews have summarized the adverse health outcomes associated with BV, ranging from preterm birth to acquisition of HIV infection. What is missing from most studies is mechanistic insight into how vaginal bacteria associated with BV may mediate these health risks. One persistent scientific conundrum arises from the observation that BV is consistently associated with risk of preterm birth, yet most (though not all) studies of antibiotic treatment for BV in pregnancy have failed to show a benefit in reducing this risk (36–38).

The Microbiology of BV: Cultivation Studies

BV is characterized by a bloom of anaerobic and facultative bacteria in the vagina, with an increase in species richness and diversity. Early cultivation studies identified numerous bacterial species associated with BV, including those in the genera *Prevotella*, *Porphyromonas*, *Mobiluncus*, and *Mycoplasma* (39). However, cultivation has not been a reliable diagnostic test for BV. In the 1950s Gardner and Dukes isolated a bacterium from women with the condition nonspecific vaginitis, now known as BV, and made the argument that this bacterium was the primary cause of BV (40). Originally named *Haemophilus vaginalis*, the isolated organism was renamed *Gardnerella vaginalis* in honor of Gardner. Subsequent studies have revealed that G. *vaginalis* is indeed present in most women with BV but is also present in the majority of women without BV when sensitive detection methods are used (41). The failure to induce BV in most women inoculated with pure cultures of G. *vaginalis* (40) and the presence of G. *vaginalis* in women without BV provide evidence against the fulfillment of Koch's postulates for disease causation. On the other hand, high concentrations of G. *vaginalis* are a more specific indictor of BV, and this may indicate a threshold effect wherein bacterial levels that exceed a particular threshold lead to onset of BV (42). Supporting this concept is work on vaginal biofilms in women with BV showing that G. *vaginalis* is a major constituent of dense mats of bacteria coating the vaginal epithelium in some women with BV (43, 44). Accordingly, it may not be the mere presence or absence of G. *vaginalis* that is important in BV, but rather the density of bacteria, interactions with other bacterial species, and what these bacteria are doing. When highly sensitive PCR detection methods are used, the presence of G. *vaginalis* has very poor specificity for BV (41). In addition, high concentration of G. *vaginalis* can be found in some women without BV during menses (3).

The Microbiology of BV: Broad Molecular Surveys

Our understanding of the microbiology of BV has been greatly expanded through the use of nucleic acid–based

microbial detection and identification methods. The primary method employed in this approach has been broad-range 16S rRNA gene PCR with sequencing of amplicons (15, 16, 45, 46), though other phylogenetically informative genes have also been used as PCR targets, such as *cpn60* (47, 48). A nucleic acid amplification-independent approach relies on high throughput metagenomic sequencing of community DNA to identify microbes and their genes; this approach is tractable but has been little used to study BV. One advantage of these molecular approaches is that fastidious or uncultivated bacteria are revealed, providing a more complete census of microbes. However, biases exist with each approach, and this is also true for broad-range PCR methods where the consensus primers used to bind conserved DNA sequences may not efficiently amplify the DNA from all species in a way that represents the true abundances in the sample (18, 49). It is the intervening variable sequence between primer binding sites that provides the phylogenetic resolution to identify genera and species, but first, amplification must occur in the target gene.

There have been many large studies analyzing the vaginal microbiota of women with BV, and usually comparing the microbiota to women without BV or in some cases to women who were designated asymptomatic. These studies highlight the fact that women without BV tend to have vaginal bacterial communities dominated by lactobacilli, whereas women with BV have vaginal bacterial communities that are more diverse, with many putative anaerobes, and have more evenness based on sequence reads (i.e., less likely to show domination of a single bacterial species, though this can occur in BV) (16). Some investigators have reported that diverse anaerobic bacterial communities can occur in asymptomatic women, but these women usually had BV by Gram stain, and unfortunately, Amsel criteria were not assessed (15). We know that a large fraction of women with BV by Gram stain are asymptomatic (27).

Several new bacterial species have been identified or detected in the human vagina using molecular surveys and associated with BV, including *Atopobium vaginae*, *Sneathia amnii*, *Sneathia sanguinegens*, an *Eggerthella*-like bacterium, two novel *Megasphaera*-like bacteria, several *Prevotella* species, and several bacteria in the *Clostridiales* order that we have designated BV-associated bacterium 1, 2, and 3 (BVAB1, BVAB2, BVAB3) (45, 46, 50).

The microbiology of BV is heterogeneous, and this is demonstrated in a study of 220 women in Seattle who had their vaginal swab subjected to broad-range 16S rRNA gene PCR with pyrosequencing (Fig. 1) (16). Some women with BV had vaginal bacterial communities dominated by *Prevotella* species, some by BVAB1, and some by *Sneathia* species. Other women had vaginal bacterial communities that were more even, without species domination. All women with BV had highly complex communities of bacteria, which contrast to the *Lactobacillus* genus domination in women without BV. It is not clear why these different bacterial consortia form in BV, and nor is the nature of the cross-species interactions, but it is clear that BV is never a mono-microbial process.

Bacterium-specific quantitative PCR (qPCR) has demonstrated that onset of BV is associated with acquisition of BV-associated anaerobes, and antibiotic treatment for BV is associated with depletion of these anaerobes. Interestingly, treatment with antibiotics does not usually lead to

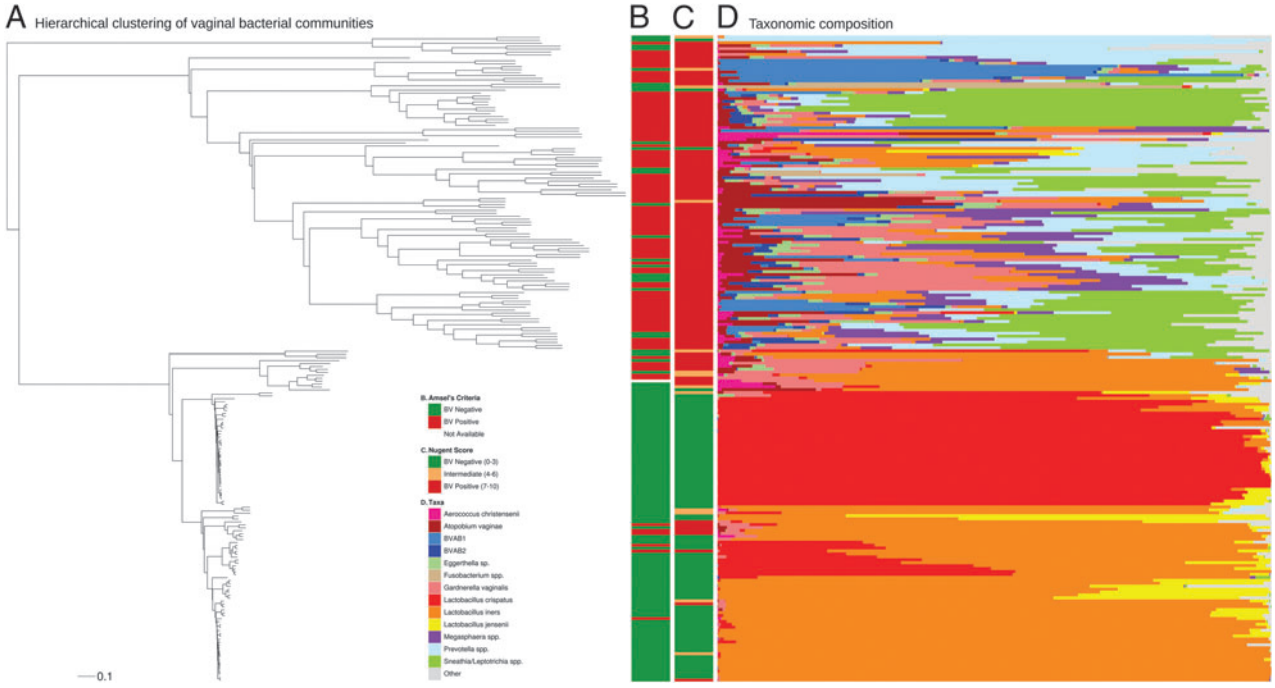

FIGURE 1 Vaginal bacterial communities in all women. (A) A novel variant of hierarchical clustering was used to generate a clustering tree depicting the bacterial diversity in 220 women with and without BV. The scale bar represents KR distance, a generalization of UniFrac. The colored bars represent bacterial taxa that were most abundant in each sample. Less abundant taxa are grouped in the "other" category. BV status by Amsel criteria (B) and Gram stain (C) is provided for all women in the two vertical bars. In the absence of BV, the vaginal microbiota was dominated by *Lactobacillus crispatus* or *Lactobacillus iners*. Women with BV had more diverse bacterial communities (16).

eradication of G. *vaginalis*, though concentrations usually decrease with therapy (51). It is notable that the antibiotics deemed to be most effective in BV are metronidazole and clindamycin, which have excellent coverage against anaerobic bacteria. These antibiotics are effective when given vaginally or orally, though there is a high rate of BV recurrence after antibiotics are stopped. Metronidazole is not a reliable antibiotic for treating G. *vaginalis* infection (39).

Diagnostics for BV

There is a need for rapid, objective, and accurate diagnostic tests for BV given the limitations of (Amsel) clinical criteria and vaginal fluid Gram stain interpretation (e.g., Nugent criteria).

Currently available technologies include the BD Affirm VP III test, which uses a nucleic acid probe to detect a high concentration of G. *vaginalis* in a nonamplified assay (52–54). The assay can be completed in under an hour. The rationale behind this assay is that G. *vaginalis* may be present in women without BV but is not likely to be detected in this platform given the nonamplified format and low assay sensitivity or high detection threshold. In contrast, women with BV tend to have high concentrations of G. *vaginalis*, and thus the assay has reasonable specificity for BV. The flaw in this approach is that some normal women experience very high concentrations of G. *vaginalis* during menses and yet do not have BV, so there may be some false-positive results.

The OSOM BVBLUE assay is a point-of-care diagnostic that operates by producing a chromogenic compound in the presence of sialidase activity. Sialidase is known to be elevated in vaginal fluid from women with BV, and several bacterial species may produce these enzymes. The assay can be completed in 10 minutes. This assay has performed well with some patient populations (55, 56) and less well in others (57).

The Potential for Molecular Diagnostics in BV

Several published studies have tested the utility of species-specific PCR assays for the diagnosis of BV using either conventional PCR assays or qPCR. One of the earliest such studies was published by van Belkum et al. in 1995 and showed that G. *vaginalis* was present in 10 of 11 women with BV but present in 40% of women regardless of BV status, highlighting the poor specificity of G. *vaginalis* PCR for BV (58). Similarly, in 2001 Schwebke and Lawing analyzed the prevalence of *Mobiluncus* in vaginal samples from 74 women and noted a sensitivity of 84.5% for BV. *Mobiluncus* was also detected by PCR in 38% of women without BV, again showing limited specificity for BV, though the species *Mobiluncus curtisii* was more specific (59). In 2002 Obata-Yasuoka described a multiplex PCR assay to detect the three bacterial Gram stain morphotype-genera (*Gardnerella*, *Bacteroides*, and *Mobiluncus*) associated with BV by Gram stain, demonstrating a sensitivity of 78.4% and a specificity of 95.6% compared to Nugent score (60).

The advent of advanced molecular methods for characterizing the vaginal microbiota in women with BV has led to the identification of many additional bacteria that could be used as markers of BV (45, 46, 61). For example, in 2004 Verhelst et al. and Ferris et al. used species-specific PCR assays to show some utility of A. *vaginae* as a marker of BV (45, 62). Bradshaw et al. (50) applied PCR assays for A. *vaginae* and G. *vaginalis* to vaginal samples from 358 women and demonstrated a sensitivity of 96% and specificity of 77% for A. *vaginae* PCR in diagnosis of BV. The PCR assay for G. *vaginalis* was 99% sensitive but only 35% specific, showing once again that the presence of G. *vaginalis* is a poor indicator of BV. Sha et al. used quantitative PCR to show that using a selected threshold for concentration of bacterial DNA could improve the specificity of the PCR assay for G. vaginalis (63). Similarly, Menard et al. linked quantity of bacterial DNA to diagnosis of BV, with a focus on A. *vaginae and* G. *vaginalis* (42, 64). Use of quantitative cutoffs to define positive test results was essential to improve specificity in these studies, with some expected decline in diagnostic sensitivity.

Fredricks et al. used a panel of conventional species-specific PCRs applied to vaginal samples collected in a cross-sectional study of >200 women, with sequence confirmation of the amplicons to assure amplification of the intended bacterial target. PCR detection of either a vaginal *Megasphaera* species or BVAB2 yielded a sensitivity of 99% and a specificity of 89% for diagnosis of BV compared to Amsel clinical criteria, and a sensitivity of 95.9% and a specificity of 93.7% compared to Nugent criteria for diagnosis of BV (41). Of note, the subjects in this study were drawn from two clinics in Seattle, Washington, and the performance characteristics may differ in other populations of women. Zozaya-Hinchliffe et al. also found an association between detection of *Megasphaera* species by qPCR and presence of BV, but in a cohort of women with mucopurulent cervicitis (65). The results of a more extensive panel of qPCR assays targeting vaginal bacteria and applied to vaginal samples from 37 women were described by the same group in 2010, again showing the utility of *Megasphaera* species and many other bacteria as markers of BV, though the study population was of limited size (66).

How do bacterium-specific PCR assays for BV perform in populations of women outside the United States and Europe? Almost every study published to date shows that the prevalence of vaginal bacteria is different in different studies, and this likely reflects differences in study populations and risks for BV, the DNA extraction methods employed, and different PCR assays performed with different DNA detection thresholds and different specificities for the bacterium being targeted. Nevertheless, studies from China (67), Africa (68, 69), and Greenland (70) suggest that the vaginal bacterial species present in these women are generally similar to what is found in the United States and Europe. It does not appear that that there are vaginal bacterial species that are unique to a given population of women. More recent studies have confirmed the value of key bacterial species as indicators of BV, have highlighted the value of quantification of bacterial DNA by PCR to identify DNA thresholds indicative of BV rather than low-level colonization, and have illuminated the value of using multiple species rather than a single target to inform the diagnostic algorithm (71–74).

Is PCR-based diagnosis of BV ready for deployment? There are "reference" labs that are currently offering many of these species-specific PCR assays to clinicians. Determining the true value of these assays requires having well-defined protocols (such as DNA extraction and quality control) and assays with validated lack of cross-reactivity with other species. Furthermore, we need a much better understanding of how the performance of these PCR assays for diagnosis of BV differs in different populations of women. For instance, does one need to use different assay targets or different DNA thresholds to indicate a positive result when used in a population of commercial sex

workers with high risk for BV seen at an STD clinic compared to college women seen at a student clinic? There have been few large studies investigating these questions, and no studies have compared large numbers of women in different settings.

The ability to diagnose BV using PCR is very exciting because it offers the potential to combine these BV PCR assays (41) with other nucleic acid–based assays in a single platform for detecting multiple genital tract infections simultaneously, such as infection with *Trichomonas vaginalis*, *Chlamydia trachomatis*, *Neisseria gonorrhoeae*, herpes simplex virus, or C. *albicans*. Metagenomic methods that rely on sequencing whole-community DNA offer another avenue for characterizing vaginal microbial populations, but there are few studies using this as a diagnostic approach, and it is not currently practical outside a research setting.

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Microbial Communities of the Male Urethra

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13

HISTORICAL UNDERSTANDING OF THE MICROBIAL COMMUNITIES OF THE MALE URETHRA

Traditionally, the male urethra in healthy men was believed to be sterile or only transiently colonized by microorganisms. This view reflected technical limitations in studying this site that were not easily surmounted until methods for cultivation-independent bacterial identification became available. First, while the obvious analogous compartment to the male urethra may be the female urinary tract, given the function of the penis, the male urethral compartment is most often compared to the vaginal microenvironment. A simple Gram stain of these two body sites demonstrates substantial differences. Vaginal samples from healthy women typically contain significant numbers of epithelial cells and a large number of microorganisms including *Lactobacillus* and *Candida* species among others (see chapter 12). In contrast, it is unusual to identify similar numbers of microorganisms by simple Gram stain of urethral smears obtained from healthy men. An unintended consequence has been that far less effort has gone into characterizing the relatively sparse microorganisms that are routinely observed, and ignored, in urethral specimens from healthy men. As described later in this chapter, recent cultivation-independent surveys of male urethral swab, urine, and semen specimens have confirmed that the male urethra harbors smaller absolute numbers of microorganisms than the vagina but have also identified a surprisingly diverse array of bacterial taxa, many of which correspond to pathogens and commensal organisms found in the vagina. This has led to the reemerging idea that the microbiology of the male urethra may be more complex than is appreciated and that the composition of urethral microbial communities, collectively the urethral microbiome, might play a role in male urethral health and disease analogous to the role of the vaginal microbiome.

There are multiple challenges associated with culture-based methods for detecting microorganisms in the male urethra. Some of these are related to the form and function of this body compartment. While the vagina is exposed, via sexual activities or hygiene practices, to outside influences, it also remains a partially protected environment with little efflux. In contrast, the male urethra is repeatedly flushed by urination and ejaculation. This results in lower organism loads and enrichment for organisms that can adhere to and/or invade the urethral epithelium. Since little is known about the composition of the normal microbiome of the male urethra, optimal media and conditions for the cultivation of urethral microorganisms are often unknown. Illustrating this, five of the most common sexually transmitted bacterial pathogens of the male urethra (*Neisseria gonorrhoeae*, *Chlamydia trachomatis*, *Trichomonas vaginalis*, *Mycoplasma genitalium*, and *Ureaplasma urealyticum*) can only be cultivated using highly specialized cultivation methods. Additionally, the necessary culture conditions for these five pathogens are highly divergent, ranging from culture on agar media, culture in broth, and tissue culture often requiring days to weeks to cultivate organisms.

The physiological form of the penis presents challenges, compared to the vagina, regarding collection of adequate specimens for cultivation of organisms. Sampling of the male urethra, which provides the best specimens for microbial culture, is painful and provides very limited specimen volume. In clinical practice, urethral sampling is never performed in healthy men and is typically only performed when men present with urethritis or other urinary tract complaints. Coupled with the development of rapid and specific nucleic acid amplification tests (NAATs) for diagnosis of many sexually transmitted infections (STI) that function well with urine (1), this clinical limitation led to a dramatic decline in the collection of male urethral specimens from which residual portions might be used for research into the microbiome of this compartment. Although residual urine specimens are still routinely collected and retained by many STI research groups, the acidity of urine makes this specimen inferior to urethral swabs for culture of many fastidious urethral microorganisms. Further, fastidious organisms usually do not survive in stored urine specimens.

Despite the challenges associated with urine and urethral specimens, attempts to culture male urethral bacteria began in the 1950s and expanded widely by the 1970s. This was driven by three factors. First, it began to be appreciated that a significant proportion of men who presented with urethritis failed to respond to penicillin treatment and were negative for *N. gonorrhoeae*. This resulted in development of the name of the syndrome nongonococcal urethritis (NGU) when no other etiologic agent of urethritis was identified (2–4). Second, a broader range of media and methods for culture of anaerobes became widely available (5–7). Finally, this was the era when many microorganisms, now recognized as causal, were first

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associated with NGU including C. *trachomatis* (8, 9), M. *genitalium* (10), U. *urealyticum* (11–14), herpes simplex virus (15), and T. *vaginalis* (16). Success triggered the search for additional NGU-associated pathogens that continues today. However, since most of the early studies of urethral bacteria focused on identifying agents of NGU when incidence of this syndrome was rapidly increasing, the studies often suffered design weaknesses including imprecise clinical definitions, lack of case-control and treatment studies, and investigation of one or a few targeted microorganisms (17–23). Nonetheless, by the mid-1970s it was generally appreciated that aerobic bacteria including *Staphylococcus*, *Streptococcus*, *Lactobacillus*, *Haemophilus* (subsequently determined to be *Gardnerella vaginalis*), and a handful of poorly differentiated anaerobes at least periodically colonized the healthy male urethra.

A case-control study of men with and without NGU by Bowie et al. in 1977 provided the foundation upon which many more contemporary studies of the male urethral microbiome have been built (13). Goals of the study were to identify proportions of NGU cases that could be attributed to C. *trachomatis* and U. *urealyticum* and to identify other urethral pathogens possibly associated with this syndrome. Corresponding urine, urethral swab, and postprostatic massage specimens from 69 men with well-defined urethritis and 39 controls without urethritis were subjected to broad-range anaerobic and aerobic culture. The results confirmed that C. *trachomatis* infection was strongly associated with NGU (8, 24, 25) and provided early evidence that U. *urealyticum* was more frequent in men with nongonococcal nonchlamydial urethritis (NGNCU) than in similar men without urethritis (14). No significant association between NGU or NGNCU and other bacteria was found, but the results provided one of the first pictures of the urethral microbiome in sexually active adult men. Aerobes were almost ubiquitous, and *Staphylococcus epidermidis*, various *Corynebacteria* spp., lactobacilli, and G. *vaginalis* were each detected in specimens from at least half the men in one or more of the NGU, NGNCU, and control subgroups. Aerobic lactobacilli, G. *vaginalis*, and α-hemolytic streptococci were enriched in the controls compared to the combined NGU group (NGU and NGNCU). Anaerobes were also cultured from 91% of the control group. These included multiple *Bacteroides* spp. and undifferentiated lactobacilli, *Propionibacterium* and *Eubacterium*, and Gram-positive cocci. Gram-negative bacteria were more frequent in the control group than in the combined NGU group, and complete absence of anaerobes was significantly less frequent in the control group (9%) than in the NGNCU group (34%). The authors were unable to draw firm conclusions concerning the significance of these differences but proposed that altered microbiomes in men with NGU might predispose them to infection with urethral pathogens. This important hypothesis continues to guide studies of the urethral microbiome today.

Culture-based investigations of the male urethral flora declined in the 1980s, likely because attention was focused on NGU pathogens that were becoming more firmly established such as C. *trachomatis*. Enthusiasm may also have been diminished by preliminary associations of NGU with various urethral microorganisms including *Bacteroides ureolyticus* (26–28), G. *vaginalis* (29–32), and *Clostridium difficile* (33) among other organisms, which were subsequently determined to be more likely to be commensals in later case-control and treatment studies (13, 14, 32–41). Nonetheless, these studies employed improved cultivation approaches that expanded the list of bacteria known to colonize the urethra.

A key question that remained unanswered until the 1980s was, What was the origin of nonpathogenic microorganisms in the adult male urethra? Although indirect evidence for sexual transfer of a variety of bacterial vaginosis (BV)–associated taxa from men to women, and of G. *vaginalis* specifically, existed (42–44), the respective roles of sexual exposure, maternal transfer, auto-inoculation and/or environmental reservoirs in establishment of the urethral microbiota were unclear. A cross-sectional, cultivation-based study of 16 adolescent men who denied previous sexual activity (group 1), 42 young men who reported sexual activity and who did not have pyuria (elevated white blood cell counts in urine) (group 2), and 32 young men who reported sexual activity and had pyuria (group 3) provided some of the first answers (45). Urine and urethral swab specimens from the young men were cultured for both anaerobic and aerobic bacteria and for specific STIs including C. *trachomatis*, N. *gonorrhoeae*, U. *urealyticum*, and *Mycoplasma* spp.

Aerobic bacteria were isolated from every subject, and similar numbers of species were isolated from individuals in the three groups. Diptheroids, coagulase-negative staphylococci, lactobacilli, and streptococci were abundant in all three of the groups. Since these taxa are common on the penile skin and glans, the authors speculated that these represented normal flora of the distal urethra. Interestingly, α-hemolytic streptococci were more abundant in group 1, suggesting that these might be commensal and displaced by bacteria subsequently introduced through partnered sexual activity. Anaerobes were isolated from approximately 80% of the specimens in each of the groups, but greater numbers of anaerobic taxa were isolated per specimen in sexually active groups 2 and 3. *Peptostreptococcus* and *Bacteriodes* spp. were almost exclusively isolated from groups 2 and 3; many of these resembled vaginal bacterial associated with BV. G. *vaginalis* was frequent in specimens from groups 2 and 3 but was only identified in a single group 1 specimen. *U. urealyticum* and *Mycoplasma* spp. were observed frequently and were exclusively identified in specimens from the sexually active young men, supporting the hypothesis that these organisms may be sexually transmitted. No anaerobes were statistically associated with NGNCU, consistent with previous studies (13, 27, 46). In summary, this landmark study established that the adolescent male urethral microbiota is established prior to initiation of partnered sexual activity and is additionally shaped by subsequent sexual exposures.

A few detailed cultivation-based studies of male urethral specimens have been periodically reported since the late 1980s. A study by Mazuecos and colleagues (92) identified bacteria in urethral specimens from 110 adult men; 35 did not have urethritis, 17 had gonococcal urethritis, and 58 had NGU. Streptococci were more frequent in healthy men than men with NGNCU, diversity in the gonococcal urethritis group was substantially lower than other subgroups, and Gram-negative anaerobes were more frequent in men with urethritis than controls. Bacteria found previously in vaginal specimens were identified including *Veillonella*, *Porphyromonas*, *Bacteroides*, *Clostridium*, and *Prevotella* spp. A study by Willen and colleagues of specimens from the vas deferens, prostatic secretions, semen, urethra, and coronal sulcus from 97 healthy men showed that the upper male genital tract (prostate gland and vas deferens) is usually sterile, as is semen, until it passes through the urethra

(47). Over 80% of the semen samples collected from these men yielded positive bacterial cultures, and the same species of bacteria were often isolated from corresponding prostatic secretion, semen, and urethral specimens. Over 70% of isolates identified in coronal sulcus swabs were detected in urethral specimens, indicating that there is substantial cross-talk between these habitats. However, only 1 of 61 surgically collected vas deferens samples yielded viable bacteria.

Krieger reported similar results from localization studies of bacteria in serial urine and postprostatic massage specimens collected from men with prostatitis (48). Gram-negative and Gram-positive bacteria were detected relatively frequently, but these only rarely localized specifically to the prostate fluid specimens, indicating that the organisms originated in the lower genital tract, usually in the urethra (48). The same group detected bacterial 16S rDNA in approximately 77% of surgically collected prostate specimens in another study, but these specimens rarely yielded bacteria in culture (49). A more recent study of 18 healthy men and 24 men with persistent nonspecific urethritis differentiated closely related *Corynebacterium* spp. and *Staphylococcus* spp. in urethral specimens (50). Bowie's original observation that lactobacilli were more frequent in urethral specimens from healthy men compared to men with urethritis was also confirmed, because 100% of the healthy men and none of the men with persistent, nonspecific urethritis were colonized with lactobacilli (14). Correlations between the loss of urethral microbial diversity and persistent nonspecific urethritis, and positive association of persistent nonspecific urethritis with opportunistic bacteria including micrococci, enterococci, and *Staphylococcus aureus* were also reported (50). Provocatively, staphylococci isolated from the men with persistent nonspecific urethritis had higher decomplementary activity, which was defined as the decrease in complement activity under the influence of culture supernatants, than did isolates from the no-urethritis control men. This illustrates that finer differentiation of urethral microorganisms can differentiate pathogenic and nonpathogenic microorganisms from broader higher taxonomic divisions that show weak or no disease association. This could be particularly pertinent to identification of new agents of NGU considering that differentiation of species of commensal and pathogenic *Mycoplasma* spp. (*Mycoplasma hominis* vs. *M. genitalium*) and *Ureaplasma* spp. (*U. urealyticum* vs. *Ureaplasma parvum*) resolved conflicting data concerning roles of specific members of these genera in this syndrome (51–56). Collectively, the cultivation-based studies described above reinforced the idea that the upper genital tract of healthy men is usually sterile but that the distal urethra contains a diverse and dynamic microbiota.

CULTIVATION-INDEPENDENT CHARACTERIZATION OF THE MALE URETHRAL MICROBIOME

Cultivation-independent characterization of the urethral microbiome could not be accomplished without two key technological advances. First, PCR made specific amplification of rare bacterial nucleic acids in complex milieus of clinical specimens feasible for the first time (57). Second, Pace and colleagues showed that knowledge of a bacterium's 16S small subunit rRNA sequence (16S sequencing) was often sufficient to identify the organism, even if the bacterium could not be cultivated (58). The coupling of degenerate 16S PCR with high-throughput DNA sequencing has driven the subsequent rapid evolution of cultivation-independent approaches for characterization of bacterial communities in general. Excellent reviews of this topic for the reader interested in more detail have been written by Clarridge (59) and Köser (60).

Riemersma and colleagues were the first to characterize urethral microbial communities using a cultivation-independent 16S-based approach. Despite the small size of their study, it was significant for its novelty and findings (61). Urine was collected 2 weeks apart from five healthy men and five men with NGNGU. Men in the latter group were treated with azithromycin, which is effective against *Chlamydia* and potentially *Mycoplasma*, at enrollment. Bacteria in urine were identified using a degenerate 16S PCR and restriction fragment length polymorphism (RFLP) approach where representative restriction fragments were sequenced. Reagents were analyzed to differentiate potential contaminants which included *Pseudomonas* spp. in the local water source. In the control group 71 RFLP types were identified, and 84 were identified in the urethritis group. This study detected much greater microbial diversity than had been found using cultivation-dependent methods in previous studies. RFLP types were frequently identified in only one of the two corresponding urine samples, indicating that many bacteria were not stable residents of the microbial communities. Sequences from *M. genitalium* and *Haemophilus parainfluenzae*, known urethral pathogens, were each detected in one urine specimen from a participant with NGNCU. Interestingly, one RFLP type was detected in all five healthy men but in none of the men with urethritis; however, this bacterium was not identified. Many RFLP types corresponded to taxa that had been cultured from urethral and urine specimens in previous studies, including streptococci, *Corynebacterium*, *Veillonella*, and *Gemella*.

The potential of broad-range 16S sequencing for investigation of the urethral microbiome was suggested by early studies of vaginal bacteria in women with and without BV (62–66) (see also chapter 12). Although it had long been appreciated that BV was associated with replacement of vaginal lactobacilli with a population of mixed anaerobes, these studies revealed that remarkable microbiological diversity was associated with this syndrome (67, 68). Although many taxa associated with BV corresponded to anaerobic taxa that Bowie and others had previously identified in specimens from men with NGNCU, another 5 years passed before male specimens were similarly analyzed. One reason for this delay may have been the belief that bacterial loads were too low in male specimens or the difficulty associated with collection of male urethral samples. Some researchers investigated previously described members of the urethral microbiome in men with NGU using panels of sensitive PCR probes for known urethritis- and BV-associated organisms. In a study by Masue and colleagues urine specimens from 161 men with urethritis were analyzed using multiplex PCR for 16 known and suspected urethral pathogens (69); 43.6% of the specimens from these symptomatic men tested negative for all organisms. This once again highlights the need for further research in this area to examine the entire microbiome rather than a subset of known organisms to identify the causative agent or mechanisms involved in this large proportion of cases of NGU.

To study the urethral microbiome among a broad cross-section of men, Dong and colleagues assessed the relationship between organisms detected in corresponding urethral

swabs and urine samples (70). This study was conducted among attendees of a sexually transmitted diseases clinic in the midwestern United States. Urethral swabs were collected first, followed by collection of a urine specimen (≤60 ml) from 32 men. Of these 32, 10 were diagnosed with one or more STIs (*C. trachomatis*, *N. gonorrhoeae*, and/or *T. vaginalis*), while the remaining 22 were not, so the analyses were stratified based on STI diagnosis and urethritis. Microbiome analysis was based on 16S rRNA pyrosequencing of the V1 to V3 regions, and bacterial taxa were assigned using Ribosomal Database Project classifier v2.2 (71). In the STI and no-STI groups, as well as the urethritis and no-urethritis groups, the relative abundance of the most common genera was similar in corresponding urethral swab samples and urine specimens. Analysis of intra-individual variation also demonstrated that corresponding urine and swab specimens from any individual in the study were usually more similar to one another than to any other study specimen. These data suggest that for larger studies, urine is a useful sample type that provides data, particularly on the population level, that is representative of male urethral microbial communities.

In addition to validating the use of urine as an appropriate specimen for microbiome analysis, the study by Dong et al. demonstrated that in men without urethritis and/or STI the male urogenital tract supports diverse bacterial taxa. These included known STIs (e.g., *N. gonorrhoeae* and *Mycoplasma* spp.) as well as a number of genera associated with BV in women (e.g., *Prevotella*, *Atopobium*, *Sneathia*, *Streptococci*, *Veillonella*, *Aerococcis*, *Anaerococcus*) (72, 73). Based on these findings, additional research was conducted with samples obtained from sexually transmitted disease clinic attendees who did not have urethral symptoms during their clinic visit. In this study, urine samples were used to assess the microbial communities of 19 men who had fewer than five polymorphonuclear cells on Gram's stain smears, 8 of whom had an asymptomatic STI (74). Similar to the findings from men with urethritis, in this population of men without urethral complaints the microbial communities comprised a high diversity of organisms, with more than 70 genera identified across all specimens. *Lactobacillus*, *Streptococcus*, and *Staphylococcus* spp. were more common in men without a concomitant STI, while in men with an STI *Prevotella*, *Gemella*, *Sneathia*, and *Leptotrichia* spp. were more common.

These feasibility and proof-of-concept studies led to a longitudinal assessment of the urogenital microbiome in young men recruited from community settings. Adolescents between the ages of 14 and 17 were targeted for participation since this is an important developmental phase during which, among other things, the sexual repertoire is expanding, and thus organisms may be introduced into the urethral niche. Behavioral data were collected on a daily basis, and urine samples were obtained monthly for up to 2 years. Microbiome data were obtained using full-length 16S rRNA gene PCR and Sanger sequencing and three independent 16S rRNA gene subregion pyrosequencing approaches. The recurring collection of urine specimens allowed the first assessment of stability of the microbial community composition in healthy individuals. In the preliminary analysis of data from 18 young men, variations in organisms identified, and their relative abundance, were observed over the first 3 months of follow-up (75). Despite this variation, samples from any given individual were more similar to each other than to samples from other participants.

In addition to analysis of variation over time, samples from this cohort also revealed interesting features of urethral microbial communities among healthy young men. Perhaps not surprisingly, the urethral microbiome is similar to that of other mucosal surfaces: *Lactobacillus*, *Prevotella*, *Streptococcus*, and *Staphylococcus* were found in the majority of urine specimens. Sexual activity appears to influence the communities since, in this small population, *Mycoplasma*, *Ureaplasma*, and *Sneathia* were identified only among men who had engaged in partnered sexual activity. Additionally, some young men who reported no (lifetime) partnered sexual experiences were colonized with organisms routinely associated with BV in women (e.g., *Gardnerella*, *Atopobium*, etc.). Further research is needed to elucidate the role these organisms in the male urethra may play on downstream risk of BV in women.

Circumcision status was captured during this study, and this factor appears to influence the communities of both the coronal sulcus (see reference 75 for additional information about the microbiome of this habitat) and the urogenital microbial communities. For example, urine specimens from circumcised participants were enriched in aerobes and putative cutaneous taxa including *Corynebacterium* and *Staphylococcus*, whereas only specimens from uncircumcised participants contained *Prevotella*. For many years there has been strong epidemiologic data regarding the reduction in risk of HIV acquisition as a result of circumcision, but the mechanism of this protection is still not understood. Emerging epidemiological links between circumcision and STI susceptibility in men (76) suggest that the composition and dynamics of male urogenital tract microbiomes could have ramifications for health and susceptibility to infectious diseases in both men and women. The finding that circumcision influences the colonization of the urogenital compartment of healthy young men may lead to improved understanding of organisms that provide a beneficial or protective effect or those that result in increased susceptibility to infection.

POTENTIAL FOR DIAGNOSTICS AND EPIDEMIOLOGIC APPLICATIONS

As a result of the incredible pace of growth in the field of next-generation sequencing (NGS) and microbiome analysis, the length and accuracy of reads are increasing and the costs of sequencing are decreasing. This has led to development of both research tools and clinically accessible, often highly automated, assays for a variety of health-related conditions. The landscape of clinical diagnosis has been radically altered by the application of NGS and other molecular tools in ways that make the future of clinical laboratory science dynamic and exciting (77, 78). The influence of NGS will not require that every clinical laboratory begin to perform sequencing, and all of the necessary analysis to interpret the data generated, for every analyte or condition of interest. While in some cases sequencing may be of great benefit clinically, the majority of advances are going to result from an improved understanding of the urethral microbiome.

The "-omics" revolution, which includes genomics, transcriptomics, proteomics, and metabolomics, will provide information that can be used to impact the future of molecular medicine (Table 1). An important caveat of genomic approaches is that they do not differentiate bacterial nucleic acids from viable and nonviable bacteria. Also, the sensitivity of PCR-based diagnostics and 16S sequencing

TABLE 1 Potential diagnostic and clinical utility

Input: human samples			
Genomics (DNA-based)	Transcriptomics (mRNA-based)	Proteomics (protein-based)	Metabolomics (metabolic products-based)
Pathogen discovery	Drug resistance potential	Biomarkers	Environmental effects
Pathogen detection	Quantification	Drug resistance activity	Drug development
Quantification		Immuno-modulators	
Epidemiologic surveillance			
Biomarkers			
Output: bioinformatics			

approaches can be a limitation in specimens that contain low bacterial loads, as is the case with most urethral specimens. Contaminating bacterial nucleic acids in PCR reagents and specimen collection tubes and from laboratory workers are real risks with these approaches. Transcriptomics and proteomics are critical to understanding the gene expression and protein profiles that are involved in the phenotype (rather than the genotype) characterization of organisms and may help overcome some of these limitations. For example, a drug-resistance gene may be present in the genome, but it may not be expressed, and thus the organism may remain sensitive to the compound in question despite the presence of the gene. Information regarding phenotypes may be highly relevant to decisions related to targeted treatment regimens. These studies may be particularly relevant to vaccine development in the STI field (79). Metabolomics, the newest in the field of -omics, is highly relevant to understanding organism-host interactions and whether those interactions are more likely to be protective or associated with disease. The need for applied data from these newer -omics is clear, but as yet little has been done as it relates to the male urethral habitat.

Genomics is the oldest, and thus often the most advanced, of these fields of study. Genomics can (i) improve our understanding of a healthy microbial environment, (ii) identify new pathogens associated with disease, (iii) provide improved diagnostic targets for known pathogens, (iv) identify biomarkers for risk of future infection, (v) identify drug resistance genes in known pathogens, and (vi) identify targets for novel drug development. Examples of the potential for prevention, diagnostic, and treatment applications of these types of data related to the male urogenital tract are described below.

An illustration of the impact of sequencing on our understanding of the male urethral microbiome is the evolution of our understanding of the role of *Ureaplasma* in NGU. For many decades investigators have attempted to understand the role of these organisms in the development and persistence of NGU as mentioned above (80). With additional sequence information, Robertson and colleagues proposed that the species formerly known as *U. urealyticum* in fact comprised two species (*U. parvum* and *U. urealyticum*) (81). New epidemiologic data are shedding light on the fact that *U. parvum* may be very common in healthy men, while *U. ureaplasma* is associated with NGU (82). The disease-specific focus of many of the recent studies does not allow us to draw conclusions regarding the role, if any, of *U. parvum* in maintaining a healthy urethral environment.

The potential application of information generated by microbiome analyses is already being realized in research settings. Manhart and colleagues have used PCR assays developed for identification of BV-associated organisms in an effort to identify organisms related to NSU in men (39). Urine from 157 men with NSU and 102 controls was tested using these assays. In this study, *Leptotrichia* and/or *Sneathia* spp. were significantly associated with urethritis and accounted for 15% of NSU cases. Interestingly, other organisms associated with BV (BVAB-2, BVAB-3, and *Megasphaera*) were found only in men with *Leptotrichia* and/or *Sneathia* spp. Future research will determine whether *Leptotrichia* and/or *Sneathia* spp. are causative agents of urethritis and would be appropriate targets for a diagnostic assay or whether these organisms may be useful as a diagnostic biomarker of a group of other organisms that cause disease in men. This information may have clinical utility in the near future or may influence the direction of researchers interested in this area of study. However, the limitation of this type of approach is that it focuses on (i) previously identified pathogens and (ii) only men with disease.

The ability to identify the most common and most abundant organisms in healthy men using microbiome analyses provides an opportunity to define the constituents of a healthy urethral environment. Our understanding of the vaginal microenvironment has taught us that the presence of certain microbes (i.e., lactobacilli) is essential to maintaining vaginal health, and we may find an analogous situation in the male urethra. Currently few studies have attempted to determine the microbial community structures in healthy men other than as a comparison, or control group, in studies of men with urethritis. In the study of adolescent men described in the preceding section, we have begun to develop an emerging picture of the composition and stability of the urethral microbiome in healthy young men (75).

An interesting secondary analysis of data from this project was made possible by the high rate of chlamydial infection observed during the longitudinal follow-up of these young men who were routinely asymptomatic. Eleven incident chlamydial infections were identified among the first 54 participants, 78% of whom had not reported any lifetime sexual exposure at enrollment. The Sorensen's Index of similarity was increased in the samples collected at the visit during which infection was identified compared to the sample collected 1 month prior to infection. Similarly, the total numbers of sequences from samples at enrollment and 1 month postinfection were higher than the numbers obtained from the infection and 1 month preinfection visits (Fig. 1). Further analysis demonstrated the appearance and disappearance of certain taxa in relation to the identified onset of chlamydial infection. Of particular

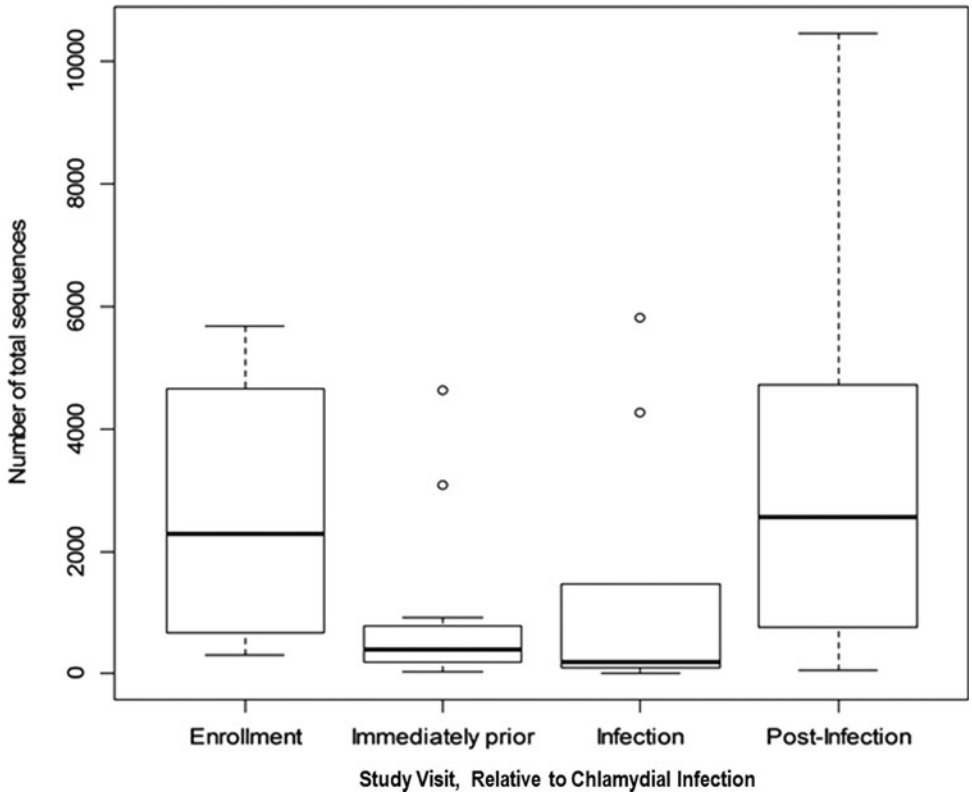

FIGURE 1 Sequence diversity before, during, and after an STI. Total number of sequences obtained from samples collected at baseline, 1 month prior to infection with *Chlamydia trachomatis*, at the time infection was detected, and 1 month posttreatment.

interest in this study was the fact that some taxa (i.e., *Veillonella*) appeared for the first time concurrently with chlamydia and remained following antibiotic treatment (Fig. 2).

A well-described epidemiologic feature of chlamydial infection is that one of the most relevant predictors of current infection is a history of previous infection. As a result, the Centers for Disease Control and Prevention recommend follow-up screening within 3 to 6 months of treatment for a diagnosed infection (83). Although the sample size is extremely small ($n = 11$) for this evaluation, it is possible that the altered microbial composition has either lost a protective member of the community or gained organisms that result in an environment that is more receptive to infection. The data suggest that further longitudinal studies using microbiome analyses may help to identify mechanisms through which chlamydial infection alters the susceptibility of the host to future infections. It is important to recognize that such studies will have to take host immune factors into account as well as the microbial community profiles since it is likely that susceptibility to STIs in general, and chlamydia specifically, is affected by multifactorial influences. With this caveat in mind, this study illustrates the potential for application of microbiome analysis to our understanding of the complex relationship between commensal and pathogenic organisms. In much the same way that the absence of lactobacilli in vaginal samples is considered to be a biomarker of vaginal disease, future

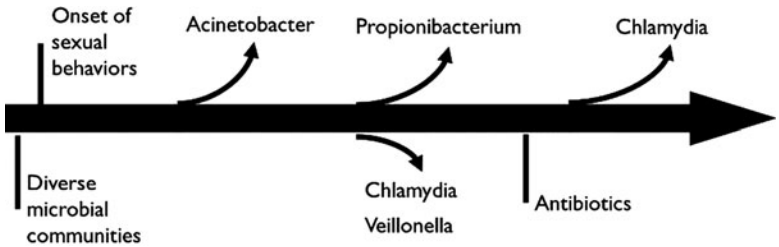

FIGURE 2 Exemplar timeline of community composition changes associated with STI. Example of microbial community changes associated with a common STI (*C. trachomatis*). The male urethral microbiome is diverse and complex prior to onset of partnered sexual behaviors. In a longitudinal study, for some men genera routinely found in the urogenital compartment are notably missing prior to and during infection with chlamydia (e.g., *Acinetobacter* and *Propionibacterium*, P values ≤ 0.05), while others (e.g., *Veillonella*) are first detected during chlamydial infection but remain following antibiotic treatment.

research may identify biomarkers that predict risk of future chlamydial infections or other STIs.

C. trachomatis also provides an example of the utility of NGS as it applies to improving diagnostic assays. First-generation NAATs were widely introduced in the late 1990s throughout the United States and Western Europe. An interesting evolutionary phenomenon that occurred in Sweden was detected in 2006 (84). The Swedish chlamydial screening program used two NAAT platforms at this time. These platforms utilized different amplification targets, both of which were located on the cryptic plasmid. Positivity rates began to decline in counties using one of the NAATs, while rates remained steady in counties using the other assay. Subsequent testing revealed a high number of false-negative results in the counties with apparent decreases in the positivity rates. Sequencing revealed a 377-bp deletion in the new variant that encompassed the primer target region for the assay that had begun to have a high false-negative rate (85, 86). This is the first description of an evolutionary response to diagnostic, rather than antimicrobial, pressure. NGS is a tool that can rapidly identify such changes, and in this case, the second generation of most chlamydia NAATs contains two target regions to avoid this problem in the future for this organism.

Unlike *C. trachomatis*, which is considered to have limited genetic exchange with other organisms due to its intracellular lifestyle, *N. gonorrhoeae* is recognized as having a high degree of genetic flexibility (87, 88). This often complicates diagnosis since target regions are subject to mutations (89). Further, the issue of drug susceptibility is a key factor in the control of *N. gonorrhoeae*. Since the first widespread use of antibiotics against this pathogen, the gonococcus has developed or acquired resistance mechanisms to all classes of compounds except cephalosporins, and only one injectable form of this compound is currently recommended for treatment of uncomplicated gonococcal infections (83). No commercially available molecular diagnostics exist at this time that can identify the presence of genes related to resistance or the transcription of those genes. As global STI control efforts become more reliant on molecular diagnostics, standardized tools that can provide information related to antimicrobial susceptibility are desperately needed. At this point, culture capacity must be maintained to fully evaluate the phenotype of organisms (i.e., drug susceptibility and resistance patterns). However, the future holds vast potential for moving toward molecular assessments based on both genomics and proteomics (90). Organisms that both contain known resistance genes and express appropriate proteins, suggesting that the mechanism is active, may be managed clinically in ways that differ from the management of infection with fully susceptible organisms. While this may never be a cost-effective public health measure, molecular tools will certainly be applied to monitoring the spread of drug resistance and improving our understanding of the epidemiology of diseases of the male urethra.

Beyond diagnostics and disease management, genomics and proteomics have and will continue to have a role in the identification of virulence factors and the development of vaccines for currently known STIs and those that may be discovered in the future. Understanding the virulence factors of pathogens is a critical step in the process of identifying new or improved targets for the treatment of vaccines. Identifying genes and proteins that are appropriate antigens for vaccines is a commonly used tool that will continue to be relied upon as we learn more about the organisms for which vaccines would be desirable. Identification of virulence factors may be used to genetically modify pathogens into attenuated organisms that may have utility as live organism vaccines. This is an area in which efforts will be substantially intensified in the years to come.

CONSIDERATIONS

While genomics and other -omics are exciting new tools in our research repertoire, it is important to remember that microbiome studies should be focused on answering specific questions and testing hypotheses as we move forward. The field has been in the exploratory stage for the last decade, with a very real need to develop an overarching understanding of microbial communities in states of health and disease. However, future studies should be hypothesis-driven, data-generating studies with translational outcomes in mind from the outset.

There are important considerations concerning the application of microbiome studies to routine epidemiology or clinical utilization for diagnostics or treatment assessment. The possibilities for application of data obtained from -omics research is often limited by our ability to process the data generated by the studies. Bioinformatics analyses of this type of research is a field that is evolving as rapidly as sequencing technologies. As more whole-genome sequences are available, and as technologies that provide deeper sequencing coverage continue to improve, the assembly of the data becomes somewhat more precise, but the data overload continues to require methods of analysis that can control for the expected false discovery rate that comes with gigabytes of data. As a result, analyses of a data set may provide very different interpretations today than we would have had 1 year ago. This results in a continual "moving target" of analyses and comparisons. Further, given the cost and technical expertise required to obtain and analyze samples for microbiome analysis, we will always have limited sample sizes. Thus, we need to exercise caution in making global statements and microbiome composition in the male urethra. Finally, microbiome analyses are confounded by the natural intra-individual variation in community composition that may occur as a result of (i) time since urination, (ii) partnered sexual behaviors, (iii) diet, (iv) consumption of vitamins or other nonantimicrobial compounds, (v) drug use, (vi) other systemic infections, (vii) hygiene habits, or (viii) any of a multitude of other possible behaviors and environmental stressors. Thus, we may never fully understand the "core" microbial community profiles that are associated with a healthy urethral environment. Hopefully we will be able to identify key microbial players (both commensal and pathogenic organisms) and begin to understand the complex interactions that result in a variety of health states.

Diagnostics that are based on NGS will have to take all of these complexities into account, and manufacturers will have to develop complex interpretative software to provide useful feedback to clinicians. While this may be an area of potential application in the near future for critical illnesses such as cancer and cardiovascular disease, it is unlikely that urethral diseases will warrant the capital investment that would be required to use NGS for detection of urethral pathogens or susceptibility profiles such as one might envision as part of a personalized medicine profile. Thus, it is likely that the most obvious clinical application of the data obtained from -omics research will be for developing new, or improving existing, diagnostic targets and perhaps monitoring the spread of antimicrobial resistance.

SUMMARY

Targeted treatment and/or more personalized health management in the future may include evaluation of an individual's microbial community profile, types, numbers, and relative abundance of bacteria, with resulting suggestions for risk management and health maintenance. It has been hypothesized that some cases of NGU may actually be the result of dysbiosis within the microbial community and the resulting inflammatory response rather than the result of a specific etiologic agent (91). Better understanding of the microbiome of the male urethra will be essential to disentangling the role of pathogens and host immune response in men. Powerful -omics tools should assist us with finally understanding the organisms that are epidemiologically associated with urethritis and with a healthy microenvironment. This may lead to new diagnostics, new treatment strategies (which may even include probiotics), and the identification of biomarkers related to onset of unhealthy immune responses. Although research regarding the male urethral habitat is a much more recent interest than research related to the vaginal microenvironment, the application of new technologies should help to improve our understanding of this body site in the near future. This will allow sexual health researchers to begin to build a more comprehensive picture of the dynamic interpersonal interchanges that lead to disruption of sexual health among both men and women.

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The Human Virome in Health and Disease

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14

Early studies of the human microbiome were directed at bacteria. However, just as the bacterial microbiota affects human health and disease, viruses have analogous interactions. Thus, the human microbiome should be thought of as having a viral component, which is designated the human virome (Fig. 1 and Table 1). The definition of the human virome is complicated by the complexity of viruses and their life cycles. Viruses may be associated with acute infections that may or may not produce manifestations of disease and in which the presence of the viral etiologic agent is transient. In other cases, viral infections are persistent with prolonged presence of the implicated virus and ongoing replication. Persistent infections may or may not be associated with disease. In addition, some viruses become latent following acute infection. During latency, the viral genome persists, but viral replication does not occur, although transcription of some viral genes may take place. Another component of the virome consists of sequences within the human genome that appear to have resulted from remote incorporation of viral elements into the human genome. Designated as endogenous human retroviruses, these sequences cannot generate infectious viral particles. They occupy approximately 4.8% of the human genome (1). Finally, bacteriophages are viruses that infect the bacteria that make up the human endogenous microbiota. While all of the forms of viral infection described above may legitimately be considered part of the human virome, this chapter will focus on the first three groups: namely, viruses that infect eukaryotic cells and are capable of independent replication. The reason for that focus is that these are the viruses that have been associated to date with human disease and are the targets of diagnostic testing.

As is also true of the nonviral human microbiome, the concept of the human virome is inseparable from the development of powerful culture-independent methods to detect viruses that have revealed much greater diversity than was previously appreciated. Unlike bacteria, for which a specific DNA molecule, namely the gene encoding 16S rRNA, can be used to achieve a census of resident bacteria, no single DNA or RNA segment or molecule can be used to detect and classify all viruses. Also, the fact that viruses typically have genomes that consist of either DNA or RNA means that comprehensive methods to detect viruses must address both genomic categories. Because of these considerations, studies of the virome require metagenomic sequencing (also referred to as "shotgun" sequencing), in which sequencing is performed on all of the nucleic acid present in a sample without prior isolation or culture of each species. Metagenomic sequencing has been greatly facilitated by the development of high-throughput sequencing methods (also known as "next-generation" or "massively parallel" sequencing) (see chapter 6).

Among the reasons for being interested in the use of high-throughput sequencing to characterize the human virome is its potential use as a viral or microbial diagnostic tool. This possibility is intriguing because of the possibility of culture-independent detection of an unlimited number of viruses and other microbes within the same diagnostic test. Also, high-throughput sequencing-based diagnosis offers the possibility of generating information in addition to the presence or absence of microbes that goes far beyond traditional culture-based or PCR-based diagnosis, including detailed identification and characterization to the level of serotype, genotype, or individual strain; detection of antimicrobial drug resistance genes; and detection of genes associated with virulence or other properties of interest. One of the prerequisites for using high-throughput sequencing as a diagnostic tool is to have information about the background pattern of microbes that will be detected in healthy individuals. This information can be supplied by studies that survey the microbiome at various body sites of interest.

After reviewing methodological aspects of virome studies, this chapter will provide a comprehensive view of studies of the human virome that have been carried out to date.

METHODS FOR VIROME STUDIES

Metagenomic sequencing is now the method of choice for defining the human virome. This method generates nucleotide sequence data from nucleic acids extracted from a sample containing mixed organisms as well as material of host origin. Thus, a metagenome is the composite of all of the genomes from a sample of mixed organisms. The steps involved in carrying out metagenomic sequencing for viruses are shown in Fig. 2 and include (i) initial sample processing (e.g., concentration, filtration, or neither) and nucleic acid extraction, (ii) library construction with possible enrichment for viral sequences (e.g., by target amplification or capture), (iii) nucleotide sequencing, typically using a high-throughput sequencing platform, (iv) primary sequence analysis, including initial processing and cleanup of raw data generated by the sequencing instrument

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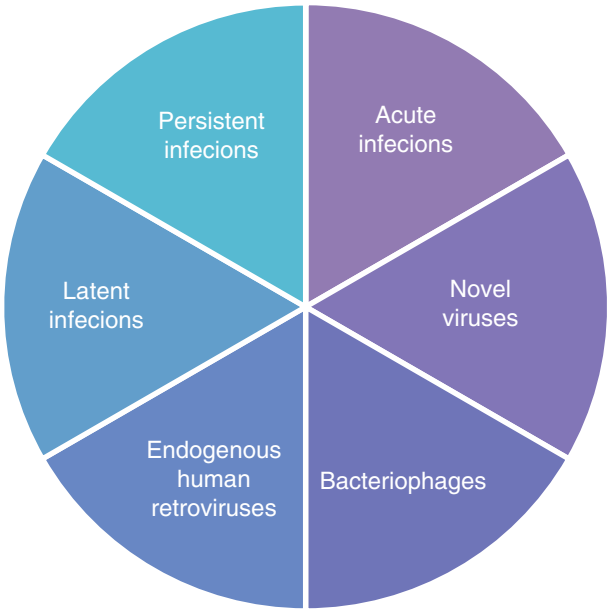

FIGURE 1 Components of the human virome.

followed by comparison of cleaned sequences with standard databases, and (v) interpretation and additional analysis.

Within this general framework, several approaches that have been used for the detection of viral sequences are also shown in Fig. 2. The first and most basic is to sequence DNA and RNA extracted directly from a clinical sample without prior processing. This method requires either a nucleic acid extraction protocol that recovers both DNA and RNA or, alternatively, separate DNA and RNA extractions. This method has been used in several studies (2–4). In some studies, DNase treatment of the sample before and/or after nucleic acid extraction has been used to increase the percentage of sequencing reads that were viral (5–8). The advantages of sequencing without efforts to enrich for viruses are that selective biases are not introduced by the enrichment procedures and that nonviral microbes can be assessed using the same sequence data. An additional advantage is that no processing steps are included that might decrease the ability to detect novel viruses that might be present in the sample. The disadvantage is that most of the sequences determined are of human or nonviral microbial origin because human and nonviral microbial nucleic acids are usually present in the sample in much greater abundance than viral nucleic acids.

Many methods have been described for virus enrichment, including ultracentrifugation, filtration, and polyethylene glycol precipitation (5–7). While detection of some viruses can be improved by prior enrichment, these methods may also introduce biases against other viruses (8). Methods that incorporate purification of virus-like particles have the advantage that all sequence reads are presumably of viral origin, theoretically allowing detection of novel viruses whose nucleic acid sequence is divergent from all previously sequenced viruses. In contrast, highly divergent viruses might be missed by methods that rely on identifying sequences as viral by matching them against databases of known viral sequences.

An alternative approach to physical enrichment of virus-like particles is to perform selective amplification of viral genomes or relevant segments prior to high-throughput sequencing. For example, to facilitate sequencing of influenza genomes, Hoper et al. (9) designed two PCR reactions for each of the eight influenza gene segments. The amplified segments include redundancy for the most variable regions. High-throughput sequencing of the PCR amplicons provides complete genome coverage. Other amplification methods that have been used include rolling circle amplification, which amplifies small circular genomes such as those of polyomaviruses or anelloviruses (10–12), and the multiple strand displacement assay (13).

An important issue with regard to metagenomic sequencing is the introduction of methodological biases that distort the distribution of sequences that are detected. This can result in failure to detect some pathogens or in distorted representation of mixed communities. Because the number of target virus sequences in clinical samples may be low, most metagenomic protocols include nonspecific amplification of target nucleic acid using random primers. The nonspecific amplification can be an important source of bias. For example, regions of high GC or AT content

TABLE 1 Components of the human virome with clinical significance

Categories	Examples	Advantages/anticipated benefits of applying high-throughput sequencing
Acute viral infections	Influenza, rotavirus, respiratory syncytial virus	Subtyping; identification of drug resistance genotypes; detection of viral variants that are missed by standard molecular tests; detection of mutations associated with increased virulence and/or transmissibility; use of human gene response to distinguish symptomatic infections from asymptomatic infections
Persistent viral infections with clinical significance	HIV, hepatitis C virus, congenital cytomegalovirus	Subtyping; identification of drug resistance genotypes; detection of viral variants that are missed by standard molecular tests; viral population analysis; ability to observe viral evolution over time; use of human gene response to distinguish symptomatic infections from asymptomatic infections
Persistent viral infections with unknown significance	Torque Teno virus and other anelloviruses; low-risk papillomaviruses	Appreciation of the presence of asymptomatic viruses and/or immune response to the viruses
Novel viruses	WU and KI polyomaviruses, VA and MLB astroviruses, Dandelong virus	Important approach for discovering viruses that are not detected by existing tests

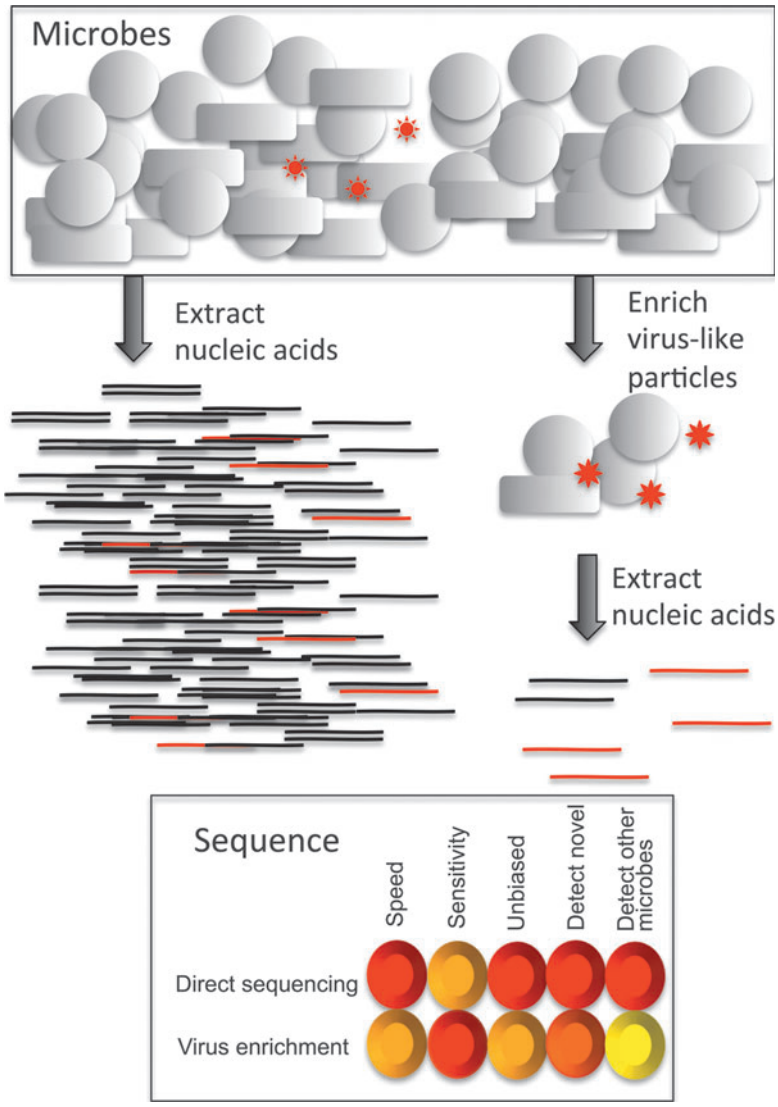

FIGURE 2 Approaches for detecting viral sequences using metagenomic shotgun sequencing. Nucleic acid can be extracted directly from a population of microbes, after which viral sequences, represented by red lines, may be a minor component of the total nucleic acid, as depicted on the left side of the figure. Sequencing libraries can be constructed directly from DNA or reverse-transcribed RNA, and rare viral sequences can be detected by ultra-deep sequencing. Alternatively, viral particles can be enriched prior to nucleic acid extraction and sequencing by filtration, DNase treatment, or ultracentrifugation. Post-enrichment, the majority of nucleic acid is from viruses, as is depicted on the right side of the figure. Relative strengths and weaknesses of sequencing by these approaches are represented by the circles in the bottom panel, with red representing relative strength, yellow representing a relative weakness, and shades of orange falling in between.

can be inefficiently amplified and thus underrepresented in sequencing libraries (14). Additives such as betaine (15) for high-GC regions and tetramethylammonium chloride (16) for high-AT regions may improve the evenness of amplification. Choices of enzyme and thermocycler characteristics have also been shown to be important sources of bias (17). These factors interacted with GC content so that different conditions were optimal for targets with different GC contents. Rosseel et al. (18) implicated annealing bias of the amplification primer as the main source of sequencing bias. Genome regions with homology to bases in the amplification primer located immediately 5′ to the random bases at the 3′ end were preferentially amplified and sequenced. In addition, increasing the number of random bases at the 3′ end of the amplification primer from 6 to 12 improved the evenness of coverage. RNA structure and GC content of the target were not important determinants of bias in that study. Amplification reactions other than PCR, including recombinase polymerase amplification, linear amplification for deep sequencing (19), and multiple displacement amplification (20) have been found to be advantageous under some conditions (14), although multiple displacement amplification has also been shown to distort quantitative analysis of metagenomes (21).

TABLE 2 Common high-throughput sequencing platforms

Platform	No. of sequence reads per run	Max sequence length	Time required for sequence run	Comment
Roche 454 GS FLX+	1 million	700–1,000 bases	23 hours	Only supported until 2016
Illumina GA IIX	640 million paired-end reads	150 bases	~13 days	Early Illumina implementation; has been replaced with the HiSeq series
Illumina MiSeq	44–50 million paired-end reads	300 bases	~55 hours	Shorter reads can be generated in less time; longer reads can help identification of more divergent viral sequences
Illumina HiSeq 2500	6 billion paired-end reads or 1.2 billion paired-end reads in rapid mode	100 or 150 bases	~11 days or ~27 hours in rapid mode	Greater depth of sequencing can be useful for detecting rare viruses; overlapping sequences can be assembled to improve detection of divergent viral sequences; comments apply to other HiSeq platforms (HiSeq 2000, 1500, 1000)
Illumina HiSeq 2000	6 billion paired-end reads	100 bases	~11 days	
Illumina HiSeq 1500	3 billion paired-end reads or 600 million in rapid mode	100 or 150 bases	~8.5 days or ~27 hours in rapid mode	
Illumina HiSeq 1000	1.5 billion	100 bases	~8.5 days	

The platform used for high-throughput sequencing can also be a source of bias. Each of the currently available high-throughput sequencing platforms has specific advantages and disadvantages (Table 2). In the early days of massively parallel sequencing, the 454 GS FLX platform from Roche 454 Life Sciences (Branford, CT) was used for virome characterization and virus discovery. This platform generates approximately 1 million sequences per run, with a sequence read length of up to 700 to 1,000 bases. The relatively long length of these sequences is advantageous for the discovery of viruses whose genomes are divergent from reference genomes. In such cases, the longer read length can help detect sequence similarities between the newly generated sequence and reference databases, which might not have been detected with a shorter read length. However, high-quality nucleic acid is required to obtain long reads, and this can be a limiting factor because samples are often not stored optimally or may be frozen and thawed multiple times prior to sequencing. The Illumina (San Diego, CA) MiSeq platform generates 50 million 300-base, paired-end sequences, meaning that 300 bases of sequence is generated from each end of each DNA fragment in the sequencing library. Therefore, if the sequencing library is constructed with fragment sizes of <600 bases, the entire contiguous sequence of the fragment can be reconstructed.

A MiSeq sequencing run generating the maximum read length requires ~55 hours. The Illumina HiSeq 2500 platform generates up to 6 billion paired-end reads, with read length of up to 100 bases in regular mode or 1.2 billion paired-end sequences of up to 150 bases in rapid mode. HiSeq runs require ~11 days in regular mode or approximately 40 hours for the maximum read length in rapid mode. A further advantage of the large number of reads generated by the Illumina platforms such as the GA IIx and the HiSeq is the higher likelihood of detecting sequences that are rare within the sequencing library. Sequencing using the Illumina GA IIx sequencer has been shown to be more sensitive for detecting viruses compared to Roche 454 using GS FLX Titanium reagents (2, 13).

A crucial step in detecting viruses in metagenomic sequence data sets is the recognition and classification of sequences, which is achieved by comparing sequence reads derived from the sequencer to reference databases. The series of informatics procedures involved in carrying out this task is referred to as the virus analysis pipeline. The most common approach is to classify sequences based on similarity to reference genomes (3). Most virus analysis pipelines incorporate a nucleotide sequence alignment step that allows high-confidence identification of viral sequences that are very similar to a reference sequence, followed by a translated alignment that allows detection of more divergent sequences with conserved protein sequences. Rapid sequence alignment tools have been developed that allow identification of viruses using this process to take place within minutes to hours. Profile hidden Markov models (22) are used to detect even more dissimilar sequences, by modeling conserved amino acid positions within a protein family. While the linear sequence of a protein may not be conserved, certain amino acid characteristics may be conserved to maintain protein fold and function. Hidden Markov models can be used to identify novel viruses by evaluating amino acid positions that are highly conserved among viral protein families (22). A limitation of using sequence similarity to identify viruses in metagenomic data sets is that currently available reference genome sets are not complete, because the sequence diversity of viruses is not fully represented. However, the number of viral genomes submitted to public databases increased nearly 9-fold from the year 2000 to 2014 (23), and improvements in technology and sequencing costs make it certain that this trend will continue. The addition of viral reference sequences will improve sequence classification, but the rapidly changing databases require adaptability, because reference genome sets used in analysis must be continuously updated. In addition, larger reference databases will require ongoing increases in computational capacity.

The richness of viral information derived from metagenomic sequence data sets varies depending on the number

of virus sequences detected and the extent and depth of virus genome coverage. In some cases, complete or nearly complete viral genome sequences can be identified, allowing for detailed genome characterization, including relevant typing or subtyping, and for analysis of specific genes that may confer virulence or drug resistance and of genes corresponding to known or predicted epitopes. In other cases, few sequence reads are identified, allowing only detection of the virus without additional characterization.

Bibby et al. (24) carried out *in silico* experiments to evaluate different processes for aligning sequence reads against relevant databases and found that sequencing read lengths of 200 nucleotides or more resulted in more accurate virus pathogen identification than read lengths of 100 nucleotides. They also found that nucleotide alignment provided more accurate identification than translated amino acid alignments and that use of focused viral-only databases resulted in better classification than use of larger databases containing diverse organisms. These findings are derived from experiments in which the virus identified is present in the reference database. For detection of viruses with differences from reference genomes, translated alignments and/or larger, more comprehensive databases may be required. In addition, use of a focused viral-only database can result in misclassification of sequences with similarity to both viral and other (e.g., human) references. This happens when, for instance, a nonviral sequence has a weak, translated alignment to the viral reference database but has a strong nucleotide alignment to its own reference genome.

THE HUMAN VIROME: GENERAL DESCRIPTION

Previous studies to define the viruses that infect humans have been based on viral culture, visualization by electron microscopy, antigen detection, nucleic acid detection, and detection of an immunologic response. Here we review studies that are based on application of high-throughput sequencing.

A recent study characterized the human DNA virome in 102 normal subjects enrolled in the Human Microbiome Project (3), providing the first view of the human virome in a large cohort of carefully screened healthy adults. Because of the methods used by the Human Microbiome Project for nucleic acid extraction and sequence library construction, the analysis was limited to double-stranded DNA viruses and single-stranded DNA viruses with double-stranded DNA replicative intermediates. Using carefully predefined procedures, subjects were sampled at up to five standard body sites (nose, skin, mouth, vagina, and stool). Sequences of multiple DNA viruses were detected in most subjects, with an average of 5.5 genera per subject. The main viral groups detected in these asymptomatic individuals were papillomaviruses, polyomaviruses, herpesviruses, adenoviruses, and anelloviruses (Fig. 3). In some cases, multiple species from a genus were found in the sample, including mixtures of papillomaviruses considered either high risk or low risk for malignancy. Viral nucleic acid was detected less frequently in stool than in the other sites. Individuals had distinct viral profiles, and follow-up studies of the same individual showed that a number of the components of the virome of an individual were stable over weeks to months. This was especially true for the roseoloviruses, which were stable in >90% of subjects, and papillomaviruses, which were stable in up to 50% of subjects. Because the study subjects were all asymptomatic healthy adults, the viruses detected can be considered to be commensal, making up the DNA component of the normal viral microbiota. The long-term effects of the presence of apparently commensal viruses on human health remain to be determined.

GASTROINTESTINAL TRACT VIROME

The gastrointestinal (GI) tract has been the subject of more virome studies than other body sites. Early studies showed that the predominant DNA viruses in the GI tract were bacteriophages, and the predominant RNA viruses were plant viruses thought to be introduced with diet, nonreplicating within the gastrointestinal tract. An early study (25) studied the stool virome in a single infant and found numerous sequences corresponding to bacteriophages but only a single sequence corresponding to a eukaryotic virus. Minot et al. (26) studied sequences from virus-like particles purified from the stool of healthy adult volunteers and found no eukaryotic viruses. In data from the Human Microbiome Project, eukaryotic viruses were rarely found in stool from healthy adults. However, roseoloviruses, papillomaviruses, polyomaviruses, adenoviruses, and circoviruses were occasionally detected (3).

Studies of individuals with diarrhea, mostly children, have shown abundant eukaryotic viral sequences. Two early studies used Sanger sequencing to carry out metagenomic analyses of stool samples from children with diarrhea. Finkbeiner et al. (27) studied samples from 12 children under 5 years of age, analyzing 384 cloned segments per sample. The analysis was directed at the detection of RNA viruses, although some DNA viruses were also detected. Of the 12 children, 10 had sequences from at least one eukaryotic virus genus, and 5 had 2 or more genera. In all, sequences from 7 families plus 3 unclassified genera were detected. In addition to recognized pathogenic viruses including rotavirus, calicivirus, adenovirus, astrovirus, and picornavirus, viruses of uncertain pathogenicity were also detected, including adeno-associated viruses, picobirnaviruses, anelloviruses, nodavirus, and mimivirus. A novel human astrovirus designated MLB1 was also discovered.

Victoria et al. (28) performed metagenomic analysis of stool samples from 35 children in Pakistan with nonpolio acute flaccid paralysis, plus 6 healthy children who were family contacts of individuals with diarrhea. Analysis was based on Sanger sequencing of 35 to 240 subcloned fragments per stool sample. In addition, high-throughput sequencing using the GS FLX 454 system was also performed on a subset of 10 samples, yielding 3,715 to 25,516 high-quality sequences per sample. Analysis of the Sanger sequencing results showed sequences corresponding to eukaryotic viruses in 29 (83%) of the 35 children with acute flaccid paralysis, with an average of 1.4 eukaryotic viruses per sample, which increased to 2.6 with high-throughput sequencing. The viruses detected were from seven virus families plus four novel virus groups. The most common viruses were single-stranded RNA viruses from the order *Picornavirales* (human enteroviruses A-C, parechovirus, rhinovirus, cardiovirus, aichivirus, cosavirus) and dicistrovirus-like virus. Also detected were double-stranded RNA viruses (rotavirus and picobirnavirus) and DNA viruses (including *Adenoviridae*, bocavirus, and anelloviruses). All six of the stool samples from healthy children also contained eukaryotic viral sequences with a predominance of viruses in the order *Picornavirales*, plus anellovirus and

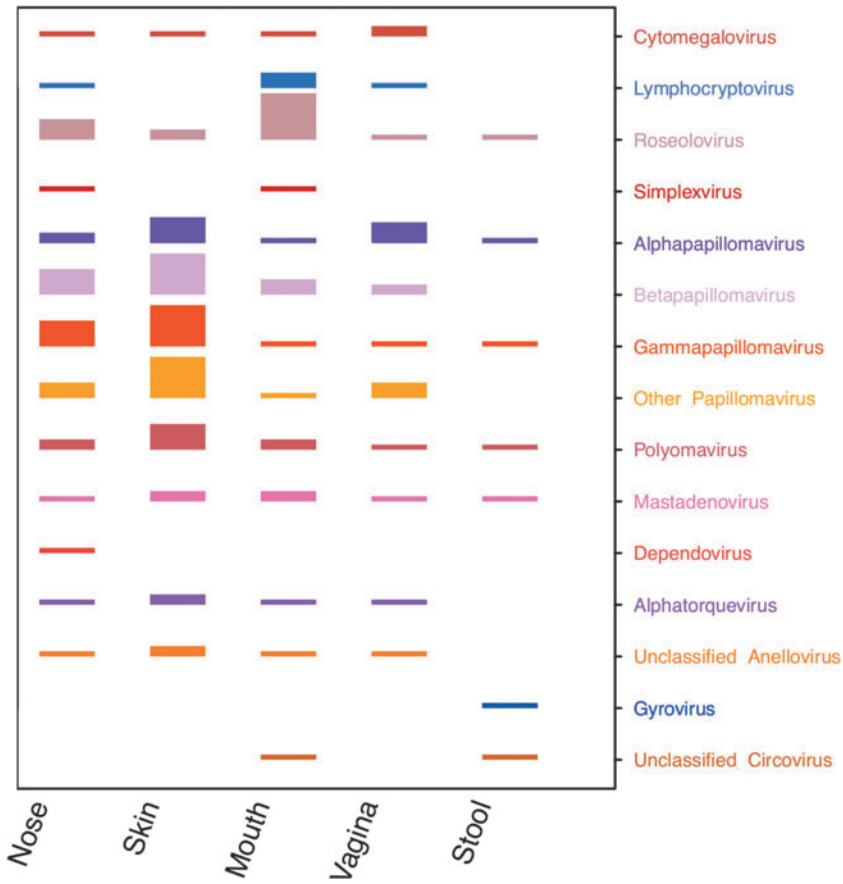

FIGURE 3 The human virome in five human body habitats. All of the viruses detected in the five body habitats are shown. Each virus is represented by a colored bar and labeled on the *y*-axis on the right side. The relative height of the bar reflects the percentage of subjects sampled at each body site in whom the virus was detected. The bar representing roseoloviruses in the oral samples reflects the maximum bar height because 98% of the individuals who were sampled in the mouth harbored roseoloviruses. Reprinted without modification from reference 3 under Creative Commons License 4.0 (http://creativecommons.org/licenses).

circovirus-like virus. Surprisingly, the percentage of all sequences that were viral was higher in the healthy children than the children with acute flaccid paralysis (49% vs. 23%).

Smits et al. (29) performed high-throughput metagenomic sequencing on stool samples from 27 patients in the Netherlands who represented either sporadic cases of diarrhea or were part of outbreaks. Most of the patients were adults, and the cause of their diarrhea was unknown after extensive diagnostic testing. Metagenomic analysis was performed on separate RNA and DNA extracts prepared from stool homogenates, followed by random PCR amplification, library preparation, and sequencing using 454 pyrosequencing. Sequences of mammalian viruses were found in stool samples from 13 patients and included picobirnaviruses in 9, anelloviruses in 2, circoviruses in 2, Epstein-Barr virus (EBV) in 1, aichi virus in 1, and bufavirus in 1.

In the largest study published to date, Holtz et al. (30) studied the stool virome of 87 young children from Melbourne and the Northern Territory of Australia. Methods involved total nucleic acid extraction from diluted stool samples followed by random PCR amplification, library preparation, and sequencing using the 454 GS FLX Titanium platform. Sequences from one or more viruses were detected in 66% of the samples, and 40% of the samples had sequences from two or more viruses. Sequences from eight virus families were detected in one sample. In all, sequences were detected from 22 virus families, of which the most common were *Anelloviridae*, *Picornaviridae*, and *Adenoviridae*. In addition to viruses anticipated to be present in the GI tract, the viruses detected also included respiratory viruses such as human parainfluenza type 3 and a number of plant viruses. PCR assays for a number of viruses were performed, and results were generally concordant with sequencing results. In addition, several novel viruses were detected but not fully characterized.

To summarize the studies of the GI tract, sequences from diverse eukaryotic virus families have been detected in stool samples from individuals with diarrhea. These viruses include those currently known to be causes of gastroenteritis in humans and some that are not currently recognized as GI tract pathogens. Viruses from outside the GI tract have also been detected. Studies done to date of individuals with diarrhea have included mainly children as subjects, and those of individuals without diarrhea have included mainly adult subjects. Thus, more studies on

children without diarrhea and adults with diarrhea are needed to complete the picture of the human GI tract virome. In addition, studies of immunocompromised subjects and subjects with chronic gastrointestinal diseases are needed.

RESPIRATORY TRACT VIROME

The occurrence of pathogenic and nonpathogenic viruses in the respiratory tract has been extensively described based on traditional methods of viral culture, antigen detection, and PCR. However, metagenomic studies are still relatively limited. The earliest application of metagenomics to the respiratory tract virome was a study comparing the DNA virome of individuals with cystic fibrosis (CF) to that of normal individuals (31). The analysis was carried out on expectorated sputum samples that were processed by filtration and ultracentrifugation to isolate virus-like particles, followed by DNase treatment to remove free DNA. Purified DNA was amplified using the multiple strand displacement assay, and high-throughput sequencing was performed using the 454 GS FLX pyrosequencing platform. Sequences from bacteriophages were prominent. Sequences from eukaryotic viruses were more common in the individuals with CF than in the normal individuals. A total of 15 eukaryotic virus families were detected in the CF samples, compared to 12 in the samples from non-CF individuals. Prominent taxa in both groups included *Herpesviridae*, *Adenoviridae*, *Papillomaviridae*, and *Poxviridae*.

One of the most extensive analyses of the respiratory tract virome was part of a study of febrile illness in children 2 to 36 months of age evaluated in a hospital emergency department for fever without a source (32). Samples from a comparison group of afebrile children without an acute illness were also analyzed. Nasopharyngeal secretions from both groups were analyzed by a multiplex PCR panel supplemented by PCR assays for several other viruses found in the respiratory tract. Thirty-one percent of the afebrile children were positive for a respiratory virus. Rhinovirus was the most common virus, detected in 16%. Other viruses detected included adenovirus, enteroviruses, human metapneumovirus, bocavirus, coronavirus HKU1, and the KI and WU polyomaviruses (KIPyV and WUPyV). A subset of 131 nasopharyngeal samples from both groups was also analyzed by high-throughput sequencing (2). Sample preparation included total nucleic acid extraction followed by random PCR amplification without any enrichment by filtration or centrifugation. Sequencing was performed using the Illumina GA IIx platform. Analysis of samples from 50 children with fever and 81 nonacutely ill, afebrile children revealed viral sequences from 17 genera, 15 of which were present in samples from febrile children compared to 9 for samples from nonacutely ill children (Table 3). Viruses from the genus *Enterovirus*, which includes the rhinoviruses, were the most common in both groups of children. Anelloviruses were detected frequently in both groups but occurred more frequently in the febrile children (33).

In another study of respiratory viruses in children, Lysholm et al. carried out a metagenomic analysis of 210 nasopharyngeal aspirates from children with acute respiratory infection, of whom approximately 70% were less than 7 years of age (5). Analytic procedures included separate DNA and RNA extractions, ultracentrifugation to isolate virus-like particles, treatment with DNase, and amplification by random PCR followed by high-throughput sequencing carried out using the 454 GS FLX pyrosequencing system. Viruses accounted for almost 40% of the total sequence reads. More than 90% of the virus sequences detected were from the families *Paramyxoviridae*, *Orthomyxoviridae*, and *Picornaviridae*. Respiratory syncytial virus and human metapneumovirus were the main paramyxoviruses detected, but wild type measles virus was also present. Rhinovirus A viruses were the predominant *Picornaviridae*, but two novel rhinovirus C viruses were also detected. In addition to the three predominant families, viruses from at least five other families were detected, including human bocaviruses, KIPyV, coronavirus OC43, anelloviruses, and human picobirnavirus. No healthy subjects were included in this study, precluding an assessment of the relative frequency of detected viruses in ill compared to healthy individuals.

TABLE 3 Viruses detected by sequencing in nasopharyngeal secretions from febrile and afebrile children 2 to 36 months of age

Virus	No. positive (% of total)	
	Afebrile (n = 81)	Febrile (n = 50)
Anellovirus	25 (31)	26 (52)
Enterovirus	17 (21)	13 (26)
Mastadenovirus	3 (4)	9 (18)
Parechovirus	0 (0)	3 (6)
Respirovirus	1 (1)	3 (6)
Metapneumovirus	0 (0)	2 (4)
Pneumovirus	1 (1)	2 (4)
Polyomavirus	0 (0)	2 (4)
Astrovirus	0 (0)	1 (2)
Bocavirus	1 (1)	1 (2)
Cytomegalovirus	0 (0)	1 (2)
Dependovirus	0 (0)	1 (2)
Influenzavirus A	0 (0)	1 (2)
Morbillivirus	0 (0)	1 (2)
Roseolovirus	3 (4)	1 (2)
Coronavirus	1 (1)	0 (0)
Pestivirus	1 (1)	0 (0)

Young et al. (34) studied the DNA virome in six lung transplant recipients undergoing posttransplant bronchoscopy, plus three HIV-positive individuals. The analytic procedures included purification of virus-like particles followed by concentration and treatment with nucleases to eliminate nonencapsidated nucleic acids. High-throughput sequencing was carried out using the Illumina MiSeq platform. The predominant virus sequences detected were from anelloviruses, which were detected in all bronchoalveolar lavage samples. Sequences corresponding to as many as 17 anelloviruses were detected in individual lung transplant recipients. Richness of anelloviruses and quantitative levels were higher in bronchoalveolar lavage samples from lung transplant recipients than those from HIV-positive individuals. The only other eukaryotic viruses detected were EBV, HHV-7, and human papillomavirus, with very low numbers of sequence reads. The low yield of large DNA viruses may have resulted in part from the filtration procedure used, which was shown to decrease the yield of these viruses. Antiviral prophylactic therapy received by the lung transplant recipients may have also

contributed to the limited detection of large DNA viruses. Detailed studies of anelloviruses in a larger number of subjects revealed that the levels of anelloviruses were much higher in lung transplant recipients than in HIV-positive individuals or in healthy control subjects. Anellovirus levels in lung transplant recipients were not correlated with clinical variables but were correlated with the deviation of the bacterial microbiome in bronchoalveolar lavage fluid from that of healthy subjects.

The studies described above begin to define the respiratory tract virome. Available methods can detect sequences from numerous eukaryotic viruses in both symptomatic and asymptomatic individuals. Viruses detected include those that are well recognized as respiratory tract pathogens, plus others of uncertain pathogenicity, especially anelloviruses. Immunosuppression may increase the level of some viruses including herpesviruses and anelloviruses. More studies are required under different clinical circumstances to achieve a clearer picture of the virome associated with health and disease in a variety of patient populations.

PLASMA VIROME

Early sequencing-based studies of viruses in plasma used DNase treatment (35) or a combination of ultracentrifugation and DNase treatment (8) to enrich for viruses prior to sequencing. These studies found anelloviruses, hepatitis G virus (GB virus-C), and the newly described parvovirus PAR4 in plasma samples from adults with symptoms of acute viral infection (35) and detected anelloviruses in plasma from healthy blood donors (8). Other studies have used high-throughput sequencing to analyze nucleic acid extracted from plasma without prior procedures to enrich for viruses. The study of the virome in young children with the syndrome of fever without a source described in the respiratory virus section (2, 32) also included plasma samples from febrile children 2 to 36 months of age and from nonacutely ill afebrile children from the same age group. Using a panel of PCR assays, this study found multiple viruses in the plasma of febrile children. Adenoviruses, HHV-6, enteroviruses, parechoviruses, and human bocavirus were the viruses detected most frequently. The PCR panel detected very few viruses in plasma samples from afebrile children. High-throughput sequencing, performed as described for the respiratory samples, was carried out on samples from 23 febrile and 22 afebrile children. Sequences from numerous viruses were detected in the samples from febrile children including adenovirus, roseolovirus, enterovirus, astrovirus, dependovirus, erythrovirus, flavivirus (GB virus C), orthohepadnavirus, and polyomavirus. Only anellovirus was detected in the samples from afebrile children. Anellovirus sequences were common in both groups but were more common in children with fever (33).

The plasma DNA virome of immunocompromised patients was analyzed by De Vlaminck et al., who studied 96 heart and lung transplant recipients with serial samples following transplantation (36). Viruses detected included members of seven orders and families, of which the predominant one was *Anelloviridae*, followed by *Herpesvirales*, *Polyomaviridae*, *Adenoviridae*, *Caudovirales*, *Poxviridae* and *Retroviridae*. The composition of the virome was influenced by antiviral therapy, immunosuppression, and time after transplantation. Not surprisingly, the proportion represented by *Herpesvirales* decreased with the use of the antiviral drug valganciclovir. The proportion represented by *Anelloviridae* generally increased with increasing levels of immunosuppression. The total viral load increased after transplantation, and this was associated with a decrease in viral loads of *Herpesvirales* and *Adenoviridae* and an increase in the *Anelloviridae* for patients who were treated with both antivirals and immunosuppressants. Notably, the presence of organ rejection correlated with a lower burden of *Anelloviridae*, suggesting that the anellovirus load in the plasma reflected the immune state of the patient, with increasing *Anelloviridae* correlating with decreased immune competence.

The plasma DNA virome was also studied using high-throughput sequencing in 10 adults with HIV/AIDS and 10 normal adults (37). The main virus sequences detected in the plasma of the individuals with HIV/AIDS corresponded to bacteriophages. In contrast, bacteriophage sequences were not detected in the plasma of normal individuals, in whom sequences corresponding to torque teno virus and other anelloviruses were predominant. Another study that compared the plasma virome in HIV-positive individuals from the United States and Uganda with low and high CD4 counts found sequences of multiple viruses in addition to HIV, including hepatitis B, C, and G; anelloviruses; and human endogenous retroviruses (37). Anellovirus sequence read counts were higher in individuals with lower CD4 counts, suggesting a loss of immunologic control.

Although the number of studies is limited, several points are emerging concerning the plasma virome. First, anelloviruses are common in both well and ill individuals. Evidence is accumulating that the level of anelloviremia may be an indicator of the immune state of the individual. Second, sequences corresponding to pathogenic viruses may be detected in the plasma of immunocompromised individuals, whether or not they are acutely ill. In contrast, in nonimmunocompromised individuals, pathogenic viruses are rarely detected in plasma of those who are well but may be detected in those with acute febrile illness. This may be more common in children than adults. Further studies are needed to achieve better definition of the plasma virome. Studies to date suggest that analysis of plasma may have diagnostic utility and could be carried out using a variety of test formats.

SKIN VIROME

As described in the section on general considerations related to the human virome, papillomaviruses and polyomaviruses are the main components of the skin virome. In a PCR-based study, Antonsson et al. (38) showed that a wide variety of papillomaviruses were detected in skin samples from healthy individuals. The distribution of types varied in individuals from different countries. PCR-based studies of infants showed that beta- and gamma-papillomaviruses were detected early in life (39). A landmark study was the discovery of Merkel cell polyomavirus (MCPyV) by Feng et al. (40) using high-throughput sequencing with digital transcriptome subtraction. This study linked the virus to Merkel cell carcinoma, a rare skin tumor that occurs primarily in individuals who are immunocompromised. Clonal integration of the viral genome in the DNA of Merkel cell carcinoma has been demonstrated, suggesting a causal role for the MCPyV in the generation of the cancer. Subsequent studies have shown that normal individuals may shed MCPyV (11) and have led to the discovery of 10 additional human polyomaviruses (11, 41, 42) usually found in cutaneous

samples. A study of the skin DNA virome of an individual with a history of previous Merkel cell carcinoma plus five healthy individuals found that sequences from three viral families (the *Polyomaviridae*, the *Papillomaviridae*, and the *Circoviridae*) were commonly detected and accounted for most of the viral sequences detected (43). The methodology employed included high-throughput sequencing using the HiSeq 2000 platform following multiple displacement amplification (20), which favors the amplification of circular genomes and may have affected the relative proportions of viruses detected in this study. Extensive diversity was detected within the three predominant virus families. Sequences from MCPyV were detected in all six subjects, and sequences from HPyV 6 and 7 were also commonly detected. Sequences of papillomaviruses were also detected in all subjects, with an average of 12 strains per subject.

Oh et al. (44) examined the skin microbiome including the DNA virome in 15 healthy adults sampled at 18 distinct skin sites. Because of low sample input, library preparation was done using the Nextera system followed by sequencing on the Illumina HiSeq platform. The subjects had viruses that included papillomaviruses, polyomaviruses (MCPyV), and poxvirus (molluscum contagiosum virus). Of the 18 sites sampled, the nares and the adjacent alar crease sites had the most virus sequences detected. These data and the study from the Human Microbiome Project described above (3) establish the *Papillomaviridae* and *Polyomaviridae* as common components of the human virome, with sequence divergence that has not yet been fully characterized. These are viruses that can be present in normal individuals but have the potential to cause disease, including warts and cutaneous malignancies.

METAGENOMIC STUDIES OF SEWAGE (THE SEWAGE VIROME)

An alternative approach to evaluating the human gastrointestinal microbiome or virome is to carry out studies on human sewage, because this allows sampling of human gastrointestinal contents on a population basis. However, a human origin for viruses and other microbes detected in sewage cannot be assumed since sewage transmission lines may be entered by insects and animals that may contribute their own microbes. Studies using traditional methods have shown the presence of numerous enteric viruses in sewage including adenovirus, norovirus, reovirus, rotavirus, and members of the *Picornaviridae* family (reviewed in reference 45). Other newly discovered viruses including picobirnavirus (46), cardioviruses, cosavirus, circoviruses, and human bocavirus have also been detected using directed PCR assays (45). The first metagenomic study of sewage was carried out by Bibby et al. (24), who performed studies on sewage sludge residuals resulting from municipal wastewater treatment, termed "biosolids." Using 454 pyrosequencing, they detected 10 human pathogenic viruses. Corrected for genome size, the most abundant human viruses were parechovirus, coronavirus, torque teno virus, herpes virus, and aichi virus. Other human pathogenic viruses detected were adenovirus, tanapox virus, orf virus, and hepatitis C virus.

Cantalupo et al. (47) used 454 pyrosequencing to analyze sewage samples from Pittsburgh, Barcelona, and Addis Ababa. In all, 234 viruses from 26 virus families were detected, with bacteriophage being predominant. More than 90% of the eukaryotic viruses detected were plant viruses. Seventeen viruses known to infect humans were detected, including adenovirus type 41, astroviruses, Norwalk and Sapporo virus, human papillomavirus 112, human bocaviruses, human picobirnavirus, aichi virus, klassevirus, parechoviruses, and HPyV6. Selective PCR testing revealed the presence of additional human viruses including JC polyomaviruses. In addition, there was evidence for multiple novel viruses, suggesting extensive diversity. In another study, Aw et al. (7) used the Illumina platform to sequence sewage samples from East Lansing, Michigan. As in other studies, bacteriophages were the predominant viruses detected. Animal and plant viruses were also detected. Human viruses represented 3% of viruses detected and included viruses from the families *Adenoviridae*, *Polyomaviridae*, *Picornaviridae*, and *Papillomaviridae*. The large majority of viral sequences could not be characterized, suggesting the presence of extensive diversity.

THE CANCER VIROME

Large sequence-based studies of cancer have begun to characterize the cancer virome. Tang et al. studied RNA sequencing data generated by The Cancer Genome Atlas Research Network from 4,443 tumors from 19 cancer types (48). They found that tumorigenic viruses were common in several tumor types (cervical, head and neck, liver), but were not associated with other tumors (breast, brain). Cervical cancer was the most highly virus-associated, with nearly all cases associated with high-risk papillomaviruses (96% positive for papillomaviruses in this study). Head and neck cancers were also associated with papillomaviruses in 14.1% of the cases. Hepatitis B virus was found in 11 of 34 hepatocellular carcinomas (32%). Although hepatitis C virus is also associated with hepatocellular carcinomas, it was only detected in one sample, likely due to the methods used for sample preparation. In addition, recurrent integrations were observed in or near six cancer genes: *ERBB2*, *PVT1* and *LOC727677* long noncoding RNAs downstream of the oncogene *MYC*, *RAD51B*, *MLL4*, and *FN1* in cervical, head and neck, and liver cancers.

A comprehensive characterization of bladder carcinomas by The Cancer Genome Atlas Research Network found viral DNA in 7 of 122 tumors (6%) (49). In one case, human papillomavirus 16 was integrated into *BCL2L1* (an apoptosis regulator). In another case, the BK polyomavirus (BKPyV) was integrated into *GRB14* (a signaling adaptor protein for receptor tyrosine kinases). These findings suggest that viruses may have a role in the development of a small proportion of bladder cancers.

Gastric cancers have been found to be highly associated with infectious agents, including EBV. In fact, the EBV-positive tumors were classified as one of four subtypes of gastric tumor (50). The EBV-positive tumors were associated with hypermethylation of gene promoters. EBV-positive tumors were also highly associated with mutations in *PIK3CA* (80% of tumors compared to 3 to 42% of tumors in other tumor subtypes), the gene for the catalytic subunit of the PI3 kinase, which has many roles in the cell including regulating cell growth and division. EBV correlated inversely with mutations in the tumor suppressor *TP53*. The viruses described in this section up to this point are all well-known human viruses, but as discussed above in the section on the skin virome, a novel virus was found to be associated with cancer in 2008 (40). Merkel cell polyomavirus was found in 80% of tumors evaluated, and the virus was clonally integrated in 75% of those

cases. This raises the possibility that additional unknown viruses may be discovered to be etiologic agents of cancer. Viral causes of cancer are of great interest, particularly in light of the fact that the viruses that have been shown to be associated with tumors (papillomaviruses, polyomaviruses, EBV) are viruses that are common in healthy individuals (3). An important question is why these viruses cause cancer in some people but not others.

CONCLUSIONS

The development of high-throughput nucleotide sequencing has made it possible to undertake comprehensive studies of the human microbiome. Studies of the human virome, the virus component of the microbiome, are much less advanced than studies of the bacterial component. Virome studies are complicated by the varied life cycle of viruses that includes persistent and latent states of infection for some viruses. Nevertheless, the metagenomic methods required for studies of the virome are now advancing rapidly, and pictures of the virome at various body sites are emerging. These studies define the "virus space" of humans and are the foundation for further studies of the role of viruses in human health and disease. They also provide baseline information that is required for the application of molecular detection methods including high-throughput sequencing to the process of diagnostic virology. Further studies are required to define the effects of age, geography and other environmental exposures, and the immune state of the human host. An anticipated spinoff of studies of the human virome is the discovery of novel viruses, some of which may be linked to human disease.

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section III

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Molecular Detection of *Staphylococcus aureus* Colonization and Infection

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15

HISTORY OF SCREENING FOR *S. AUREUS* AND MRSA

Prevention and control of infection with *Staphylococcus aureus*, including methicillin-resistant *S. aureus* (MRSA), have been a major health care focus since at least the middle of the 20th century (1). By 2005, mortality from MRSA in the United States exceeded that due to infections from salmonella, tuberculosis, influenza, and HIV combined (2, 3). Since that time, global efforts to reduce MRSA infection rates appear to have had some impact in both Europe and the United States (4–6). However, the improvement has not been uniform, with selected academic medical centers not realizing a reduction in disease (7), and at least one large state (Illinois) reporting an increase in overall MRSA infection reflected in their most recent data, with 11.7 MRSA infections/1,000 hospital discharges in 2009, 11.6 in 2010, 10.7 in 2011, and then up to 14.2 in 2012 (8).

Performing a PubMed search on the keywords "MRSA and infection control" in early 2016 detected more than 5,300 articles published since 1980. The conclusion of the first article in this list stated that "during a five-year period . . . Standard barrier nursing methods were unsuccessful. In the subsequent 6 years all patients were nursed in an isolation unit; the number of patients infected with methicillin-resistant *Staph. aureus* had fallen from 130 to 14" (9). While much discussion continues over the role of active surveillance testing (AST) for prevention of both MRSA and *S. aureus* infection (10), it is clear that this approach is widely used and has been very successful (11). One of the reasons that AST for MRSA should be expected to work is that the greatest risk factor for developing clinical disease is first to become colonized in the nares with MRSA (12). People who are either colonized or infected with MRSA are equally likely to have their skin and surrounding environment contaminated with this organism, so it can be readily contracted by others coming close to or touching them (13). Therefore, strategies to limit transmission that consider both colonized and infected persons are most likely to be successful (14).

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MEDICAL UTILITY

The medical impact of *S. aureus* infection on society is important for three critical reasons. First, *S. aureus*, both methicillin-susceptible strains (MSSA) and MRSA, is a major cause of health care-associated infection, which was outnumbered only by *Clostridium difficile* infection as the most commonly reported cause of U.S. health care-associated infection in 2010 (15); it is the most frequent cause of pneumonia and surgical site infection (SSI) and the third most common etiology for nosocomial bloodstream infection (BSI), only after *Candida* species and coagulase-negative staphylococci. Second, this infection results in substantial morbidity and mortality, with MRSA bacteremia resulting in death nearly twice as often as BSI from MSSA (16, 17), leading to a prolonged length of hospital stay (18); SSI from MRSA has more than 3-fold the mortality compared to MSSA disease (19). Finally, infections from these pathogens result in considerable excess cost to the U.S. health care system, usually estimated in the tens of thousands of dollars per patient (19–21), again with MRSA being more costly than MSSA infection (19).

Nasal Screen for MRSA Control

Performing AST for MRSA has generally involved the swabbing of either the nares alone or a combination of nares/throat/axilla/inguinal/rectal/perirectal areas and then testing those collected swabs as a single sample (22, 23). In the past these were processed using either nonselective agar (sheep's blood agar or chocolate agar) or selective medium (mannitol salt agar). More recently, chromogenic media and rapid molecular testing (PCR) have become available (22, 23).

The general rationale for performing AST is to appropriately place MRSA-colonized patients into contact precautions to effectively provide a barrier between the affected patient and health care workers and diminish acquisition of MRSA directly from the patients and their surrounding environment. By preventing the health care worker from even transiently acquiring MRSA, there is less likelihood of transmission to other patients who may develop MRSA clinical infection after they become colonized. There are three main issues to resolve when planning an MRSA AST program: (i) what body site(s) to test, (ii) what test to use, and (iii) whom to test. When testing a single body site, the nares is generally chosen because the anterior nares is the usual colonized site for

S. aureus, including MRSA, in humans. However, while it is the most common colonization site, it can miss up to 35% of MRSA-colonized individuals. Kunori and colleagues suggested that culturing the nares and any wound (e.g., clinically infected site) could approach 100% sensitivity (24), which seems a prudent approach for most AST programs. Two large studies from the United States have clearly demonstrated that broadly applied, active surveillance with rapid testing of nasal swabs can be associated with a dramatic reduction in clinical MRSA infection (25–28).

When deciding what test to use, the laboratory routinely assesses test cost and processing workflow in the final decision. One of the biggest challenges for the acute care hospital is deciding how sensitive a rapid test needs to be to provide a reliable result soon enough to impact MRSA control by implementing contact precautions for sufficient hospitalization time and to affect MRSA spread. Most laboratories now choose between commercial chromogenic agar and a real-time PCR as their test of choice. Both approaches can offer a result within 24 h of sample receipt by the laboratory; however, the sensitivity of a 24-h test is approximately 80% with agar medium (29), whereas it exceeds 95% for most commercial real-time PCR assays (30). Robicsek and colleagues suggested that the overall mean percentage of days patients colonized or infected with MRSA were cared for under contact precautions was a key element in determining the success or failure of an MRSA AST program (25), and Peterson subsequently argued that this needed to meet or exceed 80% in most settings (10). Since the average length of stay for U.S. acute care hospitals is 4.8 days (31), this implies that testing needs a sensitivity approaching 100% if the turnaround time from sample receipt to reported result approaches 1 day. In support of rapid testing, a systematic review and meta-analysis found a significant reduction in risk for MRSA bloodstream infections (0.54, 95% CI 0.41 to 0.71) when comparing nursing units using rapid screening tests and those not undertaking screening (32) but concluded overall that applying screening was more important than the actual test used.

The final decision is that of whom to test. Debate over whether universal surveillance is required, as opposed to a more targeted approach, continues, with a comprehensive Agency for Health Care Research and Quality (AHRQ) review suggesting the issue is not yet resolved (33). Subsequently, Kavanagh and colleagues pointed out that 41 of 43 studies included in this review demonstrated reduction of clinical disease associated with AST and argued that the evidence was compelling that using surveillance as an approach for MRSA control was effective (11). One reason for considering universal AST, at least at the beginning of the program, is that it is now difficult to predict which patients are at high risk for MRSA colonization simply by where they are cared for in the hospital. McKinnell and colleagues found that several conditions—including health care contact, previous health care-associated pathogens, and select comorbid conditions (open wounds, congestive heart failure, diabetes, chronic obstructive pulmonary disease, renal impairment, and immunosuppression)—and intensive care unit (ICU) admission were not a risk factor for MRSA colonization (34). Importantly, if one does choose to perform universal admission AST, it does not need to continue indefinitely. Robicsek and colleagues demonstrated the development of electronic prediction rules for the NorthShore University HealthSystem's universal AST program, whereby they could achieve 90% detection of MRSA-colonized individuals by testing only 50% of admissions (35)—a change in approach that was implemented on January 10, 2012, to reduce testing costs while maintaining control of MRSA nosocomial clinical infection.

Presurgical *S. aureus* Screening and Decolonization

Currently, the other major application for *S. aureus* surveillance, both MSSA and MRSA, is in the preoperative patient, where *S. aureus* clinical disease is a significant risk for postoperative SSI. The usual practice is to perform nasal swabbing for *S. aureus* in the period before surgery (no more than 30 days) and then decolonize those patients positive for *S. aureus* using a typical regimen of intranasal mupirocin twice daily for 5 days accompanied by chlorhexidine bathing. In this setting either culture or molecular testing can be used, with the caveat being that a less sensitive test will detect fewer patients. Since up to 1/3 of adults are carriers of *S. aureus* in the anterior nares (24), sensitivity can be important in maximizing the number of patients detected when preoperative testing is undertaken. The benefit of this practice is significant, particularly for operations involving prosthetic implants and cardiovascular surgery.

Colonization with *S. aureus* raises the risk of SSI several fold (36), so it is not surprising that decolonization can have a clinically meaningful impact. In a before-after trial of 1,495 prosthetic joint recipients reported by Hacek and colleagues, nasal screening followed by decolonization of those nasally colonized with *S. aureus* reduced the overall SSI rate from 1.7% to 0.77% (55%) and was cost-effective (37). In a subsequent randomized, double-blind, placebo-controlled, multicenter trial of 6,771 surgery patients that were screened and then decolonized using mupirocin and chlorhexidine in the intervention arm, a similar outcome was found (38). The effect of mupirocin-chlorhexidine treatment for deep SSIs was highly significant (relative risk, 0.21; 95% CI, 0.07 to 0.62). Interestingly, the time to the onset of infection also was shorter in the placebo group than in the intervention group ($P = 0.005$). Both of these studies used PCR as the assay for detecting *S. aureus* colonization via nasal swabbing. The hypothesis about the effect of this practice is that mupirocin decolonization effectively removes *S. aureus* from the nares for at least 30 days after treatment (39) and therefore eliminates the potential for patients to contaminate their surgical incision by rubbing their nose and then the operative site for the important time period following surgery when the incision may not be fully closed.

A third investigation focused only on MRSA and involved a prospective, controlled, interventional cohort study using 33 surgical wards in 10 hospitals from nine countries in Europe and Israel (40). They found that in cardiothoracic, neuro, orthopedic, plastic, and vascular surgery wards their strategy 2 (MRSA screening, contact precautions, and decolonization) was associated with decreased MRSA clinical cultures (15% monthly decrease, adjusted incidence rate ratio [aiRR] 0.85, 95% CI 0.74 to 0.97) and MRSA clinical infections (17% monthly decrease, aIRR 0.83, 95% CI 0.69 to 0.99). This investigation used culture of pooled nasal and perineal swabs that was plated onto chromogenic medium as well as incubation overnight in an enrichment medium followed by a

second plating to increase test sensitivity. Economically, the practice of preventing SSI by treating *S. aureus* colonization has been demonstrated as cost-effective (41) due to the high cost of caring for patients with *S. aureus* SSI.

LABORATORY DETECTION OF *S. AUREUS*

Premolecular Methods for Detecting *S. aureus*

This testing relied on culture, either using direct plating or combining broth enrichment with solid agar detection. Historically, most media contain an indicator to distinguish *S. aureus*, inhibitory substances to suppress other organisms, and antistaphylococcal antibiotics to select for methicillin resistance (42). Indicators used in screening media systems have included a carbohydrate, most commonly mannitol, and a pH indicator, traditionally phenol red, to highlight potential MRSA colonies. Recently, chromogenic indicators have replaced the other compounds (42, 43). Inhibitory substances included NaCl, ciprofloxacin, polymyxin B, aztreonam, tellurite, and desferrioxamine. Time and temperature of incubation can vary among the studies (42). Since 2010 the use of molecular techniques for direct detection of MRSA has become more commonplace (43), with many commercial assays available (to be subsequently reviewed in this chapter). Currently, real-time PCR assays are available that detect MRSA directly from swabs and selected clinical samples, as well as those assays that target both MRSA and MSSA (43).

Development of Molecular Targets from the *S. aureus* Genome

Early in the 21st century whole-genome sequencing was applied to *S. aureus*, and insights into virulence, toxigenicity, and antibiotic resistance rapidly emerged (44). A clear picture emerged of what happens to MSSA when it changes to MRSA upon the acquisition of staphylococcal cassette chromosome *mec* (SCC*mec*), which is a genomic island encoding methicillin resistance. All SCC*mec* elements reported to date carry the *mec* gene complex (*mec*) and are inserted at the integration site sequence for SCC, which is located at the extremity of the SCC element (45). This understanding led to the development of real-time multiplex PCR assays that could detect MRSA directly from clinical specimens, initially nasal swabs, in less than 1 h (46). The general design of these includes multiple primers specific to the different SCC*mec* right extremity sequences in combination with primers and probes specific to the *S. aureus* chromosomal orfX gene sequences located to the right of the SCC*mec* integration site (46). This discovery and assay development remain the basis of most rapid molecular tests for MRSA and MSSA today (47).

Early Molecular Assays

The first molecular test for *S. aureus* that was available for clinical testing was the Gen-Probe Accuprobe *Staphylococcus aureus* Culture Identification Test for testing positive blood cultures in the early 1990s (48). This test hybridizes a chemilluminescent labeled, single-stranded DNA probe for the 16S rRNA to form a DNA:RNA hybrid that specifically detects *S. aureus*. It provided species identification in a relatively short time (<1 h versus 12 to 16 h for culture) but still required additional susceptibility testing for methicillin resistance.

The initial PCR tests were in-house laboratory-developed assays that targeted a unique portion of the *S. aureus* genome, such as the *femA* gene (49). Other targets such as the *nuc* or *spa* genes have also been used (50). The limitation of this approach was that while it was satisfactory for detecting *S. aureus*, there was no ability to separate MRSA from MSSA, so culture of the specimen was required to determine if the detected isolate was methicillin resistant. Some have tried to multiplex these *S. aureus*-specific targets with *mecA* to detect the presence of MRSA, but this approach has generally been unsatisfactory when the specimen being tested has a high likelihood of containing methicillin-resistant coagulase-negative staphylococci (MR-CoNS), which harbor the same *mecA* gene and are typically present in nasal swabs (46). The exception to this problem of interference from MR-CoNS is detection of MRSA and MSSA in signal-positive blood culture bottles. Paule and colleagues found that the problem could be resolved by first determining if the amplification efficiency of the *femA* and *mecA* PCR reactions had similar amplification efficiencies (51). When that was the case, then dividing the *femA* cycle threshold (C_T) value by the *mecA* C_T value would give a C_T ratio that could differentiate an MRSA result from a mixture of MSSA and MR-CoNS. They used this strategy to call a *femA*-detected sample positive for MRSA only if the *femA*/*mecA* C_T ratio was between 0.9 and 1.1 (51), which provided an assay with 100% sensitivity and specificity for accurately detecting MRSA and MSSA. Another interesting early approach was to use magnetic beads in an immunomagnetic enrichment assay with biotinylated antispa antibody and streptavidin-coated paramagnetic beads to remove *S. aureus* from MR-CoNS followed by amplification of the optimized sample using primers for *femA* and *mecA* (52). While the sensitivity of this test was 100%, the specificity of the assay was only 64% (52), suggesting the difficulty of completely removing MR-CoNS from clinical samples.

Initial Commercial PCR

The first of the modern era molecular diagnostic tests for MRSA surveillance was that of Infectio Diagnostic Inc., with its IDI-MRSA Test, which was FDA cleared in March 2004. While this test was labor-intensive compared to newer versions of this assay, it was able to detect MRSA in the presence of other bacteria directly from nasal swabs (46). Since NorthShore was about to launch the first multihospital, universal surveillance program for MRSA control in North America, this test was chosen over the in-house-developed test that required culture of positive *femA*-positive specimens to determine which were MRSA. Testing began in August 2005, and the large-scale evaluation of the initial test found that it was very reliable, with a sensitivity of 98% and a specificity of 95% during high-volume testing (53). With this initial assay, one medical technologist could comfortably perform approximately 100 tests per day using a modified achromopeptidase lysis method—a very high volume for most microbiology testing at the time.

Peptide Nucleic Acid Fluorescence *In Situ* Hybridization

Peptide nucleic acid fluorescence *in situ* hybridization (PNA-FISH) is another application of molecular diagnostics that has been applied to *S. aureus* diagnostics (54), primarily on signal-positive blood culture bottles (55). The

PNA-FISH assay detects *S. aureus* and CoNS in 3 h and has performed well in a large comparative trial (921 blood cultures positive for bacteria) with culture-based matrix-assisted laser desorption ionization-time of flight mass spectrometry as the reference identification method (56). Importantly, in the near future this method will be able to be used directly on signal-positive blood culture bottles (57), and at that time will be the faster and less expensive of these two approaches. Currently, the PNA-FISH assay begins with a 90-min staining procedure that consists of smear preparation and fixation, hybridization with PNA probe, washing off of unbound probe, and interpretation of the slides (58). Subcultures of the original specimen onto solid media are required for confirmation of colony morphology, to detect mixed cultures, and to perform susceptibility testing (56); thus, PNA-FISH cannot distinguish between MSSA and MRSA. The main limitations of the PNA-FISH method have been relatively long turnaround time, the lack of an automated reader, and the inability to provide susceptibility test results, which has led to relatively low penetration of these methods into clinical microbiology laboratories. A recent update is the *Staphylococcus* QuickFish BC that, according to the manufacturer, is capable of identifying *S. aureus* within 20 min; similar FISH hybridization assays using beacon-based technology are also commercially available (59, 60), but all still do not have the capability to distinguish between MSSA and MRSA.

Current Commercial Assays

The list of commercial assays for the molecular detection of MSSA and/or MRSA is continually expanding, as shown in Table 1. Some are modified versions of earlier releases, occasionally under new branding, but all seek to decrease the frequency of false negatives and false positives or decrease the hands-on effort necessary to perform the testing.

All nucleic acid tests rely on sequence recognition for specificity, including capture oligonucleotides, primers for targeted PCR, and/or fluorescently labeled hybridization probes. Sequence variants between strains that occur within a targeted sequence recognition site will disrupt the assay and may yield a false-negative result. For example, the original IDI-MRSA Test, now known as BD GeneOhm MRSA achromopeptidase (ACP) assay, was designed to target six distinct *mec* right-end joining-region (MREJ) genotypes in the SCC*mec* cassette, whereas over 20 MREJ genotypes have now been reported in the literature (45, 61). Therefore, many nucleic acid amplification (NAAT) assays use multiple primer sets (one test uses 18 primer pairs) to ensure that most genotypes are targeted, reducing the number of false negatives reported. As additional sequence variations are identified, it would be advantageous to add appropriate oligonucleotides, if necessary, to avoid missing these rare variants; however, modifications like this are not permitted in FDA-cleared kits and would require additional validation experiments.

Another source of false-negative results is the recently discovered *mecA* variant labeled first as $mecA_{LGA251}$ and now known as *mec*C (62). Identified as part of the novel SSC*mec* type XI, the methicillin-resistant transpeptidase produced by *mec*C has ~63% amino acid identity to the penicillin-binding protein 2a (PBP2a) encoded by *mecA* (63). With only ~69% similarity between the two DNA sequences, the *mec*C gene sequence is not detected by molecular tests targeting the *mecA* gene. Since these are primarily livestock-associated strains (64), only a few commercial assays currently include the *mec*C gene as a target in their multiplex assays. However, in Denmark, where *mec*C frequency in human MRSA infections has been retrospectively determined, an increase in frequency was seen, from 1.5% prior to 2010 to 1.9% and 2.8% in 2010 and 2011, respectively (65), and therefore *mec*C may become an important target in the near future.

As previously mentioned, an early goal of testing was to detect MRSA without also falsely reporting as positive those specimens containing both MSSA and MR-CoNS. The IDI-MRSA test accomplished this goal by having one PCR primer targeting MREJ in the SCC*mec* cassette and the other primer targeting the *orfX* sequence specific for *S. aureus* (66). Any successful amplification would indicate both sequences on the same chromosome. No amplification would occur if the two targets were on separate chromosomes, as would be the case for a specimen containing MSSA and MRCoNS. However, using this stratagem produced different false-positive results when the specimen harbored an "empty SCC*mec* cassette"—the SCC*mec* remnant without the *mecA* gene itself (67). To significantly reduce the empty SCC*mec* cassette problem, many of the current commercial assays detect both the MREJ-orfX target and the *mecA* gene in a multiplex reaction.

With the realization that screening for MSSA colonization in preoperative patients is as clinically important as MRSA screening, contemporary molecular assays have been designed to detect and identify both targets. Finally, several modern technologies can efficiently identify dozens, even hundreds, of targets, leading to the commercial availability of pathogen panels, particularly for the detection of microbes responsible for sepsis.

Today, commercial assays for MSSA, MRSA, and several other clinically relevant pathogens are as varied as their numbers. Brief descriptions of some important elements of the FDA-cleared assays are listed below, and the sensitivity, specificity, and limit of detection for both FDA-cleared and Conformité Européenne-In Vitro Device (CE-IVD) marked assays are listed in Table 2 (56, 59, 60, 68–101). As with all *in vitro* diagnostic tests, positive and negative predictive values are highly dependent on the prevalence of the disease in the population, making accurate comparisons difficult and therefore predictive values are not included in this review.

BD GeneOhm and BD MAX

The first commercial PCR assay for MRSA has been modified to improve the test's accuracy and to reduce the labor-intensive preparation of the original test. The BD GeneOhm MRSA ACP assay employs molecular beacon chemistry, initially performed in the real-time PCR SmartCycler (Cepheid, Sunnyvale, CA) instrument to screen nasal swab specimens within 3 h. Each SmartCycler can perform 16 individual analyses after a brief achromopeptidase lysis step; it requires minimal benchtop space, and the SmartCycler instruments can be daisy-chained for screening programs requiring more capacity. This platform is also used for the expanded BD GeneOhm StaphSR, which detects both MRSA and MSSA (using *nuc* as an *S. aureus*-specific target) in positive blood cultures. Although not FDA-cleared for detection use with nasal swabs, the success of the same chemistry in the BD GeneOhm MRSA ACP assay indicates that the StaphSR could be successful in screening nasal swabs for both MRSA and MSSA after proper laboratory validation. However, at least two studies

TABLE 1 Commercial assays for MRSA/MSSA molecular testing

Manufacturer	Name	Registration status	Specimen type
Abbott Ibis Biosciences, Abbott Park, IL	PLEX-ID BAC Spectrum BC	CE-IVD marked	Blood culture
AdvanDx, Inc., Woburn, MA	*Staphylococcus aureus* and/or other *Staphylococcus* species PNA FISH Culture	FDA cleared/CE-IVD marked	Blood culture
	S. aureus PNA FISH	FDA cleared/CE-IVD marked	Blood culture
	e*Staphylococcus* QuickFISH BC	FDA cleared/CE-IVD marked	Blood culture
Amplex Diagnostics GmbH, Gars Bahnhof, Germany	eazyplex MRSA	CE-IVD marked	Nasal swab
	hyplex MRSA	CE-IVD marked	Nasal or skin/soft tissue swabs
	hyplex MRSA plus	CE-IVD marked	Nasal or skin/soft tissue swabs
Analytik Jena AG, Jena, Germany	VYOO	CE-IVD marked	Whole blood
BD Diagnostics (GeneOhm Sciences Canada, Inc.), Franklin Lakes, NJ	BD GeneOhm MRSA ACP	FDA cleared/CE-IVD marked	Nasal swab
	BD GeneOhm StaphSR	FDA cleared/CE-IVD marked	Blood culture
	BD MAX MRSA	FDA cleared/CE-IVD marked	Nasal swab
	BD MAX MRSA XT	FDA cleared/CE-IVD marked	Nasal swab
	BD MAX StaphSR	FDA cleared/CE-IVD marked	Nasal swab
BioFire, Salt Lake City, UT	FilmArray Blood Culture Identification Panel	FDA cleared/CE-IVD marked	Blood culture
bioMérieux, Inc., Marcy l'Etoile, France	VITEK MS	FDA cleared/CE-IVD marked	Blood or media culture
Bruker, Fremont, CA	MALDI Biotyper	FDA cleared/CE-IVD marked	Blood or media culture
Cepheid, Sunnyvale, CA	Xpert MRSA	FDA cleared/CE-IVD marked	Nasal swab
	Xpert MRSA/SA Blood Culture	FDA cleared/CE-IVD marked	Blood culture
	Xpert MRSA/SA Nasal Complete	FDA cleared/CE-IVD marked	Nasal swab
	Xpert MRSA/SA SSTI	FDA cleared/CE-IVD marked	Skin/soft tissue swabs
EliTechGroup Epoch Biosciences, Puteaux, France	MRSA/SA ELITe MGB	FDA cleared/CE-IVD marked	Nasal swab
Hain LifeScience, Nehren, Germany	FluoroType MRSA	CE-IVD marked	Nasal or skin/soft tissue swabs
	GenoQuick MRSA	CE-IVD marked	Nasal or skin/soft tissue swabs
	GenoType *Staphylococcus*	CE-IVD marked	Blood or media culture
	GenoType MRSA	CE-IVD marked	Blood or media culture
	GenoType MRSA Direct	CE-IVD marked	Nasal or skin/soft tissue swabs
Hologic Gen-Probe, San Diego, CA	AccuProbe *S. aureus* culture identification	FDA cleared	Blood or media culture
miacom diagnostics, Düsseldorf, Germany	hemoFISH Gram (+) Panel	CE-IVD marked	Blood culture
	hemoFISH *S. aureus*/CNS Screen	CE-IVD marked	Blood culture
	hemoFISH Masterpanel	CE-IVD marked	Blood culture
Mobidiag, Espoo, Finland	Prove-it Bone&Joint	CE-IVD marked	Body liquids, bone or tissue biopsy
	Prove-it Sepsis	CE-IVD marked	Blood culture
Molzym Molecular Diagnostics, Bremen, Germany	SepsiTest	CE-IVD marked	Whole blood
	UMD-Liquid	CE-IVD marked	Body liquids
	UMD-Swab	CE-IVD marked	Skin/soft tissue swabs
	UMD-Tissue	CE-IVD marked	Biopsies
	UMD-Universal	CE-IVD marked	Multiple
Nanosphere, Inc., Northbrook, IL	Verigene Gram Positive Blood Culture (BC-GP)	FDA cleared/CE-IVD marked	Blood culture
QIAGEN GmbH, Hilden, Germany	artus MRSA/SA QS-RGQ	CE-IVD marked	Nasal swab
Roche Molecular Diganostics, Pleasanton, CA	cobas MRSA/SA	FDA pending/CE-IVD marked	Nasal swab
	LightCycler SeptiFast MGRADE	CE-IVD marked	Whole blood
	LightCycler SeptiFast *mecA* MGRADE	CE-IVD marked	Whole blood
	LightCycler MRSA Advanced	FDA cleared/CE-IVD marked	Nasal swab
Seegene, Seoul, Korea	Magicplex Sepsis Real-time	CE-IVD marked	Whole blood

TABLE 2 Analytic performance of commercial assays for MRSA/MSSA molecular testing

Name	Specimen type	% *S. aureus* sensitivity	% *S. aureus* specificity	% MRSA sensitivity	% MRSA specificity	MRSA limit of detection (CFU/ml)	Reference
artus MRSA/SA QS-RGQ	Nasal swab	89	96	94	97	62–310	PI
BD GeneOhm MRSA ACP	Nasal swab	NA[a]	NA	92	95	130–576	68
BD MAX MRSA	Nasal swab	NA	NA	93	96	273–645	69
BD MAX MRSA XT	Nasal swab	NA	NA	93	98	64–343	PI
BD MAX StaphSR	Nasal swab	98	95	97	99	64–344	70
cobas MRSA/SA	Nasal swab	94	94	93	98	650–700	PI/FDA submission
eazyplex MRSA	Nasal swab	NA	NA	NA	NA	10	NA
LightCycler MRSA Advanced	Nasal swab	NA	NA	84–95	96–99	240	71, 72
MRSA/SA ELITe MGB	Nasal swab	96	95	92–100	95–100	165	73
Xpert MRSA	Nasal swab	NA	NA	63–99	96–100	58	71, 74–77
Xpert MRSA/SA Nasal Complete	Nasal swab	91	96	91–92	99–100	NA	73
FluoroType MRSA	Nasal or skin/soft tissue swabs	NA	NA	100	96–99	3–68	78
GenoQuick MRSA	Nasal or skin/soft tissue swabs	NA	NA	57	100	14,000	79
GenoType MRSA Direct	Nasal or skin/soft tissue swabs	NA	NA	95	99	20–30	80
hyplex MRSA	Nasal or skin/soft tissue swabs	100	100	98	84	NA	81
hyplex MRSA plus	Nasal or skin/soft tissue swabs	100	100	100	100	NA	PI
UMD-Swab	Skin/soft tissue swabs	NA	NA	NA	NA	NA	None
Xpert MRSA/SA SSTI	Skin/soft tissue swabs	99–100	90–98	99–100	72–100	NA	82, 83
UMD-Tissue	Biopsies	NA	NA	NA	NA	NA	None
Prove-it Bone&Joint	Body liquids, bone or tissue biopsy	91	90	NA	NA	NA	84
UMD-Liquid	Body liquids	NA	NA	NA	NA	NA	None
BD GeneOhm StaphSR	Blood culture	99	97	100	98	10	85
FilmArray Blood Cullture Identification Panel	Blood culture	93–100	99	95–100	97–100	NA	86, 87

TABLE 2 Analytic performance of commercial assays for MRSA/MSSA molecular testing (*Continued*)

Name	Specimen type	% *S. aureus* sensitivity	% *S. aureus* specificity	% MRSA sensitivity	% MRSA specificity	MRSA limit of detection (CFU/ml)	Reference
hemoFISH Gram (+) Panel	Blood culture	94–100	100	NA	NA	NA	59, 60
hemoFISH Masterpanel	Blood culture	NA	NA	NA	NA	NA	None
hemoFISH *S.aureus*/CNS Screen	Blood culture	NA	NA	NA	NA	NA	None
PLEX-ID BAC Spectrum BC	Blood culture	100	100	94	96	NA	88, 89
Prove-it Sepsis	Blood culture	95	99	NA	NA	11–600	90, 91
Staphylococcus aureus and/or other *Staphylococcus* species PNA FISH Culture	Blood culture	99	82	NA	NA	NA	56
S. aureus PNA FISH	Blood culture	100	99	NA	NA	NA	PI
Staphylococcus QuickFISH BC	Blood culture	99	100	NA	NA	NA	PI
Verigene Gram Positive Blood Culture (BC-GP)	Blood culture	>98	>98	>98	>98	NA	87, 91
Xpert MRSA/SA Blood Culture	Blood culture	100	100	98	100	250–350	91
AccuProbe *Staphylococcus aureus* Culture Identification	Blood or media culture	74–100	98.7–100	NA	NA	NA	92–94
GenoType MRSA	Blood or media culture	NA	NA	100	100	NA	95
GenoType Staphylococcus	Blood or media culture	97	NA	92	NA	10,000	96
MALDI Biotyper	Blood or media culture	>76	>96	NA	NA	NA	91
VITEK MS	Blood or media culture	100	97	NA	NA	NA	97
LightCycler SeptiFast *mecA* MGRADE	Whole blood	38–43	NA	NA	NA	NA	None
LightCycler SeptiFast MGRADE	Whole blood	38–43	88	NA	NA	3–30	91, 98, 99
Magicplex Sepsis Real-time	Whole blood	37–65	77–92	NA	NA	NA	100, 101
SepsiTest	Whole blood	11–95	85–96	NA	NA	NA	91, 98, 99, 101
VYOO	Whole blood	38	NA	NA	NA	5–100	91, 99
UMD-Universal	Multiple	NA	NA	NA	NA	NA	None

[a]NA, not available or not applicable.
[b]PI, manufacturer's package insert.

revealed the failure of the BD GeneOhm StaphSR to detect either MRSA or MSSA in blood cultures when the strains contained MREJ types different from the six recognized in the BD GeneOhm assays (102, 103). With the newer platform from BD Diagnostics (Franklin Lakes, NJ), the BD MAX MRSA uses a slightly different probe chemistry (5′ exonuclease probes commonly referred to as TaqMan probes) but targets the same six MREJ types as the BD GeneOhm assays.

Newer assays for this platform, namely the BD MAX MRSA XT and the BD MAX StaphSR, currently target 11 MREJ types to avoid missing many known MRSA strains. Both assays also target both the *mecA* and *mecC* genes to avoid detecting empty cassettes; these improvements were designed to aid in the detection of MRSA strains. Like BD GeneOhm StaphSR, the BD MAX StaphSR assay targets *nuc* as the *S. aureus*-specific target to detect MSSA in the specimens. The BD MAX instrument has a much larger benchtop footprint than the SmartCycler but can perform 24 individual analyses from cell lysis through detection, including different assays in the same run. There is less hands-on time for the BD MAX than the GeneOhm, and results are available within 2.5 h. All three BD MAX assays are FDA cleared for nasal specimens only, and no validation studies analyzing other specimen types have been published.

Biofire FilmArray Blood Culture Identification Panel (BCID)

This is one of several newer assays that detect not only MRSA and MSSA in signal-positive blood culture bottles but detect multiple pathogens that can cause sepsis. The BCID panel detects 19 bacteria (both Gram-positive and Gram-negative species), 5 yeasts, and 3 antibiotic-resistance genes (*mecA*, *vanA/B*, and bla_{KPC}). It is a nested multiplex real-time PCR assay performed on the FilmArray instrument, a small benchtop closed system with only 2 min of hands-on time that processes a single sample from start to finish within 1 h (104). Details on the precise targets and probe chemistry for MRSA or MSSA in the BCID assay are not publicly available. The FilmArray BCID assay is FDA cleared for positive blood culture bottles only; no validation studies analyzing other specimen types have been published.

Cepheid GeneXpert

The GeneXpert systems are random-access, single-sample instruments handling everything from sample to result, which facilitates placement in both low- and high-capacity moderately complex, real-time environments with various configurations containing 1 to 80 modules. The smaller systems are small benchtop models, whereas the Infinity-48s and the Infinity 80 are large floor models. There are four Xpert assays that are FDA cleared for the detection of *S. aureus*—both MRSA and MRSA/SA Nasal Complete for nasal specimens, MRSA/SA Blood Culture for positive blood culture bottles, and MRSA/SA SSTI for skin and soft tissue swabs. Additional applications have been evaluated and are listed in the tables. The assays require very little hands-on time, and results are available in approximately 1 h. All four use TaqMan probe real-time PCR targeting six types of SCC*mec* cassettes to detect MRSA, with three tests that also detect MSSA targeting the *spa* gene. As with early versions of MRSA assays from other manufacturers, the Xpert MRSA assay was prone to falsely identifying empty cassette strains as MRSA-positive (105–108), but the other Xpert assays now also target the *mecA* gene itself. However, the number of SCC*mec* types recognized by all Xpert assays has not been increased, which could lead to some strains being falsely identified as MSSA (73, 106, 109).

Epoch Biosciences MRSA/SA ELITe MGB

This assay is a two-step procedure best used in high-throughput environments employing batch processing. The first step requires a cell suspension of bacteria from nasal swabs, followed by DNA extraction in the automated bioMérieux NucliSENS easyMAG instrument. The second step is real-time PCR in the Applied Biosystems 7500 Fast Dx. Both instruments are placed on the benchtop and therefore have a relatively large footprint. Batches of 24 specimens are processed and resulted within 3.5 h. The detection chemistry used is the triplex-forming minor-groove-binding (MGB) hybridization probes targeting conserved sequences in *ldh1* to identify *S. aureus* and in *mecA* to identify methicillin resistance. The fluorescence of the unbound probe remains quenched while in solution, and fluorescent signal is emitted when the probe is hybridized to the correct complementary sequence. The C_T value for both targets is measured, and similar values are indicative of MRSA but can also indicate the presence of both MR-CoNS and MSSA if they are present at the same density. Different relative values or a signal only for *ldh1* is indicative of MSSA. In one study, the MRSA/SA ELITe MGB detected MRSA isolates containing *mecA* variants including *mecC* not identified by Xpert MRSA/SA (73). The MRSA/SA ELITe MGB is FDA cleared for nasal specimens only, and no validation studies analyzing other specimen types have been published.

Nanosphere Verigene

Another of the newer assays that detects multiple pathogens in addition to MRSA and MSSA is the Verigene Gram Positive Blood Culture (BC-GP). Performed on the single-sample Verigene small benchtop instrument that uses gold nanoparticle probes as part of the multiplexed microarray detection system, the BC-GP detects 12 Gram-positive bacteria and 2 antibiotic-resistance genes (*mecA* and *vanA/B*). The instrument performs cell lysis, DNA extraction, multiplex PCR, and microarray hybridization within 2.5 h. The Verigene BC-GP assay is FDA cleared for signal-positive blood culture bottles only, and both the Verigene BC-GP and the FilmArray BCID performed equally well in one comparison (87). A 30- to 41-h improvement in turnaround time compared to standard microbiology testing has been documented (110, 111). No validation studies analyzing specimen types other than positive blood cultures have been published.

Roche LightCycler and cobas

The only FDA-cleared assay for MRSA detection provided by Roche Molecular Diagnostics (Pleasanton, CA) is the LightCycler MRSA Advanced Test. The bacteria from nasal swab specimens are first lysed using heat and mechanical beating to release the DNA in a MagNA Lyser instrument, followed by real-time PCR performed in the LightCycler 2.0 instrument using fluorescence resonance energy transfer probes targeting the SCC*mec*/*orfx* region.

Both instruments are benchtop models capable of processing up to 30 specimens, with results available within 2 h and 41 min of hands-on time, compared to 2.5 h with 75 min of hands-on time for BD GeneOhm MRSA (112). Both are significantly quicker than culture, which requires 24 to 48 h for results (112).

Roche also produces three CE-IVD marked assays, not yet available in the United States, that detect MRSA: the cobas MRSA/SA for the analysis of nasal swabs (currently pending FDA clearance) and the LightCycler SeptiFast *mec*A MGRADE and LightCycler SeptiFast MGRADE assays for direct testing of whole-blood specimens, the last targeting a total of 25 blood pathogens. The cobas MRSA/SA assay is designed to detect eight MREJ types of MRSA and the *S. aureus*-specific capsular polysaccharide enzyme gene to recognize MSSA on the high-throughput cobas 4800 instrument (a floor model) with results for 6 to 94 nasal swab specimens available in 1.5 to 4 h. Roche obtained FDA 510(k) approval for the cobas MRSA/SA Test in January 2015, but availability in the U.S. is uncertain. Like the LightCycler MRSA Advanced Test, the LightCycler SeptiFast assays are performed on the LightCycler 2.0 instrument, but the latter are designed to analyze 1.5 ml of whole blood without culture or a lysis step. The assay targets a region of the internal transcribed spacer between the 16S and 23S genes of bacteria or between the 18S and 5.8S rDNA sequences of fungi that differentiate 19 bacteria and 6 fungi, with a subsequent test for the presence of a *mec*A gene. Although validated for whole blood to detect sepsis, the LightCycler SeptiFast has been used to detect pathogens in patients with infectious endocarditis (113), urinary tract infections (114), and purulent body fluids (115).

IMPLICATION OF MOLECULAR TEST CHOICE

There are three main components for decision making when approaching what to use for controlling MRSA and reducing *S. aureus* SSI, once the choice has been made to use screening as a tool for reducing infections resulting from these pathogens. A critical aspect is how testing will impact laboratory staffing (e.g., workflow) and reagent cost. Selecting a method that requires minimal hands-on time or one that is potentially automatable with a "sample in–answer out" format can help lessen the pressure on increasingly limited expert staff availability as well as fit into future lab design that likely will involve advanced robotics (116). Table 3 lists the parameters that are important to laboratory workflow and that are known for the commercial assays reported in this review. With laboratory budgets under pressure for reducing expenses, the test cost can become an important aspect of assay choice. The current manufacturer's suggested retail price for molecular diagnostics ranges from $26 to $42 per test (30), and while discounts are available, these costs can be significant to an organization when thousands of assays are performed annually.

A second issue is the cost to the hospital that is not directly related to laboratory expense but results from other charges accrued related to test performance. For example, an MRSA surveillance test with lower specificity can generate significant expense due to unnecessary patient isolation compared to a test with better specificity performance (30). Contact isolation (precautions) can range from $60.40 to $118.84 per day (117–119). This implies that for each 1% lower specificity at a hospital where the mean length of stay is 5 days, the additional isolation cost generated when performing 10,000 samples will range from $30,000 to $60,000 (30), a significant additional expense for even a few percent difference in specificity.

Evidence of patient benefit should be a major driver of the final choice, and it can differ between surgical *S. aureus* infection prevention and MRSA control programs. For surgical infection prevention, sensitivity is key since if colonization with *S. aureus* (MSSA and MRSA) is not detected, then decolonization will not be performed and the risk of a postoperative SSI increases (37, 38). In this setting NAAT is preferred due to the relative insensitivity of culture (29), even though testing can be done several days in advance of surgery for nonurgent or emergent cases.

Improved prevention of MRSA infection has driven much discussion during the past decade, ranging from advocacy of universal admission testing for detecting all those colonized (10, 11, 25–28) to doing no testing and simply decolonizing everyone with mupirocin and chlorhexidine (120). Perhaps the two best studies to compare for this decision are the large multicenter U.S. trials by Jain and colleagues from the Department of Veterans Affairs (26) and that by Huang et al. in the Hospital Corporation of America (HCA) hospitals (120). At the end of their programs, the overall Veterans Affairs ICU MRSA infection and BSI rates compared to those of Huang et al. were 0.62 versus 2.1 infections/1,000 patient days and 0.09 versus 0.47 BSI/1,000 patient days, respectively; in these two measures the VA demonstrated 3- to 5-fold lower infection rates when using universal MRSA surveillance and contact precautions for patients found to be colonized with MRSA. Also, a pair-wise group comparison of risk ratios found no statistical difference between decolonization versus screening and isolation (risk ratio of 0.9234; 95% CI 0.7311 to 1.1662; $p = 0.5034$) when coagulase-negative (skin) staphylococci were removed from the analysis in the HCA study (120), suggesting no benefit from universal decolonization compared to surveillance and targeted intervention. Table 4 includes a recent analysis of our NorthShore University HealthSystem data compared to the HCA study, which further supports the utility of a targeted approach for MRSA using NAAT methodology.

CONCLUSIONS AND FUTURE DIRECTIONS

At this time it is clear that surveillance for *S. aureus* (both MSSA and MRSA) can be very useful in improving patient outcome and that molecular diagnostic tests have a significant role to play. Real-time PCR test platforms are likely to become more numerous and easy to use, resulting in lower costs and facilitating further penetration into medical care practice. Within the next decade novel technologies are likely to emerge that will further enhance detection of these organisms and lower costs. In the summer of 2014 the U.S. Department of Health and Human Services released a 5-year funding opportunity announcement titled "Partnerships for Diagnostics to Address Antimicrobial Resistance of Select Bacterial Pathogens (R01; RFA-AI-14-019)" with the goal of funding 10 to 15 sites for developing "diagnostics that will enable rapid, sensitive, specific, culture-independent detection of high-priority antimicrobial-resistant Gram-negative bacterial pathogens." Additional goals of this funding opportunity are to attain a diagnostic test time of <3 h for reporting species and resistance profile information that is easy to use (i.e., closed sample-to-answer system with minimal operator training and expertise required), random access, and

TABLE 3 Laboratory workflow for commercial assays for MRSA/MSSA molecular testing

Name	Instrument	Footprint[a]	Single/random access/batch[b]	Maximum samples in 8 h per instrument	Result requires interpretation	Time to result (h)	Hands-on time (min)
artus MRSA/SA QS-RGQ	QIAsymphony SP/AS & Rotor-Gene Q	Floor and medium countertop	Batch	72	No	4–8	20
cobas MRSA/SA	cobas 4800	Large countertop	Batch	384	No	1.5	20
LightCycler SeptiFast MecATest MGRADE	LightCycler 2.0	Large countertop	Batch	32	Yes	6	15
LightCycler SeptiFast Test MGRADE	LightCycler 2.0	Large countertop	Batch	32	Yes	6	15
LightCycler MRSA Advanced Test	MagNA Lyser and LightCycler 2.0	Large countertop	Batch	128	Yes	2	15
Magicplex Sepsis Real-time Test	Nucleic acid extraction, conventional and real-time PCR	Large countertop	Batch	24	Yes	5.5	NA[c]
MRSA/SA ELITe MGB	NucliSENS easyMAG and 7500 Fast Dx Real-Time PCR instrument	Large countertop	Batch	120	No	3.5	<15
Prove-it Bone&Joint	Prove-it StripArray System	Small countertop	Batch	96	No	7	120
Prove-it Sepsis	Prove-it StripArray System	Small countertop	Batch	96	No	4	60
SepsiTest	SelectNA/ SelectNA plus and thermal cycler and sequencing instruments	Large countertop	Batch	NA	Yes	8–12	45
UMD-Universal	SelectNA/ SelectNA plus and thermal cycler and sequencing instruments	Large countertop	Batch	NA	Yes	8–12	45
GenoQuick MRSA	Thermal cycler	Small countertop	Batch	Thermal cycler dependent x 3	Yes	2.5	NA
GenoType *Staphylococcus*	Thermal cycler and Twincubator or GT-Blot 48	Large countertop	Batch	60 or 240	Yes	4	NA
GenoType MRSA	Thermal cycler and Twincubator or GT-Blot 48	Large countertop	Batch	60 or 240	Yes	4	NA
GenoType MRSA Direct	Thermal cycler and Twincubator or GT-Blot 48	Large countertop	Batch	60 or 240	Yes	4	NA
BD MAX MRSA	BD MAX	Large countertop	Random access	96	No	2	6
BD MAX MRSA XT	BD MAX	Large countertop	Random access	96	No	2	6
BD MAX StaphSR	BD MAX	Large countertop	Random access	96	No	2	6
BD GeneOhm MRSA ACP	SmartCycler	Medium countertop	Random access	32	No	3	NA

TABLE 3 Laboratory workflow for commercial assays for MRSA/MSSA molecular testing (*Continued*)

Name	Instrument	Footprint[a]	Single/random access/batch[b]	Maximum samples in 8 h per instrument	Result requires interpretation	Time to result (h)	Hands-on time (min)
BD GeneOhm StaphSR	SmartCycler	Medium countertop	Random access	32	No	3	NA
Xpert MRSA	Xpert	Small to large countertop versions	Random access	6–480	No	1.25	3
Xpert MRSA/SA Blood Culture	Xpert	Small to large countertop versions	Random access	8–640	No	1	3
Xpert MRSA/SA Nasal Complete	Xpert	Small to large countertop versions	Random access	8–640	No	1	3
Xpert MRSA/SA SSTI	Xpert	Small to large countertop versions	Random access	8–640	No	1	3
FluoroType MRSA	BioShake iQ and FluoroCycler	Small countertop	Single or batch	48	No	2.5	NA
hyplex MRSA	Thermal cycler and microplate reader or ELISA processor	Large countertop	Single or batch	288	Yes	2.5	5 or 30 for 96 samples
hyplex MRSA plus	Thermal cycler and microplate reader or ELISA processor	Large countertop	Single or batch	288	Yes	3.5	5 or 30 for 96 samples
FilmArray Blood Culture Identification	FilmArray	Small countertop	Single	7	No	1.2	3
hemoFISH Gram (+) Panel	Fluorescent microscope	Large countertop	Single	80	Yes	0.5	10
hemoFISH Masterpanel	Fluorescent microscope	Large countertop	Single	80	Yes	0.5	10
hemoFISH *S. aureus*/CNS Screen	Fluorescent microscope	Large countertop	Single	80	Yes	0.5	10
S. aureus and/or other *Staphylococcus* species PNA FISH culture	Fluorescent microscope	Large countertop	Single	NA	Yes	1.5	NA
S. aureus PNA FISH	Fluorescent microscope	Large countertop	Single	NA	Yes	2.5	NA
Staphylococcus QuickFISH BC	Fluorescent microscope	Large countertop	Single	NA	Yes	0.35	NA
AccuProbe *Staphylococcus aureus* Culture Identification	GEN-PROBE LEADER Luminometer	Large countertop	Single	Thermal Cycler/ sequencer dependent	Yes	<1	NA
MALDI Biotyper	MALDI Biotyper CA	Medium countertop	Single	1,568	No	1	<5
VYOO Sepsis	Multiple real-time PCR platforms	Medium to large countertop	Single	NA	NA	8	NA
PLEX-ID BAC Spectrum BC	PLEX-ID	NA	Single	NA	NA	12–16	NA
Verigene Gram Positive Blood Culture (BC-GP) Test	Verigene Reader and Processor SP	Small countertop	Single	3	No	2.5	5
VITEK MS	VITEK MS	Floor	Single	384	No	0.2*	<5

[a]Small countertop (<30 in. wide), medium countertop (30–60 in. wide), large tabletop (>60 in. wide), or floor.
[b]Although all assays can be used for a single specimen, "batch" is used if that is the most efficient practice. Similarly, random access is reserved for instruments that are capable of performing more than one assay in a single run and not for instruments capable of performing only one test at a time.
[c]NA, not available.

TABLE 4 Comparison of health care-associated infection outcome of universal decolonization (120) with concurrent data from NorthShore University HealthSystem (authors' unpublished data)

Data on:	Universal decolonization results (120)	NorthShore targeted isolation results	Comments
Scope/population	18 months/29 ICUs, 101,600 patient days	38 months/4 ICUs, 55,350 patient days	Similar hospital types
Outcome	No screening and decolonize all	Screen all and only isolate positives	
Rate of MRSA clinical isolates	2.1 per 1,000 patient days	0.3 per 1,000 patient days	NorthShore data based on 342,000 hospital-wide patients
Rate of all cause ICU bacteremia	3.6 per 1,000 patient days	1.0 per 1,000 patient days	NorthShore rate 3.6-fold lower
Rate of MRSA bacteremia	0.47 per 1,000 patient days	0.018 per 1,000 patient days	NorthShore rate 26.1-fold lower
Cost	$40 per patient[a]	$27–$37 per patient (includes all MDROs)	NS cost range based on test price for all assays used

[a]Range of prices for 5 days of Bactroban Nasal from 11 online pharmacies is $135.48 to $145.44 (http://www.goodrx.com/bactroban-nasal), as of October 24, 2014.

non-NAAT-based. With such a stimulus we should expect novel assay development that will enhance our current capacity.

An example of an anticipated new innovation is GeneWeave Biosciences' sample in–answer out diagnostic system called the vivoDx, which includes a universal reagents-on-board test kit for MRSA surveillance. The assay is developed around Smarticles, which are highly engineered phages developed to target a desired bacterial species, group, or family, and a substrate reagent that induces light production at the end of the assay read by a photomultiplier tube within the vivoDx instrument, all in under 4 h (121). Interestingly, the test results are inoculum independent, so this assay is expected to work for various bacterial infections and provide identification and susceptibility directly from clinical samples. FDA clearance trials for this new technology began in late 2015, with the MRSA test expected to be commercially available in 2016.

S. aureus was first described in modern medicine 135 years ago by the Scottish surgeon Alexander Ogston, who recognized it as the prominent cause of pus in postoperative infection (http://www.antimicrobe.org/h04c.files/history/S-aureus.pdf). Over the decades it has caused countless infections and eventually adapted to the development of antibiotics by becoming resistant to most of them. With progress in diagnostic microbiology, first using culture and more recently with the availability of rapid molecular diagnostics, we better understand how to detect and prevent infections from this pathogen. We now have a wide array of NAAT that assist in the management of these infections, and the future holds promise for even newer technology to deploy in the fight against this organism.

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Molecular Diagnostics for *Clostridium difficile*

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16

Clostridium difficile is the most important cause of health care-associated diarrhea in developed countries (1–3). During the past decade, the emergence of an epidemic C. *difficile* strain, termed PCR-ribotype 027 or North American pulsed-field gel electrophoresis type 1 (NAP1), has been associated with dramatic increases in the incidence and severity of C. *difficile* infection (CDI) in North America and Europe (1, 4). In the United States, C. *difficile* now causes more hospital-onset infections than any other pathogen (~80,000 infections annually), resulting in an estimated 14,000 deaths each year (2, 3). Moreover, these numbers greatly underestimate the true burden of CDI because up to 75% of cases in the United States now have their onset in long-term care facilities or the community (2).

In the context of these changes in the epidemiology of C. *difficile*, efficient and accurate strategies to diagnose CDI are crucial to guide management and prevent transmission. Efficient testing for CDI may reduce delays in initiation of isolation and treatment for confirmed CDI cases, while also allowing rapid discontinuation of empirical therapy and isolation when testing is negative. Accurate testing strategies can prevent unnecessary isolation and treatment due to false-positive results and lack of isolation and treatment due to false-negative results. In this chapter, we review the diagnostic methods available for CDI testing, focusing primarily on molecular methods. We also highlight clinical factors that are essential to consider in efforts to optimize the efficiency and accuracy of CDI testing.

GENERAL CONCEPTS

The diagnosis of CDI is usually based on a combination of clinical symptoms consistent with the diagnosis and laboratory tests confirming the presence of a toxigenic strain or toxin in stool. In the absence of a positive laboratory test, histopathologic or endoscopic findings may be used to support the diagnosis (5, 6). According to the European Society of Clinical Microbiology and Infectious Diseases guidelines, an episode of CDI is defined as "(i) a clinical picture compatible with CDI and microbiological evidence of toxin producing C. *difficile* in stool without evidence of another cause of diarrhea or (ii) pseudomembranous colitis" (5). A similar definition is given by the Society for Health Care Epidemiology of America and the Infectious Diseases Society of America: "A case of CDI is defined by the presence of symptoms (usually diarrhea) and either a stool test positive for C. *difficile* toxins or toxigenic C. *difficile*, or colonoscopic or histopathologic findings revealing pseudomembranous colitis" (6).

Clinical symptoms are required for the diagnosis of CDI because asymptomatic carriage of C. *difficile* is common, particularly in health care facilities, and treatment is not recommended for carriers (7–12). For example, in a multicenter study in France, the prevalence of carriage among asymptomatic hospitalized patients was 5% (12). In a more recent multicenter study in Canada, the incidence of asymptomatic carriage among hospitalized patients was 7% (10). Rates of asymptomatic carriage are typically even higher in long-term care facilities, with as many as 51% of asymptomatic residents carrying toxigenic C. *difficile* strains in outbreak settings (13). Education of clinicians and nursing staff on appropriate selection of patients for testing is essential because currently available tests do not accurately distinguish CDI from asymptomatic carriage of toxigenic C. *difficile*. It is also recommended that laboratories only accept unformed stools (defined as stools taking the shape of the container) for testing unless ileus due to C. *difficile* is suspected (14). In addition to the detection of asymptomatic carriers, inappropriate testing can also result in false-positive test results in the absence of carriage of C. *difficile* when enzyme immunoassays for toxin are used due to suboptimal specificity of these tests (15, 16).

Performance of a test of cure after CDI treatment is not recommended (5, 6, 14). It has been demonstrated that PCR and glutamate dehydrogenase (GDH) tests often remain positive after resolution of symptoms, and ~20% of patients may have positive tests at the end of treatment (17). Moreover, after discontinuation of metronidazole or vancomycin treatment, as many as 56% of patients who remain asymptomatic have positive cultures 1 to 4 weeks after treatment cessation (18). Resolution of diarrhea is the appropriate criteria for assessing the efficacy of treatment.

Repeat testing during the same episode of diarrhea is a common practice that should also be discouraged. Repeat testing increases the possibility of false-positive results and does not substantially increase the yield of true positives (19, 20). For example, Aichinger et al. (20) found that the gain (defined as the rate of negative results that converted to positive) of repeat testing was only 1.9% and 1.7% for enzyme immunoassay (EIA) for toxins A and B and PCR, respectively. In practice, some laboratories prohibit repeat

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testing for 5 or 7 days after an initial negative test to avoid inappropriate repeat testing.

LABORATORY METHODS FOR *C. DIFFICILE* TESTING

The methods used for the diagnosis of CDI have rapidly evolved over the last 10 years. The available laboratory tests fall into three groups depending on their target: methods that detect free toxins A and B in stools, methods that detect the presence of a toxigenic strain, and methods that detect the presence of C. *difficile* in stools (Fig. 1) (21). Two methods are regarded as reference methods: the stool cytotoxicity assay (CTA), which detects free toxins in the feces, and toxigenic culture, which detects toxigenic C. *difficile* strains (21).

Stool Cytotoxicity Assay

The CTA is the historical "gold standard" approved by the U.S. FDA. This test was first described by Chang et al. and is still considered a reference method for the diagnosis of CDI (22, 23). A stool filtrate is inoculated on cell culture, and a specific cytopathic effect (rounding of the cells) is observed after 24 to 48 h of incubation. The cytopathic effect is mainly due to the toxin B-associated cytotoxic activity, which is 10^3 to 10^4 times more potent than that of toxin A (24). This method is sensitive (limit of detection of toxin B is around the picogram of toxin) and inexpensive (25).

Although CTA has some advantages, there are several drawbacks. CTA requires cell culture facilities, and it is technically difficult to perform. CTA also takes much longer than many current rapid tests (25). CTA results are not available before 24 to 48 h, and in the case of a positive result, a confirmatory test must be performed with neutralizing antibodies to ensure the specificity of the cytopathic effect. In addition, CTA is not standardized, and the technique varies according to the type of cells used, the dilution of the stool, and the incubation period.

It is possible that more rapid CTA-type assays will be available in the future. For example, a real-time cell analysis assay (ACEA Biosciences) based on electronic impedance technology was described in 2010 for quantitative detection of toxin B in stool samples (26). The system provides automated data acquisition in real time and is amenable to a high-throughput, on-demand platform.

Toxigenic Culture

Toxigenic culture is also considered a gold standard for CDI diagnosis. This is a two-step method, with the initial step being isolation of C. *difficile* in culture (27). In the second step, the capacity of the strain to produce toxins *in vitro* is determined by cytotoxicity assay (by inoculating colonies in broth and testing the supernatant on cell culture). An enzyme-immunoassay for toxins A and B can be used as an alternative method to confirm toxin production, although this application is not systematically validated by the manufacturers. Finally, PCR targeting *tcdA* and/or *tcdB* after DNA extraction from colonies could also be used to detect the presence of the genes encoding for toxins.

Different selective media, either homemade or commercially available, can be used and are usually derived from the cycloserine cefoxitin fructose agar medium (28). The media generally contain antibiotics (cycloserin and cefoxitin) to ensure selectivity and sometimes taurocholate or lysozyme to promote germination of spores and enhance sensitivity of the media (29, 30). Chromogenic media are

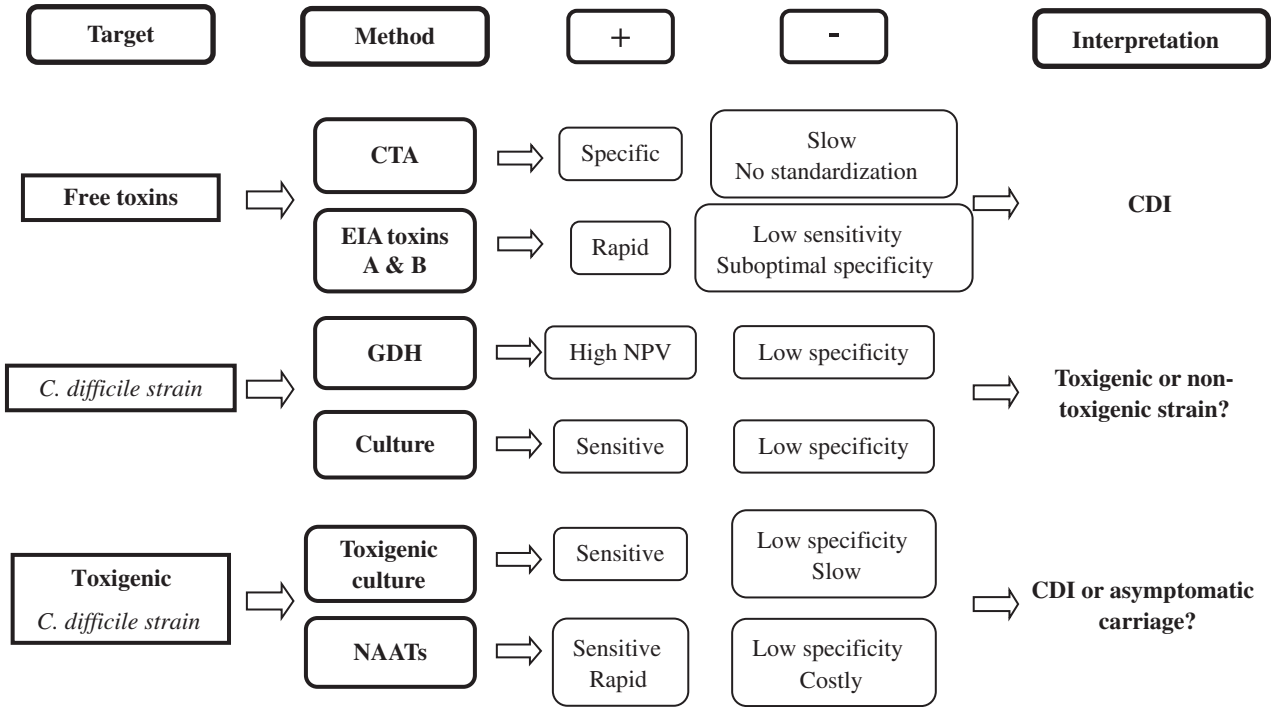

FIGURE 1 Different diagnostic methods and their targets. +, advantages; –, disadvantages; NPV, negative predictive value. Adapted from reference 25.

now available and allow an easy and more rapid identification of *C. difficile* due to the black color of the colonies (31). Ethanol or heat shock can be performed before plating to reduce the endogenous flora and to optimize the recovery of *C. difficile* strains (32). Sensitivity is about 2,000 bacteria per gram of stool. After 48 h in an anaerobic atmosphere, colonies are easily identified by their odor due to the production of *para*-cresol and their typical aspect (large gray colonies). They exhibit a green "chartreuse" fluorescence under UV light. Biochemical methods (e.g., RapID ANA II System, Thermo Fisher Scientific, Waltham, MA), gas liquid chromatography, latex agglutination, or matrix-assisted laser desorption ionization-time of flight mass spectrometry can be performed to confirm presumptive identification.

Although toxigenic culture is a very sensitive method, the time required (2 to 5 days) for testing is too long for routine clinical use. Toxigenic culture remains invaluable for epidemiological purposes and for antimicrobial susceptibility testing. In addition, toxigenic culture is commonly used as a gold standard for comparison with new testing methods.

Glutamate Dehydrogenase

GDH is a metabolic enzyme produced in relatively large amounts by *C. difficile* strains. Its detection is simple and rapid using EIA tests (mostly well-type EIA or membrane-type EIA), and numerous tests are available to detect this enzyme in stools. EIAs for GDH are sensitive (about 88 to 89% compared to the culture) (21). The advantage of these tests is their high negative predictive value, which allows the diagnosis of CDI to be ruled out by a negative result. In the meta-analysis conducted by Shetty et al., the negative predictive value varied between 94.6 and 100% (33). However, tests that detect GDH in stools lack specificity. A positive result indicates the presence of *C. difficile* but cannot predict the toxigenicity of the strain. In addition, GDH can be produced by some organisms other than *C. difficile* (34). A second test must be used to confirm the toxigenicity of the *C. difficile* isolate. GDH is therefore a good screening test and can be used as the first step of a diagnostic algorithm.

It was recently reported that sensitivity of GDH detection may vary depending on strain genotype. In particular, compared to Xpert *C. difficile*, a PCR-based method, sensitivity of GDH algorithms would be significantly lower for PCR-ribotypes other than 027 (35). However, these results were not confirmed in a subsequent study that found no difference in detection ability between the PCR-ribotypes and showed that PCR was globally more sensitive (36). A third study compared the sequence of the *gluD* gene encoding for GDH and showed that sequences of 24 out of 25 PCR-ribotypes tested were identical and that the change in the remaining one did not affect immunoreactivity (37).

EIAs for Toxins A and B

EIAs targeting toxins A and B ("free toxins") in stools became widely used by laboratories because they provide rapid results and are easy to use (21). These assays usually target both toxins A and B to detect toxin A^- toxin B^+ (A^-B^+) strains. It is now widely accepted that these tests cannot be used as stand-alone tests for the diagnosis of CDI, primarily because they lack sensitivity. René et al. (38) demonstrated that sensitivity of different EIA tests for toxins A and B compared to cytotoxicity assay varied between 41 and 67% depending on the test used. The performance of EIA was even worse compared to toxigenic culture, varying between 33 and 57% (38). Similarly, Crobach et al. (23) reported that the mean sensitivities of membrane-type and well-type EIA for toxins A and B were 72% and 82%, respectively, compared to CTA, and 52% and 66%, respectively, compared to toxigenic culture.

As noted previously, the use of EIA for toxins A and B as a stand-alone test is also problematic due to the suboptimal specificity of these assays (15, 16). Suboptimal specificity is particularly an issue in settings where the prevalence of CDI is low or if a patient being tested has a low pretest probability of CDI. For example, if the specificity of an EIA for toxins A and B is 97% and the pretest probability of CDI is 3%, the positive predictive value of a positive test would be 50% (i.e., 50% of positive tests would be false positives). One example of a setting where stand-alone EIAs for toxins A and B may result in false-positive tests is in a pediatric hospital population where the prevalence of CDI is low (16). In this setting, only 72 of 112 (64%) EIA-positive stool samples had detectable *C. difficile* by culture, suggesting that about one-third of all positive tests were false positives (16).

To date, only one commercially available EIA assay combines simultaneous detection of GDH and toxins A and B (C. diff Quik Chek Complete, Techlab; Encart *Clostridium difficile* GDH et toxins A & B, Theradiag). Concordant results (GDH+toxin+ or GDH–toxin–) readily allow for diagnosis or exclusion of CDI. A second test is required in the case of discordant results. This assay was successfully used in a two-step protocol for primary screening for CDI in a study in which discordant results (GDH +toxin–) were confirmed by PCR (39). Results for 87% of the samples could be reported rapidly after the C. diff Quik Chek Complete assay. A confirmation test was required for only 13% of the samples. This finding resulted in an improved turnaround time for the detection of toxigenic *C. difficile* compared to an algorithm based on GDH screening followed by CTA. Conversely, Goldenberg et al. (40) did not find any benefit in terms of sensitivity, specificity, and costs from coupling GDH with EIA for toxin as the first part of a testing algorithm when compared with an algorithm based on GDH detection alone.

Nucleic Acid Amplification Tests

NAATs are the most recent tests developed for the diagnosis of CDI. Most of these methods target a conserved region of the gene coding toxin B (*tcdB*) (Simplexa *C. difficile* Universal Direct [FOCUS Diagnostics], BD GeneOhm Cdiff Assay [BD Diagnostics], BD MAX Cdiff [BD Diagnostics], Portrait Toxigenic *C. difficile* Assay [Portrait, Great Basin], ProdesseProGastro Cd assay [Gen-Probe]), whereas others target the toxin A gene (*tcdA*) (Illumigene [Meridian Bioscience] and AmpliVue [QUIDELMolecular]) (Table 1) (21, 41, 42). Some molecular methods are multiplex PCR and detect several genes (RIDAGENE *Clostridium difficile* & Toxin A/B [R-biopharm; *tcdA*, *tcdB*, and genes specific for *C. difficile*], Xpert *C. difficile*/Epi [Cepheid; *tcdB*, binary toxin gene and deletion in position 117 in the *tcdC* gene], GenoTypeCDiff [HAIN LIFESCIENCE; *tcdA*, *tcdB*, binary toxin genes, genes responsible for fluoroquinolone resistance, deletion in *tcdC* including deletion in 117, specific gene for *C. difficile*], or Verigene CDF test [Nanosphere Inc., Northbrook, IL; *tcdA*, *tcdB*, binary toxin

TABLE 1 Comparison of current diagnostic methods

Diagnostic method	Target	Principle	Turnaround time
Stool cytotoxicity assay	Free toxins B (and A)	Cytotoxicity on cell culture	24–48 h
Toxigenic culture	Toxigenic *C. difficile*	Culture and *in vitro* determination of toxigenicity	2–5 days
EIA for GDH	*C. difficile*	EIA	15 min–2 h
EIA for toxins A and B	Free toxins A and B	EIA	15 min–2 h
Molecular tests			
Simplexa *C. difficile* Universal Direct	*tcdB*	Real-time PCR	1 h
BD GeneOhm Cdiff Assay	*tcdB*	Real-time PCR	1.5–2 h
BD MAX Cdiff	*tcdB*	Real-time PCR	1.5–2 h
Portrait Toxigenic *C. difficile* Assay	*tcdB*	Helicase-dependent amplification	1.5 h
ProdesseProGastro Cd assay	*tcdB*	Real-time PCR	3 h
Illumigene	*tcdA*	LAMP technology	1 h
AmpliVue	*tcdA*	Helicase-dependent amplification	1.5 h
RIDAGENE *Clostridium difficile* & Toxin A/B	*tcdA*, *tcdB*, CD-R	Real-time PCR	1.5 h
Xpert *C. difficile*/Epi	*tcdB*, *cdt*, *117*	Real-time PCR	<1 h
GenoTypeCDiff	*tcdA*, *tcdB*, *cdt*, FQ-R, 117, *tcdC*, *C. difficile*	DNA strip technology	5 h
Seeplex Diarrhea ACE Detection	*tcdB*, panel of virus and bacteria	Multiplex PCR system	4 h

[a]EIA, enzyme immunoassay; cdt, binary toxin genes, 117, deletion in position 117 in the *tcdC* gene; FQ, fluoroquinolone; FQ-R, genes responsible for fluoroquinolone resistance; *tcdC*, deletion in *tcdC* (−18 or −39 bp); CD-R, specific gene for *C. difficile* resistance.

gene, *tcdC* deletion) (21, 43). Finally, *C. difficile* is also included in tests that simultaneously detect a panel of other viruses and bacteria responsible for infectious diarrhea (Seeplex Diarrhea ACE Detection; Seegene), Xtag Gastrointestinal panel; Theradiag). This list is not exhaustive, and new tests are marketed regularly.

The NAATs vary according to the target and differ in the amplification method (Table 1). Use of real-time PCR predominates, whereas other methods are based on loop-mediated isothermal amplification (e.g., Illumigene) or helicase-dependent amplification (AmpliVue and Portrait assays).

Laboratory turnaround time can range from less than 1 h (Xpert *C. difficile*/Epi) to up to 4 to 5 h (GenoTypeCdiff and Seeplex Diarrhea ACE Detection) (Table 1). NAATs can be performed in batches (e.g., RIDAGENE *Clostridium difficile* & Toxin A/B) or on demand (AmpliVue) depending on the system used. Results are often interpreted using a software system, but amplified products can sometimes be visualized on strips (e.g., AmpliVue, GenoTypeCdiff). Other characteristics such as the DNA extraction method, use of internal controls, manual assays, or fully automated systems must be taken into consideration when choosing a molecular method.

A recent meta-analysis including different molecular methods showed that the pooled sensitivity was 92% compared to toxigenic culture and 87% compared to cytotoxicity assay (44). In another study evaluating the diagnostic accuracy of real-time PCR, similar results were found with an overall mean sensitivity of 90% (45). Thus, studies that compared different NAATs to a reference method were not able to show any significant difference between the different NAATs. However, these studies were not designed and powered for such conclusions. To show differences in terms of sensitivity between different methods, testing thousands of stools would be required with comparison to a reference method such as toxigenic culture.

One concern regarding NAATs is potential genetic modification of the regions targeted by the primers (21). A mutation in this region may lead to a false-negative result with such methods. Another potential limitation of the NAATs is the possible emergence of atypical strains (e.g., strains that do not produce toxin B and thus lead to a false-negative result if a molecular method detecting *tcdB* is being used).

The financial cost of molecular methods is another major consideration because the cost of these tests is high in comparison to EIA for toxin or GDH. One approach to reduce costs of testing is to use a less expensive screening test, with NAATs reserved for confirmatory testing. For example, a two-step algorithm based on an initial GDH screen with a NAAT only in the case of a positive GDH result can decrease laboratory costs relative to NAAT as a stand-alone test. Vasoo et al. (46) found that the implementation of a two-step algorithm with initial testing with a combined GDH and toxin assay followed by PCR for indeterminate results led to a significant cost saving per test compared to the use of PCR alone. The calculated cost per 1,000 tests was $13,663 using the two-step algorithm (C. diff QuikChek Complete reflexed to BD GeneOhm Cdiff Assay for discordant results) versus $26,950 and $25,000 for BD GeneOhm Cdiff Assay alone and ProdesseProGastro Cd assay alone, respectively. Culbreath et al. also found that laboratory costs were reduced when using a two-step strategy based on GDH and PCR (i.e., C. diff QuikChek Complete and Xpert *C. difficile*, cost was $70,633) compared to PCR alone (i.e., Xpert *C. difficile*, cost was $159,877) (38). In contrast, in a cost-benefit analysis that included both laboratory costs and estimated hospital costs of isolation and treatment, Schroeder et al.

(47) found that stand-alone on-demand PCR was favored in most settings, whereas on-demand PCR preceded by lateral-flow GDH testing was favored if a missed CDI case resulted in less than $5,000 of extended hospital stay costs and <2 transmissions, if lateral-flow GDH diagnostic sensitivity was >93%, or if the symptomatic carrier proportion among the toxigenic culture-positive cases was >80%.

STRATEGIES TO OPTIMIZE LABORATORY TESTING FOR CDI

Although there is general agreement that EIA for toxins should not be used as a stand-alone test, there is still considerable debate regarding strategies to optimize laboratory testing (21, 48–51). The main area of debate relates to whether it is sufficient to detect the presence of a toxigenic *C. difficile* strain or whether detection of both the organism and toxin is required for optimal testing (21, 48). In the United States, many laboratories have switched from toxin EIA to NAATs as a stand-alone test for CDI (i.e., detection of toxigenic strains but not toxin) (14). The major concern about this approach is that NAATs have suboptimal specificity because they can detect asymptomatic carriers (21, 48–51). As noted previously, rejection of formed stools by the laboratory can reduce the potential for detection of asymptomatic carriers (14). However, testing of asymptomatic carriers who develop diarrhea due to laxatives or viral infections can result in false-positive tests and unnecessary CDI treatment (15, 52). As will be discussed, although there is a potential for adverse consequences due to unnecessary treatment of asymptomatic carriers identified through inappropriate testing, it is also possible that identification and isolation of carriers may be beneficial if the risk for transmission is reduced.

In the United Kingdom, guidance published on behalf of Department of Health/Advisory Committee on Antimicrobial Resistance and Health Care Associated Infection in 2012 concluded that NAAT testing alone was insufficient and testing should include detection of toxin (53). Specifically, a two-test algorithm was recommended in which an EIA for GDH or NAAT for toxin genes is used as an initial sensitive screen, followed by a relatively sensitive EIA for toxins A and B for cases in which the screening test is positive (Fig. 2) (46, 53). This recommendation was based on a large observational study of more than 6,000 patients with diarrhea that sought to derive an optimum laboratory diagnostic algorithm for CDI (54). In comparison to patients with diarrhea but negative for *C. difficile*, patients with diarrhea and CTA positivity had increased mortality, whereas those with detection of toxigenic *C. difficile* but not toxin (toxigenic culture positive, CTA negative) did not. The authors classified patients

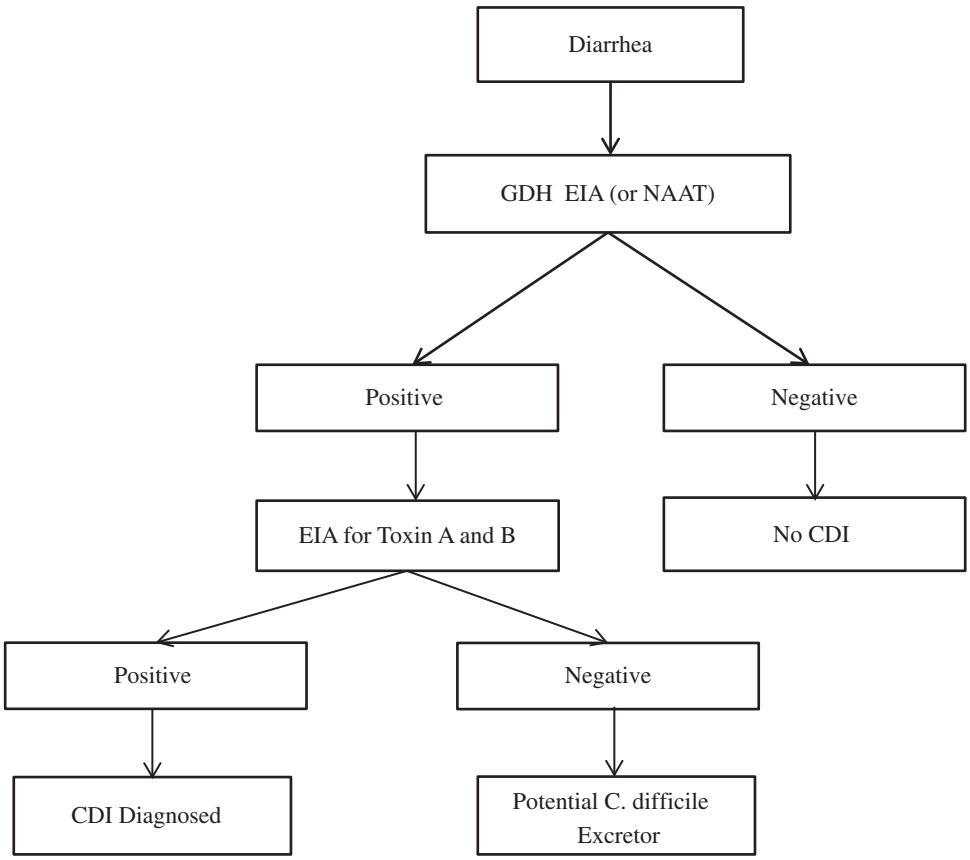

FIGURE 2 Two-step diagnostic testing algorithm for CDI from the United Kingdom Department of Health/Advisory Committee on Antimicrobial Resistance and Health Care Associated Infection (51). A relatively sensitive EIA for toxin A and B is recommended. Patients with positive GDH or NAAT but negative EIA are classified as potential *C. difficile* excretors (i.e., asymptomatic carriers with diarrhea not due to CDI). Fecal excretors may be isolated to prevent cross-transmission, but not routinely treated unless indicated based on clinical interpretation.

with positive toxigenic culture but negative CTA as potential C. *difficile* excretors (i.e., asymptomatic carriers with diarrhea not due to CDI) (54); it was recommended that such patients be isolated to prevent cross-transmission but not be routinely treated. One notable aspect of this study is that all unformed stools submitted to the laboratory from hospital patients >2 years old and from individuals in the community >65 years old were tested for CDI irrespective of whether CDI testing was requested. Thus, it is likely that many samples from patients with obvious alternative explanations for unformed stool or not meeting criteria for diarrhea (defined as three or more unformed stools in 24 hours) were tested; such testing would not be recommended in settings where NAATs are used as stand-alone tests (6, 14, 15).

Smaller studies have reached differing conclusions regarding whether patients with positive tests for toxigenic C. *difficile* but negative toxin assays are at a low risk for complications due to CDI. Longtin et al. (55) found that cases detected by PCR, but not by a three-step algorithm (GDH, EIA for toxin A and B, and CTA), were significantly less likely to develop complications (30-day all-cause mortality, colectomy, admission to ICU, and hospital readmission for CDI) compared to cases detected by PCR and the three-step algorithm (3% versus 39%, respectively). In contrast, in a prospective study of 132 patients, Guerrero et al. found that EIA-negative patients with CDI did not differ in clinical presentation from EIA-positive patients; notably, 21% of EIA-negative patients had severe CDI, including one patient who died of fulminant CDI (56). Similarly, in a study of 143 CDI patients, there was no correlation between the presence of the free toxin in stool and the severity of the disease; 42% of patients classified as having severe CDI had negative EIA for toxin despite the use of a relatively sensitive EIA (57). Finally, it has been shown that some patients harboring toxigenic strains of C. *difficile* but without free toxin in stools have pseudomembranes during endoscopic examination, suggesting that some CDI cases may be missed by reliance on toxin tests only (58, 59).

It should also be noted that asymptomatic carriers may have toxin in stool. For example, Kyne et al. (60) detected cytoxin activity by CTA in stools of 15 of 19 (79%) asymptomatic carriers, and 3 of the 4 negative specimens were from patients carrying nontoxigenic strains. Koo et al. (61) detected toxin by EIA in 3 of 11 (27%) asymptomatic carriers of toxigenic C. *difficile*. It has also been demonstrated that many patients continue to shed toxin in stool after CDI treatment (62, 63). For example, Louie et al. (63; unpublished data) found that toxin was suppressed to undetectable levels during treatment with vancomycin or fidaxomicin, but reappearance of toxin was observed in the 22 days after treatment in about 25% of stool specimens tested from patients who did not develop recurrent symptoms. Reappearance of toxin correlated with recurrent shedding of C. *difficile* in stool. Thus, if diarrhea develops in patients with recent CDI, it is important to consider that a positive toxin assay, in addition to a positive GDH or NAAT test, may be present if diarrhea occurs due to reasons other than recurrence of CDI (e.g., laxatives, viral gastroenteritis).

Taken together, the findings of these studies highlight the continued need for clinical judgment in the diagnosis of CDI. For facilities using NAATs as stand-alone tests, ongoing education of providers and nursing staff regarding appropriate indications for CDI testing is needed to reduce the risk that asymptomatic carriers will be diagnosed with CDI and treated unnecessarily (64, 65). In settings where sensitive screening tests are followed by a relatively sensitive EIA for toxins A and B, it must be appreciated that even relatively sensitive toxin tests miss some patients with CDI (38). Planche et al. (54) cautioned that the need for CDI treatment in toxin-negative patients is a clinical decision. Furthermore, Bignardi et al. (50) found that 9 of 102 (9%) GDH-positive but EIA-negative patients who had persistent or recurrent diarrhea subsequently were diagnosed with CDI based on clinical presentation and a positive GDH and EIA.

CLINICAL AND INFECTION CONTROL CONSIDERATIONS

Reducing Delays in Diagnosis

Efforts to expedite the diagnosis of CDI may be beneficial for several reasons. First, delays in diagnosis may increase the risk of transmission. Sunkesula et al. (66) found that patients with CDI presented a high risk for skin and environmental shedding at the time the order for testing was placed, suggesting that preemptive isolation of patients with suspected CDI might be beneficial. Second, if patients with suspected CDI are preemptively isolated, rapid diagnostic testing will allow timely discontinuation of isolation for those patients who subsequently test negative for CDI. Third, rapid diagnosis of CDI will reduce the time to initiation of treatment for patients with CDI and the time to discontinuation of empirical therapy in patients who test negative. Finally, efforts to expedite diagnostic testing for CDI may reduce inappropriate empirical therapy if clinicians are assured that results will be available in a timely manner (67).

Table 2 provides a summary of three recent studies that have examined the impact of interventions to expedite CDI testing (64, 68, 69). While some studies have focused on reducing delays in laboratory testing, often by switching to molecular or other rapid tests, others have included efforts to reduce delays in specimen collection and delivery to the laboratory. For example, Kundrapu et al. (64) identified a number of modifiable factors that contributed to delays (e.g., delays in communication of the test order to nursing, not providing stool collection supplies to patients in a timely fashion, rejection of specimens due to leaking of containers or incorrect labeling, and laboratory delays due to batch processing of specimens for once-daily GDH testing); an intervention to address several of these factors resulted in a significant decrease in the time required to complete CDI testing. Barbut et al. (69) found that a change from toxigenic culture and CTA to a NAAT or a two-step algorithm including GDH and NAAT resulted in a significant reduction in the time to reporting of test results and earlier initiation of treatment, reduced empiric therapy in patients without CDI, and a decrease in processing of multiple samples. The authors concluded that reducing delays in testing may improve the quality of patient management.

Reducing Inappropriate Testing and Treatment

As noted previously, a major concern regarding the use of NAATs is suboptimal specificity due to detection of asymptomatic carriers with unformed stool due to other causes (48). A number of studies have demonstrated that changing from EIA testing to NAATs may result in significant increases in the incidence of CDI (55, 70–74). For exam-

TABLE 2 Summary of three recent studies that have examined the impact of interventions to expedite CDI testing

Reference	Change in laboratory testing		Other interventions
	Preintervention	Intervention	
66	Toxigenic culture and external toxin assay	EIA for toxin A	Education of physicians
62	GDH screen with PCR confirmatory test	PCR CDI testing made priority (processing ≤2 h)	Education of nurses, physicians Change to a no-leak stool container CDI test specimens made a priority for transport to the laboratory
67	Toxigenic culture and CTA	Phase 2: PCR Phase 3: GDH followed by PCR	N/A

ple, Fong et al. (70) observed an increase in CDI incidence rates from 4.9 to 10.3 when switching from EIA to PCR. Although such increases may be due in part to detection of CDI cases that were missed by insensitive EIA tests, there is also concern that a significant proportion of the increase may be due to detection of asymptomatic carriers by sensitive NAATs. Dubberke et al. (15) found that 36% of patients tested for CDI did not meet criteria for testing because they did not have clinically significant diarrhea, including many patients who had received laxatives. Similarly, Kundrapu et al. (75) reported that in a VA hospital 22% of patients with CDI based on PCR testing either did not have clinically significant diarrhea or had a clear alternative explanation for diarrhea (e.g., laxatives).

Although there is concern that inappropriate testing may lead to treatment of asymptomatic carriers, there is limited data on effective strategies to address this issue in facilities that use NAATs as stand-alone diagnostic tests. It is likely that ongoing education of physicians and nurses along with monitoring and feedback will be necessary to reduce inappropriate testing (19, 65). Such stewardship approaches have been effective in improving adherence to CDI practice guidelines, including reducing inappropriate testing (65; authors' unpublished data).

Another potential strategy to address inappropriate testing might be to use supplementary tests that measure colonic inflammation. For example, fecal lactoferrin and fecal calprotectin are biomarkers of intestinal inflammation that has been evaluated in infectious diarrhea and inflammatory bowel disease (76, 77). In one study, fecal lactoferrin levels were higher in toxin-positive than in toxin-negative patients with positive GDH and PCR (76). Elevated lactoferrin has also been associated with moderate to severe CDI (77). In another study, fecal calprotectin levels were higher in EIA toxin-positive samples than in EIA toxin-negative but PCR toxin gene-positive patients, and both were higher than C. *difficile*-negative control patients (78). Further studies are needed to determine if measurement of fecal lactoferrin or other inflammatory markers are useful to distinguish asymptomatic carriers who do not benefit from treatment and patients with CDI who would benefit from treatment. Such studies should compare these inflammatory markers with toxin, which has been proven to be useful as a predictor of adverse outcomes (54).

False-Negative Tests Due to Empirical CDI Therapy

One of the guiding principles of infectious disease management is that diagnostic specimens should be collected prior to initiation of antimicrobial therapy whenever feasible. The rationale for this recommendation is that prior antimicrobial therapy may result in false-negative diagnostic test results, particularly cultures (79–81). Clinicians caring for patients with suspected CDI often prescribe empirical CDI therapy prior to collection of stool specimens for diagnostic testing (67). In a recent study, Sunkesula et al. (17) demonstrated that such empirical therapy may result in rapid conversion of CDI test results, including PCR, GDH, and toxigenic culture, to negative (Fig. 3). For PCR, 14%, 35%, and 45% of positive tests converted to negative after 1, 2, and 3 days of treatment, respectively. Increased age and infection with NAP1/027 strains were associated with persistent positive PCR results. For 9 CDI patients who received empirical therapy, 4 (44%) converted to negative PCR results compared to 0 of 23 CDI patients who did not receive empirical CDI treatment.

Based on these data, clinicians managing patients with suspected CDI should be aware that empirical therapy may result in false-negative CDI test results. For patients who are prescribed empirical therapy for suspected CDI with mild to moderate symptoms, it might be reasonable to stipulate that empirical therapy should only begin after a stool specimen for testing has been collected (17). If immediate empirical therapy is indicated for suspected severe CDI, efforts should be made to expedite collection of stool specimens for diagnostic testing.

Diagnostic Testing in Patients with Suspected CDI and Ileus

A small proportion of CDI cases present with no diarrhea due to ileus (6). Diagnostic testing in patients with suspected CDI with ileus is challenging because stool specimens are not available for testing. In these cases, computed tomography scans may be useful if they show findings consistent with CDI and if other intra-abdominal conditions that present similarly are ruled out. In severe CDI cases, computed tomography findings may include colonic mural thickening diffusely throughout the colon or localized, pericolonic fat changes, trapping of contrast material between thickened folds (accordion sign), and ascites (82). Sigmoidoscopy or colonoscopy may also be useful in these cases to evaluate for the presence of pseudomembranous colitis (6). Finally, perirectal swab specimens could potentially be useful as a diagnostic approach in patients with suspected CDI and ileus. In a study of 139 patients tested for CDI by PCR (Xpert C. *difficile*), the sensitivity, specificity, positive predictive value, and

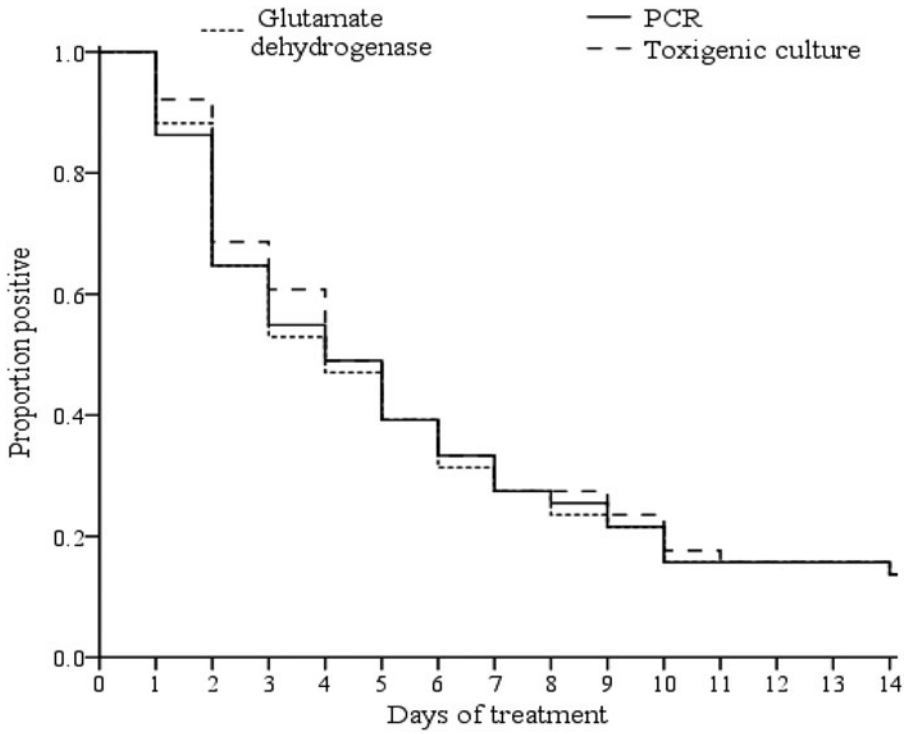

FIGURE 3 Kaplan-Meier curves showing time to conversion of CDI test results from positive to negative during CDI therapy, by test type (toxigenic culture, PCR for toxin B genes, and GDH). Reprinted from reference 17.

negative predictive value of testing perirectal swabs versus stool specimens were 96%, 100%, 100%, and 99%, respectively (83). However, because the study did not include patients with ileus, further studies are needed to confirm that perirectal swab PCR tests are useful in this setting.

Screening for Asymptomatic Carriage of *C. difficile*

Although it is known that asymptomatic carriage of toxigenic C. *difficile* is common in health care facilities, current infection control measures focus on preventing transmis-

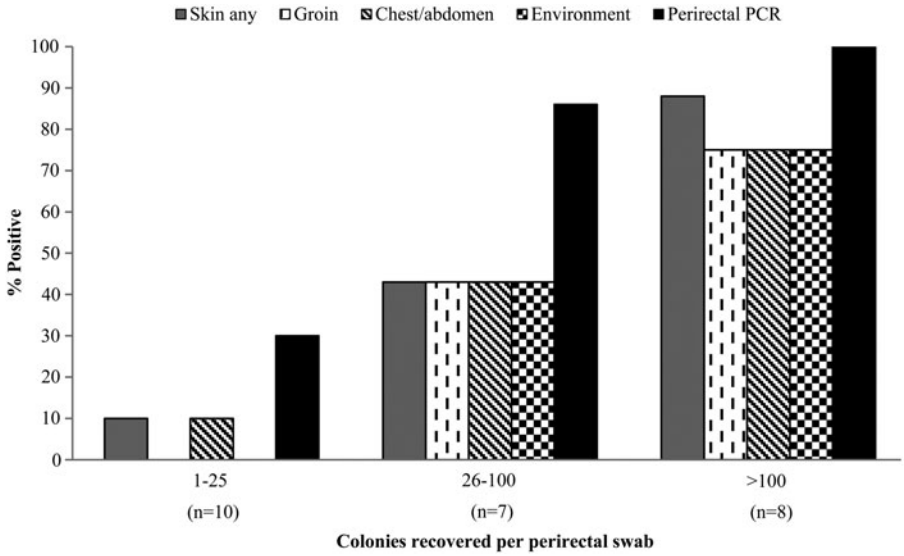

FIGURE 4 Percentage of positive perirectal PCR results and skin and environment cultures for long-term care facility residents with asymptomatic carriage of C. *difficile* based on positive perirectal cultures, stratified by the number of C. *difficile* colonies recovered per swab. Skin sites cultured included the groin and combined chest and abdomen. Environmental sites cultured included the bed rail and bedside table. Twenty-five sets of specimens were collected from 21 asymptomatic carriers. Reprinted from reference 88.

sion from CDI cases (5, 6). It is not recommended that screening cultures be used to identify asymptomatic carriers, and treatment of carriers is not recommended (5, 6). However, there is evidence that asymptomatic carriers could be an underappreciated source of transmission. For example, Clabots et al. (84) found that 84% of nosocomial acquisitions of *C. difficile* on a medical ward were linked to newly admitted asymptomatic carriers based upon restriction enzyme analysis typing. More recently, Curry et al. (85) demonstrated by using multilocus variable number of tandem repeats analysis genotyping that incident CDI cases in a tertiary care hospital were as frequently linked to asymptomatic carriers as to CDI cases (29% versus 30%, respectively). In contrast, in a small study from Oxfordshire, England, none of 13 asymptomatic carriers of toxigenic *C. difficile* were linked to subsequent transmissions to CDI cases diagnosed within 3 months, suggesting that transmission events from individual carriers may be relatively infrequent (86). Although no studies to date have demonstrated that screening for and isolating carriers reduce transmission, a recent mathematical modeling study suggested that screening for asymptomatic carriage on admission could reduce new colonization and hospital-onset CDI cases (87).

If screening for asymptomatic carriers is to be considered as a control measure in the future, there will be a need for simple and effective methods to rapidly identify carriers. Because culture for *C. difficile* is not routinely available and may require days for processing, molecular methods may be preferred. In a recent study, a commercial PCR assay (Xpert *C. difficile*) of perirectal swab specimens detected 17 of 25 (68%) asymptomatic carries of toxigenic *C. difficile*, including 93% with skin and/or environmental contamination (88). Notably, the number of colonies recovered from perirectal swabs varied widely, and a low burden of colonization was associated with a low risk of skin or environmental shedding and a low likelihood of having a positive perirectal PCR result (Fig. 4). These findings suggest that a commercial PCR assay of perirectal swab specimens could provide a sensitive and rapid means to identify asymptomatic carriers of *C. difficile* at high risk for shedding of spores. The study was conducted in a long-term care setting, and therefore additional studies are needed in hospitals.

CONCLUSION

Efficient and accurate diagnostic testing strategies for CDI are essential to guide management and prevent transmission. EIAs for toxins A and B are no longer recommended as stand-alone tests for CDI due to their suboptimal sensitivity and specificity. NAATs have been an important advance due to their sensitivity and efficiency. However, these tests lack specificity in settings where inappropriate testing results in detection of asymptomatic carriers. Use of two-test algorithms, in which a NAAT or GDH test is used as an initial sensitive screen, followed by a relatively sensitive EIA for toxins A and B for cases in which the screening test is positive, has been proposed as a strategy to improve specificity. However, some asymptomatic carriers of *C. difficile*, including those recently treated for CDI, often have positive stool assays for toxin, suggesting that toxin assays may also have suboptimal specificity. Thus, it remains important for clinicians to be aware of the performance characteristics of CDI tests used in their facility and of the need to consider clinical presentation in the diagnosis of CDI.

Efforts to improve CDI testing must address clinical factors that contribute to suboptimal testing. Delays in diagnosis are often due to delays in collection or transport of specimens to the laboratory. Inappropriate testing often occurs due to lack of knowledge on the part of clinical staff. Recent studies have demonstrated that multifaceted interventions focusing on nurses and physicians in addition to the laboratory may result in significant improvements in the timeliness and appropriateness of testing and increased adherence to management guidelines.

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Overview of Molecular Diagnostics in Multiple-Drug-Resistant Organism Prevention: Focus on Multiple-Drug-Resistant Gram-Negative Bacterial Organisms

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17

The combination of increasing rates of antibiotic resistance and the decreasing pace of new antibiotic development has become an urgent public health crisis (1). Multiple-drug-resistant bacterial organisms (MDROs) have now become common causes of health care-associated infection (HAI), the most prevalent being methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin-resistant *Enterococcus* (VRE), and a variety of multiple-drug-resistant Gram-negative rods (MDR-GNRs; organisms that are resistant to most, if not all, available antibiotic classes through myriad mechanisms, including production of β-lactamases with increasingly broad activity) (2–4).

Given the limited treatment options, prevention of MDRO infections is paramount. MDRO prevention approaches can be broadly categorized into (i) measures to improve antimicrobial use, (ii) measures to prevent transmission of MDROs, and (iii) measures to prevent infection among patients who are asymptomatic carriers of an MDRO. For example, all hospitals should have antimicrobial stewardship programs that are designed to control the emergence of multiple-drug resistance (5). Once an individual is colonized or infected with an MDRO, the Centers for Disease Control and Prevention (CDC) recommends the use of contact precautions to prevent spread in the health care setting (6). Some hospitals also practice focused decolonization of MDRO carriers in an effort to prevent subsequent infection (7). Given that routine clinical cultures identify only a minority of those who carry an MDRO (8, 9), the use of active screening for MDRO carriage is also common. Current CDC guidance recognizes active MDRO surveillance as an important "tier 2" measure, to be applied during outbreaks or when rates of a targeted MDRO are not decreasing (6).

Some of the major obstacles to both antimicrobial stewardship and active MDRO surveillance have been (i) long turnaround time (TAT), (ii) poor sensitivity of culture-based diagnostics, and (iii) poor specificity of phenotypic confirmatory testing. For example, culture-based assays for MDRO detection can take between 24 and 72 h to complete, during which infection prevention interventions such as initiation of isolation precautions will be delayed unless they are applied to all screened patients while awaiting results. Thus, rapid molecular detection of MDRO carriage has long been considered an "unmet clinical need" (10). This chapter will review advancements in the area of molecular testing for MDRO prevention with a focus on molecular approaches to MDRO surveillance. We will cover MDR-GNRs in the most detail, given that other chapters in this volume provide detailed information about MRSA (chapter 15, this volume) and VRE (chapter 18). Rapid molecular diagnostics that support expedited antimicrobial management of infected patients are discussed in other chapters (chapter 26). Limitations will also be discussed.

MDRO SURVEILLANCE AND PREVENTION: A COLLABORATIVE EFFORT

The incorporation of microbiological surveillance into MDRO prevention efforts is a complex endeavor and requires laboratories to collaborate closely with hospital infection prevention programs and patient care unit personnel (i.e., nurses, physicians, allied health, housekeeping, etc.) to ensure that interventions linked to MDRO surveillance results are implemented safely and efficiently. The steps required to improve the likelihood of success of active MDRO surveillance programs are beyond the scope of this chapter, but key elements include optimizing the TAT from specimen receipt in the laboratory to notification of results; monitoring and optimizing the initiation of infection prevention interventions (i.e., implementation of isolation precautions, decolonization); monitoring and preventing unintended adverse consequences of the interventions (e.g., reduction of clinician bedside visits or increased incidence of adverse events in patients on isolation precautions); and measurement of meaningful outcomes to evaluate the effectiveness of the MDRO surveillance program (11).

One important concept when assessing the impact of molecular diagnostics on MDRO transmission is the gap between analytic versus actual TAT. A major advantage of molecular MDRO detection is, of course, speed. However, too often we focus only on the analytic TAT of a test from

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the laboratory perspective. The meaningful TAT measure is the time from when a test is ordered to when the result is translated into action (e.g., institution or removal of contact precautions, decolonization). For an MDRO detection test to be truly considered rapid, it should provide at least same-day TAT. A rapid test with an analytic TAT of 2 h that is batched and performed once daily represents little improvement in TAT over culture-based methods. Similarly, some molecular tests require an isolated bacterial colony to perform and thus are not truly rapid, given that 24 h or more may be required to grow the organism. Thus, the most useful rapid molecular tests are those that can be performed directly on patient samples (or on enrichment broth cultures after an abbreviated period of incubation). Ultimately, if the improved analytic TAT of molecular MDRO detection tests is to provide any benefit, there must be a robust postanalytic system in place to ensure that results are translated into action. In other words, unless the performance characteristics of a given test are significantly superior, there is no reason to convert to a test with shorter analytic TAT if the actual TAT of the test will not be meaningfully reduced.

MRSA

MRSA is the most prevalent MDRO in hospitals in most countries and is currently the most common cause of HAI involving an MRDO in U.S. hospitals, accounting for 8.5% of device-associated HAIs reported to the National Healthcare Safety Network (NHSN) (12). First recognized in 1961, shortly after the introduction of methicillin for clinical use (13), MRSA has spread rapidly in hospitals and community settings worldwide in the decades since, with a few successful clones accounting for much of the spread (14). Because it is so common, MRSA is the MDRO with which we have the most experience with respect to detection of asymptomatic carriage ("active surveillance").

Methicillin resistance in *S. aureus* is mediated by an altered penicillin-binding protein (PBP2a), one that has poor affinity for β-lactam antibiotics. The PBP2a protein is encoded by the *mecA* gene, which is carried on the staphylococcal cassette chromosome *mec* (SCC*mec*) element, which integrates into the *S. aureus orfX* gene. A homologue, designated *mecC*, was first reported in livestock in 2011 but has now been recovered from humans and is only 69% identical to *mecA* at the DNA level (15).

Chapter 15 in this volume reviews a variety of MRSA detection strategies, but the most common phenotypic approach involves recovery of MRSA from a screening swab on a commercially available chromogenic agar medium (16). While these media perform well for detection of MRSA carriage, optimal sensitivity requires incubation in enrichment broth, which increases TAT time by up to 18 h (17). In addition, sampling of multiple body sites beyond the nares (e.g., throat, rectum, and others) is required to achieve optimal sensitivity (17–19). These factors should be considered when evaluating studies that compare the performance of culture-based screening methods against molecular MRSA detection assays.

As detailed in chapter 15, there has been widespread adoption of FDA-approved molecular platforms for the detection of MRSA directly from patient samples. These include the Xpert MRSA (Cepheid, Sunnyvale, CA), the BD GeneOhm MRSA ACP (BD Diagnostics, La Jolla, CA), and the LightCycler MRSA Advanced (Roche Molecular Diagnostics, Pleasanton, CA). Interestingly, despite the fact that a single gene (*mecA*) confers methicillin resistance in *S. aureus*, the development of molecular assays that detect MRSA directly from patient specimens has been challenging. First, the *mecA* gene found in *S. aureus* is highly homologous to that carried by coagulase-negative staphylococci (CoNS), and most CoNS are methicillin resistant (14, 20). Thus, the major obstacle encountered in early assays utilizing *mecA* primers was the false-positive test resulting from detection of methicillin-resistant coagulase-negative staphylococcus (MR-CoNS) in mixed samples. To overcome this limitation, widely used commercial assays amplify and detect a region at the SCC*mec-orfX* junction that does not actually contain the *mecA* gene (21). These assays demonstrated satisfactory performance characteristics (sensitivity, specificity, and agreement with culture of >90%), although some strains of methicillin-susceptible *S. aureus* (MSSA) were observed to contain this target while having "lost" the *mecA* gene through excision or mutation (the so-called *mecA* dropouts or empty cassette variants) (22). Although initially believed to be rare, these strains were soon recognized to be common causes of false-positive tests. Inclusion of *mecA* primers in these assays has largely addressed the problem, although false positives that result from simultaneous detection of an empty cassette variant of MSSA and MR-CoNS are still possible (23). Data from the MOSAR WP2 Study Team also suggest that certain MR-CoNS alone may test false positive due to homogeneity of the SCC*mec-orfX* region (24). These false-positivity issues can result in low positive predictive values (PPV) in low-prevalence populations (25, 26). For example, in a cohort of 1,891 patients (3% of whom had MRSA in culture) who were screened using the Xpert MRSA assay and broth-enriched culture, Roisin and colleagues reported a PPV of only 38% for the Xpert MRSA assay. Fifteen patients grew MSSA in culture that tested positive by the Xpert assay on isolated colonies. In addition to empty cassette variants, some had sequence homology with the SCC*mec-orfX* junction sequences targeted by the assay (27). False-positive MRSA surveillance tests may result in unnecessary initiation of isolation precautions and/or decolonization procedures, which are not without risk.

Falsely positive tests are not the only issue for current molecular MRSA assays. As widely reported, strains of MRSA with novel SCC*mec* elements and *mecA* genes can result in falsely negative tests (28, 29). The International Working Group on the Classification of Staphylococcal Cassette Chromosomal Elements recently published a guideline for reporting novel *mecA* gene homologues (30). The prevalence and range of such strains are not well understood, but ongoing surveillance and cataloguing of different *mecA* types will be necessary for molecular detection assays to keep pace with the genetic evolution of MRSA. In addition, while *mecC*-expressing strains of MRSA remain uncommon in humans, their emergence has necessitated the re-evaluation of existing assays that rely on detection of *mecA* and the development of *mecC*-specific primers to ensure that *mecA* variants are detected (31, 32).

These issues highlight the importance of retaining culture-based screening methods in parallel with molecular platforms, to monitor for emerging strains that may present problems for existing assays. In addition, bacterial isolates derived from culture-based screening can be used for molecular typing and antimicrobial susceptibility testing (which may include testing against agents used for topical decolonization, such as mupirocin and chlorhexidine [33]).

Finally, specimens from body sites that fall outside of FDA-approved indications can be tested using culture, which is important for optimizing sensitivity of MRSA screening (17–19). Thus, depending on work flow, the molecular test may be used to reduce TAT but cannot replace culture altogether.

VRE

The rapid spread of MRSA in many hospitals in the 1980s led to a significant increase in the use of vancomycin, which no doubt favored the eventual acquisition and spread of vancomycin resistance among the enterococci. VRE is second only to MRSA as a multiple-drug-resistant cause of HAI and is responsible for 3% of HAIs reported to NHSN during 2009–2010 (12). Not surprisingly, it is also the next most common target of tests developed for detection of MDRO carriage. Vancomycin resistance in enterococci is mediated by several genes designated *vanA*, *vanB*, *vanC*, *vanD*, etc., which act by modifying or removing the vancomycin-binding target D-Ala-D-Ala in the cell wall peptiglycan (34). Among these, *vanA* and *vanB* predominate among clinically significant enterococci (*Enterococcus faecium* and *Enterococcus faecalis*) and are the only two that have major epidemiological significance due to the transmissibility of the resistance genes and outbreak potential (34).

Culture-based assays for detection of VRE carriage have traditionally taken 48 to 72 h or longer, but more recently there are chromogenic agar media that perform well (sensitivity and specificity >95%) for the detection of VRE in perirectal swabs or stool with TATs of approximately 24 h (35). Commercial detection assays that utilize real-time PCR to target the important targets (*vanA* and *vanB*) have been developed and can reduce the TAT to 2 to 4 h or less.

One of the challenges related to molecular detection of VRE carriage arises from the fact that the reservoir for VRE is within the rich microbiota of the intestine, a microbial community that is profoundly impacted during hospitalization and by receipt of antimicrobial therapy (36). All *vanA* and/or *vanB* detection assays have lower limits of detection ranging from 10 to 100 CFU/ml (10, 37, 38), so it is possible that VRE may be present at levels below detection upon hospital admission and become detectable after antimicrobial receipt causes expansion of the VRE population (36). This sequence of events could be misinterpreted as VRE acquisition (which could in turn focus infection prevention efforts solely on transmission prevention activities rather than on antimicrobial stewardship). An additional problem with direct detection of VRE from fecal flora is that intestinal anaerobes may also carry the *vanB* resistance determinant (37). Because *vanB* is also less prevalent among VRE than is *vanA*, published evaluations of the performance of rapid *vanB* detection assays reveal positive predictive values as low as 1 to 10% (38–40). Rapid molecular VRE screening tests are therefore useful mainly for detection of *vanA*-bearing enterococci. Indeed, the only FDA-approved commercial assay available in the United States at present, the Xpert *vanA* (Cepheid, Sunnyvale, CA), targets *vanA*.

In contrast to active surveillance for MRSA carriage (which is now mandated in some states), little enthusiasm has developed for routine screening for the VRE carrier state. There are two main reasons for this. First, VRE are much less common as invasive pathogens than MRSA and tend to impact a smaller subset of (usually highly immunocompromised) patients (3). Second, there are no effective decolonization options for VRE carriers, as there are for MRSA carriers.

MDR-GNRS

Compared to MRSA and VRE, for which a single gene (*mecA*, *vanA*) provides a "gold standard" for MDRO detection, MDR-GNRs present a much greater challenge. A variety of resistance mechanisms can contribute to antimicrobial resistance. These include drug destruction through hydrolysis, efflux of drugs from the periplasmic space, reduction in penetration of drugs through the bacterial cell membrane, and alteration of the drug target resulting in reduced drug binding. However, the MDR-GNRs that have the greatest potential for spread are those that harbor plasmid-mediated β-lactamases. Those that are capable of hydrolyzing extended-spectrum cephalosporins and carbapenems are of highest concern.

The first report of a plasmid-mediated β-lactamase in *Enterobacteriaceae* was published in 1965. Datta et al. described the isolation of an enzyme that hydrolyzed penicillin from an isolate of *Escherichia coli* from Greece that was named TEM-1 (41). Subsequently, in 1972, Pitton described a "sulfhydral variant" β-lactamase (SHV-1) in *Klebsiella pneumoniae* and *E. coli* (42). Organisms that produce TEM-1, TEM-2 (a single amino acid variant of TEM-1), and SHV-1 hydrolyze ampicillin, carbenicillin, ticarcillin, and cephalothin but remain susceptible to oxyimino-β-lactams (including cefotaxime, ceftazidime, ceftriaxone) and monobactams (e.g., aztreonam). In 1983, Kliebe et al. reported an SHV-1-derived β-lactamase (SHV-2) in a German isolate of *Klebsiella ozonae* with transferable resistance to the oxyimino-β-lactams (43). This was followed by a TEM-2-derived extended-spectrum β-lactamase (ESBL) (TEM-3) in 1988 (44). Since that time, plasmid-mediated ESBLs deriving from SHV-1, TEM-1, and TEM-2 ESBLs have been reported around the world. In each case, 1 to 4 amino acid substitutions in the active site of the original β-lactamase led to improved hydrolysis of oxyimino-β-lactams. Subsequently, in the late 1980s, non-TEM, non-SHV ESBLs were reported in Japan and Germany. The Japanese strain was originally published with the name FEC-1, although DNA sequencing later showed that it was very closely related to CTX-M-3. The German strain was called CTX-M to denote its ability to hydrolyze cefotaxime more efficiently than ceftazidime (45, 46). CTX-M enzymes are currently the predominating ESBL worldwide (47).

There are now over 150 CTX-M enzymes that are classified into groups by amino acid sequence. They include the CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, and CTX-M-25 groups (48; http://www.lahey.org/Studies/). CTX-M, TEM-, and SHV-derived ESBLs are classified as Ambler class A β-lactamases and are members of group 2be in the Bush-Jacoby classification system (49–51). They are inhibited by clavulanate, tazobactam, and sulbactam and do not hydrolyze cephamycins efficiently. Other class A ESBLs include BEL, BES, SFO, TLA, GES, PER, and VEB, but they are thought to be less common and tend to be distributed in a regionalized manner (50, 51). Finally, β-lactamases that hydrolyze oxacillin or cloxacillin at rates >50% of benzylpenicillin distinguish the OXA family of β-lactamases. OXA β-lactamases have their own Ambler class (class D) and Bush-Jacoby groups (2d, 2de, and 2df) (49, 50). OXA-derived ESBLs are mostly derived from

OXA-10 and are weakly inhibited by Ambler class A β-lactamase inhibitors.

AmpC β-lactamases are capable of hydrolyzing penicillins, cephalosporins (including oxyimino-β-lactams), and monobactams. Notably, most AmpC β-lactamases hydrolyze cephamycins and are generally not inhibited by clavulanic acid. AmpC β-lactamases are typically inhibited by cloxacillin, oxacillin, aztreonam, and boronic acid (52). While chromosomally mediated AmpC is typically absent in many *Enterobacteriaceae* including *K. pneumoniae*, *Klebsiella oxytoca*, *Proteus mirabilis*, and *Salmonella* spp., plasmid-mediated AmpC β-lactamase production has been documented in all of these organisms (53, 54). The reverse is also true: *Enterobacter cloacae* and *Enterobacter aerogenes* and other *Enterobacteriaceae* that harbor AmpC in their genome have been reported to carry ESBL determinants (55, 56).

Until recently, carbapenems were active against most clinically encountered MDR-GNRs. In 2001, Yigit et al. reported a strain of *K. pneumoniae* from North Carolina with MICs of 16 μg/ml for imipenem and meropenem. It carried three plasmid-encoded β-lactamases: SHV-29, TEM-1, and a novel β-lactamase that was named *K. pneumoniae* carbapenemase (KPC-1) (57). Today, 21 KPC variants have been reported, but in 2008, the nucleotide sequence of KPC-1 was determined to be identical to that of KPC-2, and the former was removed from the KPC nomenclature (http://www.lahey.org/Studies/; 58). Outbreaks involving KPC-producing organisms have been reported in the United States, South America, Western Europe, Israel, and China, and these organisms have become endemic in all of these regions (59). KPC-producing organisms typically show high levels of penicillin and cephalosporin resistance and variable carbapenem resistance. Unlike other Ambler class A β-lactamase producers, KPC (like AmpC) is inhibited by boronic acid but is only weakly inhibited by clavulanate, tazobactam, and sulbactam (60). IMI-2 and GES carbapenemases are also class A carbapenemases but are encountered less frequently (59).

Ambler class B carbapenemases (metallo-β-lactamases or MBLs) can be distinguished from class A members by their ability to hydrolyze virtually all therapeutically useful β-lactams, β-lactam inhibitors, and carbapenems. MBLs do not hydrolyze monobactams, but plasmids carrying MBL determinants frequently encode ESBL determinants that lead to their hydrolysis. MBLs are inhibited by EDTA, which is a chelator of zinc, a key player in β-lactam hydrolysis in MBL. In 2009, Yong et al. reported novel MBL-producing *K. pneumoniae* and *E. coli* isolates from a patient who was hospitalized in New Delhi, India (61). This strain was named the New Delhi MBL (NDM), and it is the most recently identified MBL. Plasmids carrying the bla_{NDM} gene often bear multiple resistance determinants, creating significant therapeutic challenges. The majority of early NDM cases had epidemiological links to the Indian subcontinent, but intra-hospital outbreaks have now been reported in the United States and other countries (62, 63). The VIM (Verona integron-encoded MBL) family of MBL was first documented in *Pseudomonas aeruginosa* in Verona, Italy, in 1997, but acquired cases have been documented in various *Enterobacteriaceae* and non-glucose-fermenting organisms (64, 65). Plasmid-borne IMP-1 was also first reported in *P. aeruginosa* in Japan in 1988 (66) and later in *Enterobacteriaceae* (67). IMP carbapenemases are now particularly endemic in Taiwan and Japan, and VIM is highly prevalent in the Mediterranean countries, but strains harboring bla_{IMP} and bla_{VIM} have now been reported throughout the world (59).

OXA-48 and its relatives, by one- to five-point mutations (OXA-162, OXA-163, OXA-181, OXA-204, OXA-232), weakly hydrolyze carbapanems and third-generation cephalosporins. There are no known inhibitors for class D carbapenemases. OXA-48 originated in Turkey and was first reported in 2003 (68) but has now spread to Mediterranean Europe and Africa (69).

CULTURE-BASED SCREENING FOR ESBL- AND CARBAPENEMASE-PRODUCING ORGANISMS

Rectal swab culture using selective agar is the most practical approach for screening patients for asymptomatic carriage of MDROs. Stool, urine, wound, and sputum and other upper respiratory tract specimens have also been included in various evaluations (70). For ESBL screening with rectal swabs, there are data supporting the practice of incubating swabs in enrichment broth prior to inoculation of media (71). Recent advances in ESBL-screening media formulations have led to enhanced sensitivity for the detection of ESBL producers. The ChromID ESBL (bioMérieux, Marcy l'Etoile, France) and Brilliance Oxoid (Oxoid Ltd, Basingstoke, United Kingdom) contain cefpodoxime to select for both CTX-M and other class A ESBLs. The antibiotics present in the CHROMagar ESBL (CHROMagar, Paris, France) are not disclosed. These products also contain chromogenic substances that identify organisms, some to the species level. Grohs et al. published a side-by-side evaluation of ChromID ESBL, Brilliance Oxoid, CHROMagar ESBL, BD Drigalski agar (similar to MacConkey but containing ceftazidime), and an in-house medium containing Drigalski agar and cefotaxime to detect CTX-M-producing organisms. A total of 2,337 rectal e-Swabs (Copan Diagnostics Inc., Murrietta, CA) were collected from 267 patients, inoculated directly to agar plates using the Copan Wasp system, and incubated for 18 h (72). ESBL detection on any of the media was considered the gold standard definition of "ESBL-positive." All five types of media had comparable sensitivity of >95%. Specificity was 72.3%, 72.9%, 57.9%, 63.9%, and 59.2% for the five types of media, respectively. False positives were mainly related to the recovery of organisms producing AmpC β-lactamase and underscored the need to confirm ESBL production status in the organisms recovered from these media.

For carbapenemase detection, the CDC has published a procedure that involves overnight incubation of rectal swabs in 5 ml of trypticase soy broth with the addition of a 10-μg disk of meropenem or ertapenem. Broth is then subcultured to MacConkey agar, and any resulting lactose-fermenting colonies are selected for confirmatory testing (73). Commercially available screening media include the CHROMagar KPC (CHROMagar), ChromID Carba (bioMérieux), Brilliance CRE (Oxoid), and the Remel Spectra CRE (Remel, Lenexa, KS). The Colorex KPC (CHROMagar) is a ready-to-use version of the CHROMagar KPC. Unfortunately, culture-based screening for carbapenemase-producing organisms can be challenging due to variations in carbapenem hydrolysis. Carbapenemase-producing isolates with imipenem and/or meropenem MICs of ≤1 μg/ml are well documented and may fail to be recovered on screening media (74).

Wilkinson et al. used 130 previously characterized carbapenemase-producing isolates and examined the analytical sensitivity and specificity of Colorex KPC (CHROMagar), ChromID Carba (bioMérieux), ChromID ESBL (bioMérieux), Brilliance CRE (Oxoid), and the CDC screening protocol. The Colorex KPC, Brilliance CRE agars, and CDC protocol repeatedly failed to detect KPC, NDM, VIM, IMP, and OXA-48 carbapenemase producers at 18 h of incubation at low inocula (100 CFU/spot) (75). Detecting 125/130 of the carbapenemase producers tested, ChromID ESBL had the highest sensitivity at 96% at low inocula, but predictably, it had a specificity of only 6% due to the recovery of non-carbapenemase producers. ChromID Carba followed with sensitivity and specificity of 91% and 89%, respectively. As a group, the screening media recovered organisms in only 9%, 46%, and 78% of plates inoculated with low inocula of OXA-48, IMP, and NDM-1, respectively. This study suggested that ESBL media may be useful for screening patients with carbapenemase-producing organisms but would come at the cost of low specificity. Also, media designed to detect carbapenemase producers may have difficulty detecting isolates with low carbapenem MICs. False positives involving the growth of AmpC producers and *P. aeruginosa* were observed in both types of media. Because OXA-48 is particularly difficult to recover reliably from selective media due to weak hydrolysis of both cephalosporins and carbapenems, Nordmann et al. proposed a novel agar called SUPERCARBA with a Drigalski agar medium base, ertapenem (0.25 g/ml), zinc (to enhance expression of MBLs), and cloxacillin to inhibit ampC cephalosporinase. This medium was reported to detect OXA-48 consistently with inocula in the 10^1 to 10^2 range (76).

PHENOTYPIC CONFIRMATION OF ESBL AND CARBAPENEMASE PRODUCTION

Previously, CLSI and EUCAST recommended a two-step procedure for identification of ESBLs. It involved, first, selection of isolates that are likely to be ESBL producers based on oxyimino-β-lactam MICs, followed by inhibitor-based phenotypic confirmation. Selection of isolates required assessments of disk diffusion or MICs using broth microdilution (or agar dilution for EUCAST) with indicator oxyimino-β-lactams (typically cefotaxime and ceftazidime). Recommended phenotypic ESBL confirmatory methods harnessed the ability of clavulanic acid to bind irreversibly to the serine residue in the active site of an ESBL, disabling the enzyme's ability to bind and hydrolyze β-lactam substrates. Antibiotic susceptibility testing of an ESBL isolate with an oxyimino-β-lactam (typically cefotaxime and ceftazidime) alone and in the presence of clavulanic acid should therefore produce a decrease in the MIC or an increase in the diameter of the zone of inhibition in an ESBL-producing organism. If pursuing phenotypic confirmation of ESBL production, both CLSI and EUCAST recommend the use of broth microdilution and disk diffusion techniques. EUCAST also supports gradient testing (i.e., E-test) and the "double disk synergy" procedure (77, 78).

Unfortunately, the "screen and confirm" approach to ESBL identification and confirmation had some challenges. First, *Enterobacteriaceae* that harbor both ESBL and AmpC determinants have a propensity toward lower MICs for the oxyimino-β-lactams that may not meet ESBL "screening" criteria (79). Second, because AmpC is not inhibited by clavulanic acid, these isolates may lead to false-negative confirmatory ESBL test results. The addition of cefepime (which is stable against AmpC hydrolysis) to cefotaxime and ceftazidime in confirmatory testing can help to overcome this problem because it is stable in the presence of AmpC. Alternatively, agar plates used for disk diffusion and double disk synergy testing can be supplemented with cloxacillin to suppress AmpC β-lactamase activity. Finally, commercially available disks or tabs with ceftazidime and cefotaxime with both clavulanic acid and cloxacillin, such as those included in the Total ESBL + AmpC Confirm Kit (Rosco Diagnostica, Taastrup, Denmark), can be useful (77, 78). The AmpC + ESBL Detection Set (MAST Group, Merseyside, United Kingdom) uses a similar format, but the ESBL and AmpC inhibitors are not disclosed. Finally, false-positive ESBL confirmatory tests are well documented. KPC can be inhibited variably by clavulanic acid and may result in a false-positive result. *K. oxytoca* that harbors chromosomally mediated K1 β-lactamase may also lead to positive results, although this phenomenon can be easily recognized by the enhanced hydrolysis of cefotaxime over ceftazidime.

In 2010, CLSI therefore lowered cefotaxime, ceftriaxone, and ceftazidime MIC interpretive breakpoints, recommended that cephalosporin MIC interpretations be reported "as found," and ceased to recommend routine ESBL screening and confirmatory procedures if the revised breakpoints were implemented (80). EUCAST made similar adjustments in 2011 (81). The rationale presented included emergence of ESBLs with MICs in the susceptible range (79), PK/PD data suggesting that the optimal time above MIC of 40 to 50% of the dosage interval can be more reliably achieved with lowered interpretive breakpoints (82), animal model data suggesting superior correlation between MIC and outcome compared to resistance mechanisms (83), reduction of reporting delay while awaiting ESBL confirmatory testing results, and simplification of laboratory testing (80).

Automated antimicrobial susceptibility testing systems have also been evaluated for their ability to identify ESBLs. Studies using molecular testing to determine reference standard ESBL status with $bla_{\text{CTX-M}}$, bla_{TEM}, and bla_{SHV} PCR assays have provided mixed results. The BD Phoenix ESBL test (BD Diagnostics, Sparks, MD) appears to perform with sensitivity and specificity >95%, but in a study involving the testing of 1,129 *Enterobacteriaceae* isolates, Spanu et al. reported false negatives in six SHV-2- or SHV-5-producing isolates. Two false positives were also noted in isolates with *K. pneumoniae* and *E. coli* hyperproducing SHV-1 (84). Similarly, false-negative results have been reported in both the Vitek 2 and MicroScan Walkaway systems, with TEM-, SHV-, and CTX-M ESBL producing isolates, while AmpC β-lactamase production has been associated with false positives (84–86).

Identification and phenotypic confirmation of carbapenemase production are also challenged by highly variable inhibition by β-lactamase inhibitors and widely ranging carbapenem MICs. The latter is a problem particularly in NDM, VIM, and OXA-48 isolates (74). Like ESBL reporting, both CLSI and EUCAST recommend reporting carbapenem MIC interpretations as found using interpretive breakpoints that have been successively lowered since 2010 (80, 81).

EUCAST supports the use of inhibitor-based disk diffusion methodology to detect carbapenemase production: a meropenem disk (10 μg) with and without an EDTA or

dipicolinic acid disk, phenylboronic acid or aminophenylboronic acid disk (which inhibits KPC), a disk with both dipicolinic acid and aminophenylboronic acid (to inhibit both MBL and KPC when they are both expressed), and cloxacillin (to inhibit AmpC β-lactamases, which are also inhibited by boronic acid derivatives) (78). There are no known inhibitors for OXA-48. The KPC+MBL Confirm Kit (Rosco Diagnostica) is a commercially available kit that provides all of the above components. The Carbapenemase Detection Set (D70C; MAST Group) is also available in a similar format, but as with other MAST Group products, the identity of the KPC, MBL, and AmpC inhibitors is not disclosed. In two evaluations, the inhibitor approach confirmed KPC and NDM production nearly 100% of the time in previously characterized isolates (87, 88) but did not detect OXA-48 well. In one study, both the Rosco and MAST Group kits had difficulty detecting VIM and IMP production also (88).

The Carba NP is an alternative phenotypic method of demonstrating carbapenemase production that was originally described using a microwell format. Bacterial lysate is added to a solution containing phenol red, zinc, and imipenem monohydrate. Hydrolysis of imipenem leads to an acidic pH change, which produces a color change from red to yellow. This assay was originally reported to perform sensitivity and specificity of 100% (89–91). Tijet et al. performed the CarbaNP on 74 previously characterized carbapenemase producers and reported 29 false negatives. With a heavier inoculum than the originally recommended 10-μl loopful, 15 additional OXA-48 isolates that initially tested negative tested positive. In their analysis, the authors therefore recommended use of a heavier bacterial inoculum (92). Finally, the modified cloverleaf (Hodge) test, which was previously recommended by CLSI, has satisfactory sensitivity for the detection of KPC production but may test false negative in MBLs and OXA-48 isolates (87, 88). Also, false-positive tests are common in isolates producing AmpC β-lactamase with concurrent impermeability related to porin mutations (87). CLSI no longer recommends it for routine testing.

MOLECULAR DETECTION METHODS FOR MDR-GNR SURVEILLANCE

Laboratory-Developed Tests

PCR-based detection of TEM- and SHV-based ESBLs can be challenging because they resulted from single-point mutations of TEM-1, TEM-2, or SHV-1 β-lactamases. A practical approach for distinguishing ESBL and non-ESBL TEM- and SHV-β-lactamases is to perform DNA PCR using universal bla_{TEM} and bla_{SHV} oligonucleotide primers followed by Sanger sequencing of the amplicons. Various primer sets have been published for universal bla_{TEM} and bla_{SHV} detection using simplex or multiplex, conventional or real-time PCR approaches (93–95). The CTX-M family of ESBLs can be accurately detected using a single universal bla_{CTX-M} PCR reaction (96–101). Differentiation between the CTX-M groups (1, 2, 8, 9, and 25) has been described using conventional (99) and real-time PCR (101) approaches.

The emergence of KPC-producing and NDM-producing organisms has provided a powerful impetus for the development of molecular assays that detect bla_{KPC}, bla_{NDM}, bla_{VIM} bla_{IMP}, bla_{OXA-48}, and other carbapenemase determinants. Real-time PCR assays that detect bla_{KPC} have been described as stand-alone tests, but determination of KPC gene variants (KPC 2 to 11) has been achieved using restriction enzyme digestion of a bla_{KPC} PCR reaction product or multiplex PCR (102, 103). Similarly, real-time simplex PCR assays have been described for detection of bla_{NDM} (104, 105). Various duplex PCR assays that detect bla_{KPC} bla_{NDM} have also been described, but worldwide dissemination of various carbapenemase determinants has precipitated the development of more extensive multiplex PCR assays in both conventional and real-time formats (106–111). Finally, direct detection of carbapenemase determinants from seeded stool samples (112, 113); perianal, perirectal, and rectal swabs (114–116); and nasal swabs (117) has been described. Vasoo et al. used a duplex PCR to detect bla_{KPC} and bla_{NDM} on surveillance perianal/perirectal swabs and compared detection when used directly from the swab or following incubation in enrichment broth. They reported no difference in detection (116).

Commercial Molecular Assays for ESBL and Carbapenemase Determinants

Hyplex ID Systems

The Hyplex ESBL ID and SuperBug ID products (Amplex Biosystems GmbH, Gars, Germany) use multiplex real-time PCR followed by amplicon detection through hybridization with reverse probes that are immobilized on ELISA-microwell plates. Hybridization complexes are then detected visually using peroxidase-conjugated antibodies. Testing requires 4.5 to 6 h. The Hyplex ESBL ID detects bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{OXA}; SuperBug ID detects bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}. Ambretti et al. tested 130 previously characterized clinical isolates. SuperBug detected 70/70 isolates with bla_{KPC}, bla_{NDM}, and bla_{VIM} (no isolates with bla_{IMP} or bla_{OXA-48} were tested). Two isolates harboring ESBLs tested false positive for bla_{KPC} but had "borderline" optical density values that were close to the 0.4 cutoff (118). Kaase et al. also reported an *E. coli* isolate with bla_{GES} that tested borderline for bla_{OXA-48}. Also, 3/3 isolates with bla_{IMP} tested falsely negative by SuperBug ID (119). Finally, in a study based in Greece, Avlami et al. used the Hyperplex MBL ID (which was later expanded and replaced by the SuperBug assay) to perform direct testing on blood ($n = 90$), urine ($n = 60$), respiratory specimens ($n = 85$), and wound swabs/pus ($n = 91$). Hyperplex MBL ID detected bla_{VIM} in 72/73 cultures that grew bla_{VIM} but detected an additional 5 that did not grow in culture (120). The authors felt that isolate selection for inclusion in the study could have accounted for these results.

Cepheid Xpert MDRO

The Xpert MDRO assay (Cepheid) is performed on the GeneXpert instrument, an automated system that performs DNA extraction, real-time PCR, and detection. The Xpert MDRO assay detects bla_{KPC}, bla_{NDM}, and bla_{VIM} and requires 47 minutes to complete after buffered specimen is added to the cartridge. Tenover et al. tested 328 rectal swabs from Spain and New York (121). Ten tested positive for bla_{VIM}, and nine of these grew bla_{VIM} isolates in culture. The tenth was deemed a false-positive result. Forty-three specimens tested positive for bla_{KPC}; 40 grew in culture and 2 tested positive for bla_{KPC} by sequencing of enrichment broth that was inoculated in parallel to standard culture and Xpert testing. The final isolate was negative either on standard culture or from enrichment broth.

Sensitivities of Xpert MDRO for bla_{VIM} and for bla_{KPC} were 100% (95% confidence interval: 72 to 100%) and 100% (93 to 100%), respectively, and specificities were 99% (98 to 100%) and 99% (97 to 100%). Sixty-six simulated rectal swabs were prepared using stool matrix spiked with *K. pneumoniae* harboring bla_{NDM} at concentrations ranging from 150 to 1,800 CFU/ml. Sixty-five of the 66 tested positive for bla_{NDM} by Xpert MDRO. Data is emerging for the Xpert Carba-R assay, which is marketed as a CE IVD product and detects bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP-1}, and bla_{OXA-48}.

NucliSENS EasyQ KPC

The EasyQ KPC assay (bioMérieux) is performed on the NucliSENS EasyQ instrument, an automated system that performs real-time nucleic acid sequence-based amplification and target detection using molecular beacons. Because DNA extraction is required prior to amplification by the instrument, total testing can require up to 5 h. McEwan et al. used the NucliSENS easyMAG instrument to extract nucleic acid from 806 specimens (619 rectal swabs and 187 stools) followed by nucleic acid sequence-based amplification on the NucliSENS Easy Q instrument (122). Twenty-eight specimens tested positive by both EasyQ KPC and direct culture on ChromID ESBL agar. EasyQ KPC tested negative in two specimens that grew on culture and tested positive for bla_{KPC} using an alternative bla_{KPC} PCR assay, but one tested positive upon repeat. Nine specimens tested positive by EasyQ KPC but negative by culture. These were not resolved using alternative molecular methods. Spanu et al. tested 300 previously characterized isolates, 111 of which were KPC (123). EasyQ KPC detected 100% of KPC-producing isolates and did not test falsely positive in the 222 non-KPC-producing isolates.

Check Direct CPE and Check-MDR Real-Time Assays

Check-MDR ESBL and Check-MDR Carba Real-Time PCR (Check Points BV, Wageningen, The Netherlands) can be performed on a variety of real-time platforms including ABI 7500 (Life Technologies, Grand Island, NY), CFX96 (Bio-rad, Hercules, CA), Lightcycler 480 (Roche Diagnostics, Indianapolis, IN), m200rt (Abbott, Abbott Park, IL), and the SmartCycler II (Cepheid). For DNA target detection, these assays rely on two specific oligonucleotide ligation probes that are joined by a DNA ligase only when they match exactly with the target DNA. This approach is used to enhance specificity. The DNA probes also contain two universal primer binding sites as well as a binding site for the molecular beacon required for target detection. The Check-MDR ESBL detects ESBL CTX-M-1, CTX-M-2, and CTX-M-9 groups and the most common ESBL mutations (TEM-E104K, TEM-R164S, and SHV-G238S). Check-MDR Carba detects bla_{KPC}, bla_{NDM}, bla_{IMP}, bla_{VIM}, and bla_{OXA-48}. Both assays require 4.5 h to complete testing in addition to the time required for DNA extraction. They are appropriate for bacterial colony testing only. Cuzon et al. tested 90 previously characterized carbapenemase-producing organisms with bla_{KPC} ($n = 14$), bla_{NDM} ($n = 22$), bla_{IMP} ($n = 11$), bla_{VIM} ($n = 21$), and bla_{OXA} ($n = 21$) and 93 isolates without carbapenemase determinants and reported sensitivity and specificity of 100% for each target (124). Willemsen et al. tested 489 previously characterized isolates, 355 of which were ESBL producing and 134 of which were not (125). Discordant results were reported in 30 isolates. The Check Points CT 103 microarray was used to adjudicate discrepant results. PCR and Check Points CT 103 results were concordant in 26/30 of these cases. In the remaining four isolates, CTX-M1 and unusual variants of TEM and SHV mutations were associated with false-negative results.

In contrast to the Check-MDR ESBL and Carba assays, the Check Direct ESBL and CPE products were designed for direct testing of perianal and rectal swab specimens. The product is compatible with ABI 7500, CFX96, the LightCycler 480 system (Roche), and the Rotor-Gene Q (Qiagen, Valencia, CA) instruments as well as the BD MAX platform. Swabs are collected and transported in appropriate molecular transport medium. Each well of a NucliSENS easyMAG automated DNA extraction cartridge receives 200 µl of fluid with 5 µl of internal control, and this is followed by DNA extraction. In the Check Direct ESBL product, primers and probes required for real-time PCR reactions are provided to detect CTX-M-1, CTX-M-2, and CTX-M-9 groups and ESBL mutations SHV-G238S and SHV-G238S + E240K. bla_{KPC}, bla_{NDM}, bla_{VIM}, and bla_{OXA-48} are detected by Check Direct CPE. Testing requires 2 h. Nijhuis et al. spiked stool matrix with 62 previously characterized carbapenemase-producing isolates that represented the assay targets and 21 non-carbapenemase-producers (126). Analytical specificity was 100%. The limit of detection was reported as ranging from 2×10^2 to 2×10^4–2×10^5 CFU/ml.

Check-MDR CT Assays

Check Points BV also produces the Check-MDR CT 101, 102, and 103 assays, which use a microarray format. Check-MDR CT 101 is designed to detect bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and plasmid-mediated AmpC determinants including $bla_{CMY-1/MOX}$, bla_{ACC}, bla_{DHA}, $bla_{ACT/MIR}$, and $bla_{CMY,\ II/FOX}$. CT 102 detects bla_{KPC}, bla_{NDM}, bla_{VIM}, bla_{IMP}, and bla_{OXA-48}, in addition to the ESBL determinants from Check-MDR CT 101. CT 103 detects all of the ESBL, AmpC, and carbapenemase targets from the CT 101 and CT 102 microarrays. The Check KPC/ESBL detected bla_{TEM}, bla_{SHV}, bla_{CTX-M}, and bla_{KPC} but was expanded and replaced by CT 102. The Check-MDR CT assays also rely on ligation-mediated amplification. Ligated probes have universal primer sites and binding sites that allow hybridization to specific positions on a microarray located on CP array tubes. The tubes are inserted in a Check Points Tube Reader. Array images are generated and are analyzed using dedicated software.

The performance of the Check-MDR CT 102 has been described in studies using previously characterized bacterial isolates (127, 128). Occasional false-negative results for isolates harboring bla_{KPC} and bla_{OXA-48} have been reported. An AmpC β-lactamase-producing organism with no carbapenemase production tested false positive for bla_{VIM}. Similar studies describe the performance of Check-MDR CT 103 (129, 130). Similar to the Check-MDR-CT 102, a false-negative result was reported in an isolate of *K. pneumoniae* with bla_{OXA-48} (130). Also, while sensitivity and specificity for ESBL determinants bla_{TEM}, bla_{SHV}, and bla_{CTX-M} have been reported to be satisfactory, discordance between gold standard PCR and Check-MDR CT 103 has been noted for CTX-M subgroup $bla_{CTX-M-15}$ (131).

MDR-GNRs are highly concerning due to the limited treatment options available to patients with infections requiring treatment. Both sensitivity and specificity can be problematic in culture-based screening for MDR-GNR.

TABLE 1 Advantages and disadvantages of culture-based versus molecular-based surveillance methods

Organism	Culture-based screening		Phenotypic confirmation		Molecular-based screening and confirmation	
	Advantages	Disadvantages	Advantages	Disadvantages	Advantages	Disadvantages
All	Specialized skills are not required Bacterial isolates available for molecular typing and susceptibility testing Detection of emerging mutants of existing resistance determinants or novel families	TAT of >24 h common	Specialized skills are not required Provides some epidemiologic data for infection control purposes	Lengthens TAT unless integrated into routine susceptibility testing	May reduce TAT May increase sensitivity	May be costly Laboratory-developed tests (LDTs): require specialized skills Commercial assays: targets are proprietary, and commercial response to emerging mutants may be delayed
MRSA		Broth enrichment step required to achieve acceptable sensitivity	PBP2a detection is generally rapid, sensitive, and specific	Monoclonal antibody tests for PBP2a may have poor sensitivity in small colony variants and isolates with weak PBP2a expression	Highly automated commercial assays are available	False positives due to "*mec*A dropouts" False negatives in SCC*mec* and *mec*A mutants
VRE		Lower limit of detection makes it difficult to distinguish new acquisition from "unmasking" of carriage after antibiotic exposure	Not applicable	Not applicable	Highly automated commercial assays are available	PPV for *van*B-bearing *Enterococcus* spp. is poor due to *van*B carried by intestinal anaerobes
ESBL		Breakthough of AmpC and K1 β-lactamase producers reduces specificity of selective media More labor required for confirmatory testing Broth enrichment step required to achieve acceptable sensitivity	May perform well if AmpC suppression or detection is incorporated into testing	False negatives in isolates with both AmpC and ESBL False positives with KPC and K1 β-lactamase producers Automated expert systems can be inaccurate	Improved specificity compared to phenotypic confirmatory methods Microarrays allow efficient confirmation of TEM- and SHV-derived ESBLs with microarrays	Few assays are designed to detect targets directly from specimens PCR detection of TEM- and SHV-derived ESBL requires sequencing Microarray assays are costly
Carbapenemase producers		Reduced sensitivity of some carbapenemase-selective agar media for weak carbapenemase producers (e.g., OXA-48) Isolates with other carbapenem resistance mechanisms may grow through (e.g., AmpC or ESBL plus porin mutation)	Performance of CarbaNP assay is promising	Reduced sensitivity of inhibitor-based methods and possibly the CARBA NP with weak carbapenemase producers OXA-45 has no known inhibitors CarbaNP data performance is mostly based on LDT data. Data supporting commercial products are sparse	Improved specificity compared to phenotypic confirmatory methods Swifter detection of multiple carbapenemase determinants simultaneously Provides epidemiologic data for infection control purposes	Data are still sparse for commercial assays designed to detect targets directly from specimens Microarray assays are costly

Carbapenemases with weak hydrolytic activity against the carbapenems (e.g., OXA-48 and some strains of NDM, VIM, and IMP) can be difficult to recover from commercially available media. The burden of confirmatory testing can be particularly significant if using selective media with suboptimal specificity. Phenotypic ESBL and carbapenemase confirmatory testing methods are associated with various performance-related challenges. With an optimized work flow, molecular diagnostics performed directly on patient specimens may provide an alternative screening method with shorter TAT, improved sensitivity and specificity, and valuable epidemiological data needed to understand health care-based transmission patterns. Until recently, molecular detection of MDR-GNR determinants was largely limited to reference laboratories, but commercially available molecular diagnostic platforms are beginning to emerge, making such testing more widely accessible.

WILL ADOPTING MOLECULAR MDRO DETECTION ASSAYS REDUCE MDRO INFECTION RATES?

As the technology for molecular detection of MDRO carriers advances, the most important question is whether such technology will be helpful in reducing MDRO transmission and infection rates. From a technical, performance-based perspective, molecular detection methods have made considerable progress, particularly in the realm of MDR-GNRs. However, as we have discussed, they are presently not without weaknesses, as summarized in Table 1.

From the broader, hospital system-based perspective, the clinical impact of active MDRO surveillance rests upon the assumption that detecting asymptomatic MDRO carriers guides interventions that will either prevent MDRO transmission (e.g., contact precautions, cohorting of colonized and infected patients) or prevent infection among carriers (e.g., decolonization). When evaluating literature regarding the utility of active MDRO surveillance, one must distinguish between outbreak and endemic settings. There are many reports of active surveillance being used to help control MDRO outbreaks and/or reduce MDRO infection rates when such surveillance is introduced in response to elevated infection rates (6, 8). In these situations, active surveillance is generally instituted as just one of several interventions, and thus the incremental contribution of the detection of MDRO carriers is not quantifiable. However, it makes sense in outbreak settings to quickly define the potential reservoir of carriage and infection with the outbreak pathogen and to use active surveillance to guide other interventions such as patient isolation and cohorting. Thus, the CDC recommends active MDRO surveillance as one of the tier 2 measures to be implemented during outbreaks or when rates of MDRO infection are not declining in response to other infection prevention measures (6).

In contrast, published data to support the use of active MDRO surveillance in the endemic setting are weak and conflicting, with several recent well-designed trials demonstrating no impact of active MDRO surveillance (132–135). A pragmatic cluster-randomized trial (REDUCE MRSA) demonstrated little impact of active MRSA screening and isolation when compared to universal decolonization approaches in intensive care unit patients (135). Additionally, the MOSAR group recently reported results from a three-phase time-series and multicenter cluster randomized intensive care unit trial, finding that in the setting of sustained high levels of hand hygiene and chlorhexidine bathing, active surveillance (for MRSA, VRE, and MDR-GNR) and isolation did not reduce MDRO acquisition rates, regardless of whether rapid molecular or conventional testing was performed (134).

Notably, proponents of active MDRO surveillance have pointed to the slow TAT of MDRO detection test results for the failure of some studies to demonstrate effectiveness (136, 138). Indeed, more studies are needed to determine the impact of faster TATs for MDRO detection. We need studies that compare rapid (<24 h TAT) MDRO testing to slower (culture-based) testing, using concurrent control groups and measuring meaningful outcomes (MDRO transmission/acquisition and infection events). Such studies have been performed almost exclusively for MRSA testing

TABLE 2 Summary of studies assessing impact of rapid versus culture-based detection of MRSA carriage[a]

Study	Design	TAT difference	MRSA outcome	Major limitations
Aldeyab (140)	Nonrandomized cluster crossover trial	PCR: 19 h Culture: 52 h	No difference in event rates (acquisition + infection)	Long PCR TAT Not randomized
Hardy (141)	Nonrandomized cluster crossover trial	PCR: 22 h Culture: 79 h	Reduced acquisition rate in PCR group (0.29 vs. 0.41 per 100 bed days)	Long PCR TAT Not randomized 2-fold more unscreened in culture arm 71% decolonized in PCR arm vs. 41% in culture arm Only 17% of carriers placed in isolation rooms
Jeyaratnam (143)	Cluster-randomized crossover trial	PCR: 22 h Culture: 46 h	No difference in acquisition or infection rates	Long PCR TAT
Roisin (143)	Cluster-randomized crossover trial	PCR: 25 h Culture: 96 h	No difference in acquisition rate	Long PCR TAT Long delay between analytic TAT and actual TAT for PCR (11 vs. 25 h)

[a]Limited to those using concurrent control groups and reporting MRSA infection or colonization outcomes.

and are summarized in two recent systematic reviews (138, 139). Both reviews found the existing literature to be scant and the individual studies to have important limitations. As a result, rapid MRSA detection tests have not been convincingly demonstrated to reduce MRSA transmission or infection rates when compared with culture-based screening. As summarized in Table 2, however, some of the best-designed studies were only able to reduce the overall TAT of the rapid (PCR) tests to 19 to 25 h (140–143). This reiterates the challenge described earlier, of setting up a system to expedite all aspects of testing, including the extremely important postanalytic arm of notification and intervention.

In addition to comparative clinical studies, several groups have performed modeling studies to determine the likely impact of rapid MRSA testing on outcomes (144–146) and found that the improvement in TAT compared with culture methods provided little benefit at substantially increased cost, particularly if applied universally (i.e., to all admitted patients).

Thus, at the time of this writing there is not sufficient evidence to conclude that investments in routine rapid MDRO testing, in the endemic setting, will reduce MDRO transmission or infection rates. However, there is reason to believe that moving to rapid testing can reduce the number of contact precaution (isolation) days, particularly under policies that involve preemptive isolation either of all admitted patients or of those with a history of MDRO carriage (147). Given the negative consequences that contact precautions can have on patient satisfaction, patient safety, and cost of care (148), this impact of rapid MDRO detection should be considered in hospitals that employ preemptive contact precautions while awaiting MDRO surveillance culture results. In a randomized controlled trial, Shenoy et al. assigned patients with a history of MRSA infection or colonization to the local standard of care for discontinuation of contact precautions (three negative surveillance MRSA nasal cultures) or real-time PCR on the Cepheid GeneXpert platform. Contact precautions were discontinued more often, and a there was a significant reduction in contact precaution days in the PCR group (149).

Since MRSA is endemic in health care settings worldwide, the MRSA testing literature reviewed above does not address the potential impact of early detection of an MDRO that has not yet been detected in a given locale. The challenges of detecting epidemiologically important MDR-GNRs with available culture-based phenotypic methods make it likely that a newly emerging MDR-GNR could spread within a facility before it is discovered. Since certain MDR-GNRs, such as KPC-producing *K. pneumoniae*, can cause explosive, deadly, and difficult-to-control outbreaks, any test that could more rapidly detect carriers would be important to provide an early warning, in addition to the value of a subsequent outbreak response. Thus, a widely available molecular method that rapidly and accurately detects epidemiologically important MDR-GNRs will be an important step forward for clinical laboratories and infection prevention programs.

FUTURE DIRECTIONS

Due to many of the challenges we have discussed, rapid molecular detection of MDRO carriage remains in its infancy, with assays only widely adopted for MRSA. Future work will bring improved rapid testing options for other emerging MDROs, including new MDR-GNRs that are increasingly important public health threats. Those tests that have good performance characteristics will be useful adjuncts during management of MDRO outbreaks or when rates of a specific MDRO are elevated and not responding to standard MDRO prevention approaches (6).

Most importantly, well-designed clinical trials should be performed to help determine how these tests should be incorporated into MDRO prevention efforts, especially in endemic or nonoutbreak settings. Because endemic MDRO infection and colonization rates are associated with so many factors, detecting any incremental benefit of rapid detection of MDRO carriers requires large multicenter cluster-randomized trials. These trials are difficult to perform and resource-intensive. However, funding such studies is an essential step in determining how best to approach MDRO prevention well into the future.

Finally, we hope that the eventual increased availability of whole-genome sequencing (see chapter 3) will facilitate future MDRO surveillance and prevention efforts, in particular through earlier recognition of emerging MDROs that may be missed by commercial assays.

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Detection of Vancomycin-Resistant Enterococci

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18

BACKGROUND AND OVERVIEW

Vancomycin is a glycopeptide derived from soil bacteria that was authorized for use in 1958 for infections due to Gram-positive bacteria resistant to penicillin, erythromycin, and tetracycline. Toxicity led to its rapid replacement by methicillin in 1960; however, vancomycin use was again required by the 1970s for infections due to methicillin-resistant *Staphylococcus aureus* (MRSA) and toxigenic *Clostridium difficile*. In 1986, the first outbreak of enterococci with high-level resistance to vancomycin and teicoplanin was reported in the United Kingdom (1). A year later, infections due to vancomycin-resistant *Enterococcus faecalis* infections were identified in the United States (2, 3).

The rapid emergence of vancomycin-resistant enterococci (VRE) in the United States was fueled by the endemnicity of ampicillin-resistant *Enterococcus faecium* clonal complex 17 (CC17) in many American hospitals (4), a lack of awareness of the importance of patient colonization in propagating outbreaks, the absence of sensitive screening methods for detecting colonized patients (5), and an incomplete knowledge of the mode of transmission (6). In the late 1990s, enterococci of CC17 arrived in Europe (7–11), and hospital VRE outbreaks followed, until to date they are globally prevalent (12–16). VRE are adept at acquiring operons and readily share these *in vivo* with receptive bacterial strains encountered in the intestine or hospital environment, such that outbreaks are often polyclonal. The *vanA* and *vanB* operons are the most successful VRE genotypes, but new genotypes continue to emerge. Fortunately, transfer of *van* operons to *C. difficile* and to MRSA has remained rare, and even fully functional *vanG* vancomycin resistance genes in *C. difficile* appear to be insufficient to mediate high-level resistance (17, 18).

VRE are formidable masters of environmental survival (19), a feature that contributes enormously to their ability to cause and sustain outbreaks. Patient-to-patient transmission occurs via both health care workers' hands and fomite contamination, and transmission between patients is remarkably efficient (6, 20). Ensuring that medical equipment and surfaces are VRE free is paramount, as is prompt implementation of additional precautions for VRE-colonized patients, screening of contacts, hand hygiene, and antimicrobial stewardship (6, 20). Clinical microbiology laboratory support is critical in promptly identifying patients newly colonized or infected with VRE.

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In this context, it is useful to understand that VRE success has also been partly due to inadequacies of detection methodology (5, 21–24) and/or failures in laboratory support. Microbiology laboratories need to continuously evaluate the performance characteristics of new screening tests against the need for speed, sensitivity, and specificity. They also need to be aware of the impact of the ongoing evolution of VRE and the appearance of new resistance determinants (25), some of which mediate only borderline resistance, which may make laboratory recognition of VRE more difficult (22). Continued glycopeptide use for a variety of infections sustains the selective pressure for VRE to emerge and spread. Laboratory failures have been attributed to mutations that affect PCR gene targets (21), heterogeneous expression (26, 27), inducible or delayed phenotypes (23), and low-density (5, 24) and/or low-level expression of resistance (23) such that strains appear susceptible or fail to be isolated on selective media (24, 28–30).

This chapter elucidates the basis for intrinsic and acquired glycopeptide resistance mechanisms as well as characteristics of the various genotypes and phenotypes found in enterococci. It discusses the relationships and shared genotypic origins as these pertain to VRE epidemiology. Against this backdrop, phenotypic and genotypic VRE detection methods applicable to clinical diagnostic microbiology laboratories will be reviewed, recognizing that the general trend for unusual variant strains is to be referred for detailed characterization to reference laboratories where expertise and advanced tools are available.

CELL WALL PEPTIDOGLYCAN SYNTHESIS AND VANCOMYCIN RESISTANCE

D-Alanyl:D-Alanine Precursors

Vancomycin is bactericidal only against Gram-positive genera that produce D-alanyl:D-alanine (D-Ala:D-Ala) ligases (*ddl*) (31). Chromosomally encoded *ddl* determinants yield species-specific enzymes active in the cytoplasm that add D-alanine to the C-terminal ends of UDP-MurNAc-tetrapeptides to synthesize pentapeptides, the essential building blocks of peptidoglycan cell walls. Pentapeptides ending in D-Ala:D-Ala are transported from the cytoplasm through the cell membrane and cross-linked by transglycosolation and transpeptidation reactions to form cell wall peptidoglycan. Vancomycin forms high-affinity hydrogen bond attachments to D-Ala:D-Ala and actively blocks transglycosolation, leading to loss of peptidoglycan integrity and cell death.

D-Alanyl:D-Serine Precursors

Enterococcus gallinarum and *Enterococcus casseliflavus* are common gut inhabitants that are much less pathogenic than *E. faecalis* and *E. faecium* (32). They differ from other enterococci in that they are capable of producing peptidoglycan precursors terminating in D-alanyl:D-serine (D-Ala:D-Ser) as well as those terminating in D-Ala:D-Ala (33). Production is mediated by intrinsic VanC-type operons (32). In the *vanC1*, *vanC2*, and *vanC3* operons that are associated with *E. gallinarum*, *E. casseliflavus*, and *Enterococcus flavescens*, respectively (34), the N-terminal loop of the membrane-bound sensor protein, $VanS_C$, protrudes through the cell membrane. When this sensor protein detects vancomycin, the histidine kinase cytosolic domain autophosphorylates, leading to phosphorylation of VanR and activation of the promoter (P_H) to transcribe *vanC-vanXY-vanT* (35) and produce peptidoglycan precursors terminating D-Ala:D-Ser (33), which have low affinity for vancomycin. This inducible ability to switch precursor types enables these enterococci to grow normally in the presence of vancomycin at concentrations up to16 mg/l without incurring a fitness cost (36). However, these species are not resistant to teicoplanin, they are rarely transmitted person to person, and their intrinsic VanC-type operons are nontransmissible, and for epidemiological purposes, species with these operons are not considered to be true VRE (32).

D-Alanyl:D-Lactate Precursors

Numerous environmental organisms have vancomycin resistance mechanisms that are similar to VRE. These organisms contain VanR/VanS regulatory systems and complex *van* operons that replace D-Ala:D-Ala with D-Ala:D-Lac pentapeptides. Since vancomycin cannot bind to D-Ala:D-Lac, cell wall integrity in such organisms is retained at vancomycin concentrations in excess of 800 mg/l. These organisms include *Streptomyces coelicolor*, *Paenibacillus thiaminolyticus* (whose *vanH-vanA-vanX* exhibit >90% homology with contemporary VRE), *Paenibacillus popilliae* (37, 38), *Paenibacillus apiarius*, and *Rhodococcus* species (39) with only slightly lower homology to *vanA* operons in VRE.

EPIDEMIOLOGY AND ORIGINS OF *VAN* OPERONS IN *E. FAECALIS* AND *E. FAECIUM*

Early evidence suggested that VRE emerged in response to years of use of a vancomycin analogue, avoparcin, as a growth promoter in the European food industry. The use of copper sulfate may also have contributed, because the copper resistance *tcrB* gene has been associated with *vanA* operons in porcine *E. faecium*. During the late 1980s, *E. faecium* containing *vanA* operons was found in food and food animals in many European countries (40–42), as well as in the feces of healthy people. It was postulated that, to survive avoparcin (43), animal enterococci had acquired protective gene clusters from inherently vancomycin-resistant enteric or environmental organisms. But because VRE were also identified from animal sources in the United States, where avoparcin had not been used, alternative mechanisms had to be considered. Inadvertent introduction of VRE from Europe in animals or humans is one potential explanation. Another is that vancomycin-resistant operons were acquired in enterococci in the United States from *P. popilliae*, which was intentionally seeded into soil in the eastern United States as a biopesticide to deter Japanese beetles (37). Sequence homology and similarity in gene organization between the *P. popilliae vanF* operon (38) and the acquired *vanA* and *vanB* VRE operons make this an attractive hypothesis.

Numerous groups have identified that as many as 50% of some human populations have anaerobic fecal bacteria which contain *vanA*, *vanB*, *vanD*, and *vanG* operons, raising another possibility—that the gut microbiome may form a reservoir for exchange of *van* operons (44, 45). While VRE strain differences in antibiograms (42), *purK* alleles (46), and Tn*1546* sequences (47) provide supportive evidence that there have been multiple introductions of *van* operons into VRE, these differences do not help in identifying specific sources. Although there may have been multiple introductions, all acquired operons were originally derived from only two operon groups (48), which can be distinguished on the basis of structure, orientation, and function of resistance genes. These conform to either *vanH-ligase-vanX*-type (as in *P. popilliae*) or to *ligase-vanXY-vanT*-type operons(35), the latter most resembling *vanC* type from *E. gallinarum* and related species (Fig. 1 and Fig. 2). This relatedness of *van* operons in *E. faecalis* and *E. faecium* to those in diverse genera explains why all PCR tests lack specificity for VRE when testing is performed on samples containing broad bacterial communities such as fecal flora (49).

VAN OPERONS ACQUIRED BY ENTEROCOCCI

Over the last 20 years, clinically relevant enterococcal species have acquired a myriad of different *van* operons (25, 48). These include the pentadepsipeptide-producers *vanA* (50), *vanB* (26, 51–55), *vanD* (56), and *vanM* and the D-serine-producers *vanE* (57), *vanG* (58), *vanL* (59), and *vanN* (60). Many variants have also been identified. To date, only *vanA* and *vanB* (61) and, more recently, *vanM* (62) have been disseminated widely in clinically relevant enterococci.

Figure 2 shows relationships between acquired and intrinsic operons from plausible representative progenitors. While each genotype is associated with a phenotype, mutations or insertional inactivation in regulatory genes commonly alter these profiles. Thus, *vanS* alterations in *vanA* operons can result in isolates with *vanB* phenotypes that are teicoplanin-susceptible (30), with *vanD* phenotypes that are teicoplanin-intermediate or low-level resistant (63, 64), or with apparently glycopeptide-susceptible phenotypes (29). Although rare, vancomycin dependency during therapy in enterococci (65) and staphylococci (66) has also been described. In *E. faecalis*, *E. faecium*, or *Enterococcus avium*, or in the vancomycin-resistant *Staphylococcus aureus* VRSA-9, where mechanisms were investigated, dependency was found to be due to impairment of chromosomal *ddl* after acquisition of *vanA* or *vanB* operons resulted in the cessation of intrinsic pentapeptide production. Withdrawal of vancomycin terminates induction of all pentadepsipeptide transcription, which terminates production of cell wall precursors and results in strain death.

ACQUIRED D-ALA:D-LAC OPERONS

vanA

vanA was the first acquired D-Ala:D-Ala operon to be characterized from an *Enterococcus* species, and its dissemination

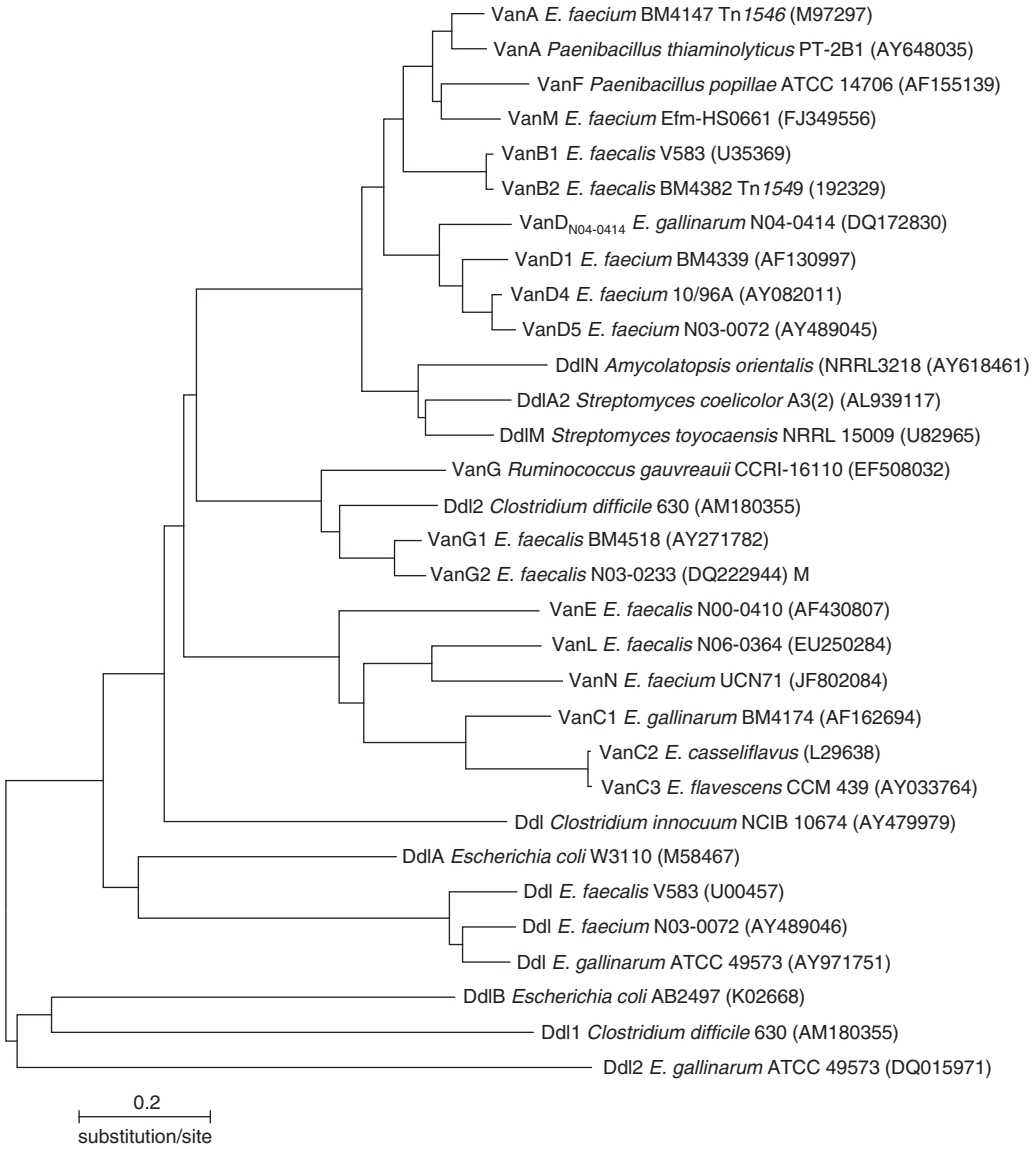

FIGURE 1 Phylogenetic tree constructed using MEGA6 (202) showing the relationships of D-Ala-D-Lac ligases, D-Ala-D-Ser ligases, and D-Ala-D-Ala ligases (Ddl). The proteins were aligned using ClustalW (203), and the tree was constructed with the neighbor-joining method (204) using 247 amino acids. The evolutionary distances were computed using the JTT matrix-based method (205), and the units are number of amino acid substitutions per site. Robustness of the data was tested by bootstrap analysis (206) using 1,000 replicates. GenBank accession numbers are shown in brackets. (Figure courtesy of David A. Boyd, National Microbiology Laboratory, Winnipeg, Canada.)

was found to be plasmid mediated (1, 50, 67). Isolates were highly resistant; MIC typically ranged from 64 mg/l to 1,000 mg/l for vancomycin and >32 mg/l for teicoplanin (68, 69). *E. faecium* dominates in *vanA* outbreaks (50), but the occasional *E. faecalis* (50, 70, 71) or other species may acquire the operon via horizontal plasmid transfer *in vivo* in the intestine (51). The *vanA* operon is an eight-gene cluster spanning ~11 kb within Tn*1546* (72). Tn*1546* is typically located in one of a variety of large conjugative multidrug-resistance plasmids, such as Inc18-related plasmids (73, 74) in *E. faecium* and pheromone-response plasmids in *E. faecalis*. *vanA* is controlled by a two-component OmpR-PhoB subclass system comprising a transcription response regulator, *vanR*, and a histidine kinase sensor, *vanS* (67, 75, 76). The N-terminal loop of VanS protrudes from the cytoplasm through the cell membrane (76). On vancomycin exposure (31, 67), the VanS histidine residue is induced to become autophosphorylated, which in turn phosphorylates VanR (77), leading to increased DNA binding of P_R and P_H promoters and initiation of *vanH-vanA-vanX* gene transcription; this in turn results in the production of pentadepsipeptides with D-Ala:D-Lac C-termini (76). *vanH* encodes dehydrogenation of pyruvate to produce the needed D-lactate substrate (78), and *vanX* encodes D,D-dipeptidases to destroy any D-Ala:D-Ala pentapeptides that continue to be produced. *vanA* also carries two accessory genes, *vanY*

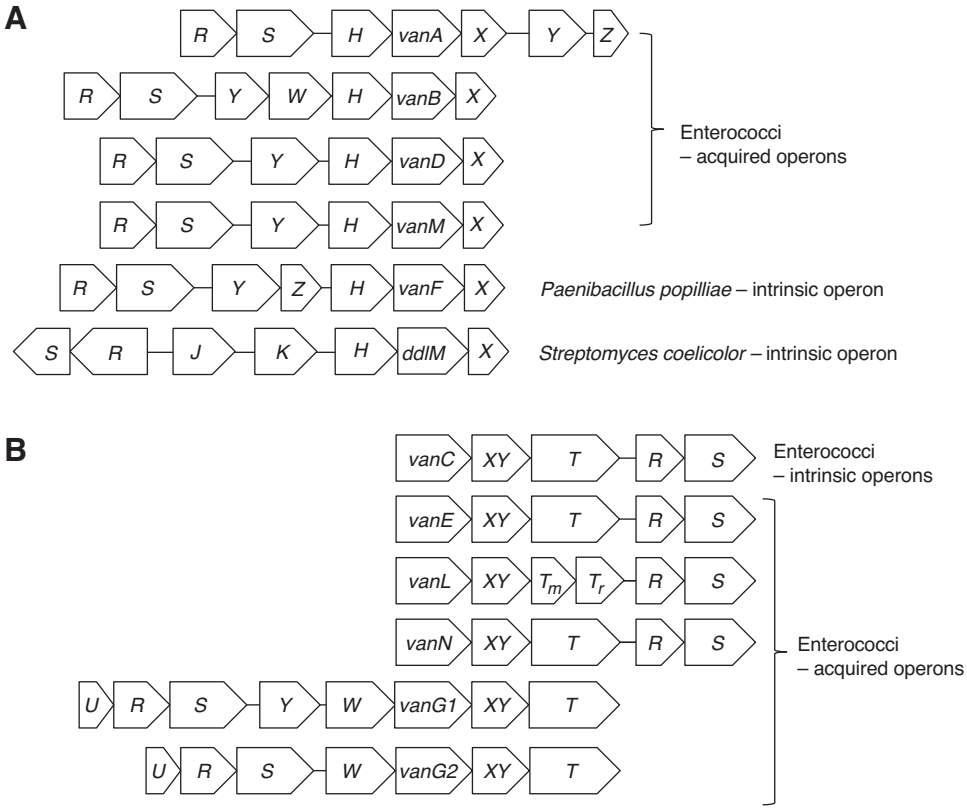

FIGURE 2 Schematic diagram of (A) the D-Ala-D-Lac operons found in enterococci and *Paenibacillus popilliae* and *Streptomyces coelicolor* and (B) the D-Ala-D-Ser operons found in enterococci. The operons are aligned at the D-Ala-D-Xxx ligase genes. Other genes are *vanU* (*U*), transcriptional activator; *vanR* (*R*), response regulator; *vanS* (*S*), sensor histidine kinase; *vanW* (*W*), unknown function; *vanH* (*H*), dehydrogenase; *vanT* (*T*), serine racemase; $vanT_m$ (T_m), serine racemase membrane domain; $vanT_r$ (T_r), serine racemase racemase domain; *vanY* (*Y*), carboxypeptidase; *vanX* (*X*), dipeptidase; *vanXY* (*XY*), dipeptidease/carboxypeptidase; *vanZ* (*Z*), unknown function but known to be involved in teicoplanin resistance; *vanK*, aminoacyl transferase; *vanJ*, teicoplanin resistance membrane protein. (Figure courtesy David A. Boyd, National Microbiology Laboratory, Winnipeg, Canada.)

and *vanZ* (79, 80). The D,D-carboxypeptidase mediated by *vanY* clears any late-stage D-Ala:D-Ala pentapeptide precursors that are missed by VanX D,D-dipeptidases (79, 81), while *vanZ* confers low-level teicoplanin resistance (76, 80). Because Tn*1546* forms stable associations with epidemic lineages, the most notable being *E. faecium* isolates belonging to multilocus sequence type CC17 with modified PBP5 mediating ampicillin resistance (82), transmission of the *vanA* operon is facilitated (4).

Recently, dissemination of *E. faecium* not recognized as *vanA*-containing VRE because of insertional inactivation of the *vanS* sensor gene has presented a new challenge (28). These strains may appear glycopeptide susceptible. However, *in vitro* studies have shown that these strains also readily switch from vancomycin-susceptible to vancomycin-resistant after exposure to subinhibitory doses of vancomycin (83), presumably mediated by an as yet unrecognized promoter. These variably expressing *vanA E. faecium* strains are particularly troubling because they are only detectable by PCR for *vanA*, which may enable them to spread unnoticed (28–30). Interestingly, while these strains are inhibited on most chromogenic agars, ~60% grow on Brilliance VRE agar (Oxoid, Nepean, Canada). However, their presence may be discounted as breakthrough because phenotypic methods will fail to confirm them as VRE (29). Other atypical phenotypes have also been described. In an outbreak in France, VRE with low-level resistance to both vancomycin and teicoplanin were found to contain a *vanA* operon in which the *vanY* accessory gene had been inactivated by the IS*16* element (63). Because the operon's DD-carboxypeptidase was not produced, the failure to eliminate late-stage D-Ala:D-Ala pentapeptides led to the unusual *vanD*-like phenotype (64). This potential for phenotype/genotype "mismatches" underscores the importance of the combined use of phenotypic and genotypic methods during investigations of VRE outbreaks (84).

In 2002, a swab from a foot ulcer of a Michigan patient yielded an MRSA isolate containing Tn*1546* complete with a *vanA* cluster (85, 86). It appeared to have transferred *in vivo* from *E. faecalis* to coisolated MRSA, creating the first clinical VRSA isolate. By 2007, eight similar events yielding VRSA had been identified in the United States (six of which were in Michigan), and such events have been identified in South Asia, Iran, and Brazil. This highlights the need for screening *S. aureus* for vancomycin resistance in patients or populations at risk of colonization with VRE.

The rarity of VRSA isolates appears to be due to plasmid incompatibilities and host barriers that inhibit gene transfer between unrelated species (87). In the first reported case of VRSA, acquisition of the *vanA* cluster by MRSA events required cooperation between different conjugative plasmids (73). A donor Inc18-like enterococcal plasmid acted as a suicide vector, releasing the transposon in the MRSA cytoplasm without itself surviving. The released transposon inserted into a recipient pSK41-like staphylococcal plasmid via "illegitimate recombination" (74). Although Inc18-like plasmids are commonly associated with Tn*1546* in *E. faecium*, *E. faecium* and *S. aureus* seldom share plasmids. VRE donor strains have been identified in only four VRSA cases and comprise three *E. faecalis* strains and one *E. avium* strain. Transposition seems to occur only during rare coincidental encounters between species of enterococci less commonly linked to VanA-Tn*1546* carriage, and only when MRSA carries compatible recipient plasmids.

Although all strains of VRSA expressed high-level vancomycin resistance, they became susceptible to cloxacillin when simultaneously exposed to oxacillin and vancomycin. When *vanA* pentadepsipeptides are cross-linked into the cell wall, the low-affinity PBP2a proteins are not used, and cloxacillin resistance is lost. Regardless, it is crucial for *vanA*-VRSA to be detected; it may only be a matter of time before these organisms acquire an escape from this paradoxical dead end.

vanB

First identified in *E. faecium* and *E. faecalis* in 1989, *vanB* was easily distinguished from *vanA* by lower levels of resistance to vancomycin (MIC: 16 to 64 mg/l) and susceptibility to teicoplanin (MIC: 0.064 to 1 mg/l) (53, 68, 69). Teicoplanin susceptibility is due to the lack of interaction between the drug and the $VanS_B$ sensor protein. However, development of teicoplanin resistance has been reported from rare cases where infections due to *vanB*-containing VRE were treated with teicoplanin (53). *vanB* genes share low sequence homologies with comparable *vanA* genes (31% ligase identity). They are similar in that they have operon regulation via a two-gene $vanR_B$-$vanS_B$ system (53), reduction of pyruvate to D-lactate by the $vanH_B$-dehydrogenase, ligation of D-lactate to C-termini of UDP-MurNAc-tetrapeptides by the *vanB* ligase, resulting in vancomycin-resistant pentadepsipeptide precursors, and concurrent hydrolysis of intrinsic D-Ala:D-Ala pentapeptides by $vanX_B$-encoded D,D-dipeptidases (52–54). However, the location and function of *vanB* accessory genes are distinct: $vanY_B$ and a gene of unknown function, *vanW*, are upstream of $vanH_B$-*vanB*-$vanX_B$ genes (52). In contrast to *vanA*, the $VanY_B$ D,D-carboxypeptidase in *vanB* is unable to eliminate late stage D-Ala:D-Ala pentapeptides and thus is susceptible to teicoplanin. These differences between *vanA* and *vanB* suggest distinct progenitors and separate evolution.

When the European *vanB* prototype strain (V583) (53) was compared to similar U.S. isolates circulating in 1993, a 3.6% sequence divergence between ligase genes was identified, and variants were designated *vanB2* (55). Another ligase variant exhibited 5% and 3.6% sequence divergences from the prototype *vanB* and *vanB2* ligases, respectively, and was designated *vanB3* (88). *vanB1* typically locates within the composite transposon Tn*1547* (52, 54, 89) or within a large chromosomally located serine recombinase-type conjugative element. By contrast, *vanB2* operons are mobilized by Tn*1549*/Tn*538* (26, 89, 90) tyrosine recombinase-type integrative conjugative elements (89), which usually integrate directly into the chromosome in the absence of plasmids. *vanB* strains of VRE have striking phenotypic variability in vancomycin resistance (vancomycin MIC: 6 to 256 mg/l), which is mediated by sequence variability in $vanS_B$ (75, 91, 92).

vanB outbreaks were first observed in Baltimore in the United States (68), but their current low prevalence in Canada (93, 94) and elsewhere in North America contrasts to their escalating "comeback" noted throughout Europe (8) from Spain (95, 96) though France (7) and Germany (92, 97) to Scandinavia (26, 90) and their predominance in VRE in Australia (44, 98, 99). However, it is important to note that there is no active surveillance system for VRE in the United States; thus, *vanB*-containing strains, which are less likely to grow on selective agar media, may go undetected in many hospitals if they are not causing overt infections. The European resurgence appears to be partly due to the acquisition by *E. faecium* CC17 of Tn*1549*/Tn*5382* harboring the *vanB2* operon (4, 9, 10, 61, 90, 96) and partly due to its mobilization in pRUM-like *repA*-containing conjugative megaplasmids equipped with *axe-txe* addiction systems (89, 97, 100). Addiction systems guarantee plasmid retention as the plasmid inserts genes into the chromosome that encode toxins lethal to the host if the plasmid-mediated antitoxin mechanism is lost. These strains often also encode virulence determinants (i.e., *hyl*, *esp*, pilus-encoding genes) to promote adherence and intestinal colonization (9, 101, 102).

As noted above, the rarity of *vanB*-containing VRE in North America may also be due in part to failure of detection. Vancomycin MICs in some *vanB* lineages commonly fall just below their established categorical threshold for resistance (91, 103). Detection also fails due to the continued use of formulations of VRE-selective agars that are insensitive (Table 1). In addition, some molecular assays (Table 2) detect *vanA* and *vanB* genes, but in some countries there is no reporting of *vanB*-positive results from those kits (i.e., BD GeneOhm VanR and Cepheid Xpert assays in the United States); this leads to the perception of a zero prevalence of *vanB* in enterococci in those regions. To further complicate VRE management and control, many *vanB2* operons have been found in fecal flora other than enterococci (e.g., *Clostridium* species) (45, 104–106); thus, it is not always possible to recover VRE from culture in PCR-positive specimens (49, 107, 108)—hence the reluctance to report their presence. However, recovery failures have since been additionally attributed to low densities in fecal flora (24), poor sensitivity of automated susceptibility testing (22), use of overly selective chromogenic agar formulations (109–115), and medium variations (116) in commercially prepared CLSI vancomycin screen agars used in the screening of clinically significant *E. faecium* and *E. faecalis* isolates (117).

vanB-containing VRE can be ruled out in PCR-positive specimens by culturing the specimens in nonselective brain heart infusion broth enrichment prior to extended incubation (36 to 48 h) on selective agars proven to support VRE with low vancomycin MIC (112, 113). The significance of PCR-detected non-VRE *vanB* in human gut microbiota remains unclear: while it has been difficult to identify their source by selective culture, functional operons have been identified in many different gut anaerobes (39, 44, 98, 104, 107, 108, 118). Both *vanB* VRE and *vanB* non-VRE prevalence appear to vary greatly in

TABLE 1 Selected recent and/or comprehensive evaluations of differential agar methods for detecting vancomycin resistance from specimens or bacterial isolates

Detection method (acronym; vendor), complexity of test	Reference	Comparator method(s)	Specimen types	Total specimens	No. *vanA* (A), *vanB* (B), other *van* genotype(s) detected/present	Sensitivity/ Specificity[a] (%/%)	Result TAT[e]
*m*Enterococcus (EV6; Difco BD) High	113	BEAV6, BVRE, BMD[a], V*Select*, MET[b], LC-VRE[c], cPCR/ sequencing	Rectal swab/stool	3,000	A: 105/126; B: 8/15	80.1/92.4	24 h
					A: 121/126; B: 15/15	96.5/83.5	48 h
					A: 125/126, B: 15/15	99.3/78.3	72 h
	112	As above	Isolates	441	At 72h: A:75/75; B: 36/36	60.8/81.2	24 h
					B1: 42/42; B2/3: 24/24	87.3/75.4	48 h
					D: 3/3; E: 4/18; L: 1/3; M: 3/3	92.2/64.7	72 h
Bile Esculin Azide (BEAV6; Oxoid) High	113	EV, BMD[a], BVRE, V*Select*, MET[b], LC-VRE[c]	Rectal swab/stool	3,000	A: 103/126; B: 8/15	78.7/84.9	24 h
					A: 109/126; B: 15/15	87.9/70.9	48 h
	112	As above	Isolates	411	At 48 h: A: 74/75; B: 35/36	90.7/60.9	24 h
					B1: 42/42; B2/3: 23/24; D: 3/3; E: 16/18; L: 1/3; M: 3/3	96.6/59.9	48 h
	162	cID	Stool	121	Combined A+B; 46/51	90.2/73.9	24 h
					Combined A+B; 47/51	92.2/72.4	48 h
	110	cID, PCR	Rectal swab	610	Only B: 106/122	86.6/81.2	72 h
	164	Spectra VRE	Stool	399	*van* not specified; 99/113	87.6/87.1	24 h
					van not specified; 106/113	93.8/72.4	48 h
	165	V*Select*, Etest	Stool	945	*van* not specified; 211/222	95.1/84	48h
Bile Esculin Azide (BEAV8, BD) High	163	Xpert *vanA/B*[d]	Rectal swab	183	Only A: 20/20	65/71.8	24 h
					Only A: 20/20	100/60.7	48 h
VRE CHROMagar (CV6, CHROMagar) High	111	BMD[a], Etest, cPCR	Isolates	144	Only B: 123/129	95.3/–	48 h
	116	BMD[a], Etest, cPCR	Isolates	150	Multicenter panel: B: 3, B1: 4, B2: 18, E: 1, G: 1	97/80	48 h

(*Continued on next page*)

TABLE 1 Selected recent and/or comprehensive evaluations of differential agar methods for detecting vancomycin resistance from specimens or bacterial isolates (*Continued*)

Detection method (acronym; vendor), complexity of test	Reference	Comparator method(s)	Specimen types	Total specimens	No. *vanA* (A), *vanB* (B), other *van* genotype(s) detected/present	Sensitivity/ Specificity[a] (%/%)	Result TAT[e]
VRE*Select* (V*Select*, Bio-Rad) High	113	BVRE, BMD[a], MET[b], LC-VRE[c], cPCR/ sequencing	Rectal swab/stool	3,000	A: 118/126; B: 9/15	90.1/79.4	24 h
					A: 120/126; B: 15/15	95.8/72.0	48 h
	112	As above	Isolates	274	At 48h: A: 49/50; B: 24/24	82.4/82.6	24 h
					B1: 28/28; B2/3: 16/16; D: 2/2; E: 3/12; L: 1/2; M: 2/2	91.9/60.9	48 h
	165	BEAV6, ET	Stool	945	*van* not specified; 222	98.7/99	28h
Brilliance VRE (BVRE, Oxoid) High	113	EV6, BEAV6, V*Select*, BMD[a] LC-VRE[c]	Rectal swab/stool	3,000	A: 119/126; B: 12/15	92.9/88.8	24 h
					A: 122/126; B: 15/15	97.2/79.8	48 h
	112	As above	Isolates	548	At 48h: A: 100/100; B: 48/48;	94.1/96.8	24 h
					B1: 56/56; B2/3: 32/32; D: 4/4; E: 22/24; L: 0/4; M: 4/4	97.8/94.6	48 h
	111	BMD[a], Etest, PCR	Isolates	144	Only B: 121/129	93.8/–	48 h
	163	Xpert *vanA/B*[d]	Rectal swab	183	Only A: 19/20	95/87.1	24 h
					Only A: 20/20	100/80.4	48 h
VREchromID (cID, bioMerieux) High	162	BEAV6	Stool	120	Combined A+B: 44/51	86.3/100	24 h
					Combined A+B: 45/51	88.2/98.6	48 h
	110	BEAV6, cPCR	Rectal swab/stool	610	Only B: 116/122	90.2/70.5	48 h
	111	BMD[a], MET[b] cPCR	Isolates	144	Only B: 12/129	96.1/–	48 h
cID version 43004	115	cPCR/sequencing	Isolates	1,341	At 48h: A: 263/300; B: 255/261; D: 6/6; E: 5/36; L: 0/6	85.8/97.4	24 h
						86.9/94.8	48 h
	116	BMD[a], cPCR	Isolates	210	Multicenter panel: B: 3, B1: 4, B2: 18, E: 1, G: 1	98/95	48 h
Spectra VRE (Remel) High	164	BEAV6, ET, VT2	Stool	399	*van* not specified	98.2/99.3	24 h
						99.1/96.5	48 h

[a]BMD, broth microdilution, to CLSI standards (117).
[b]MET, MacroEtest method using 2 MacFarland suspension incubated for 48 h on brain heart Infusion agar containing casein (141).
[c]LC-VRE, LightCycler VRE kit from Roche Diagnostics.
[d]Xpert *vanA/B*, GeneXpert *vanA/vanB* kit from Cepheid.
[e]TAT, turnaround time.

TABLE 2 Selected recent and/or comprehensive evaluations of molecular assays for detecting vancomycin resistance genes from specimens or bacterial isolates

Detection method (acronym; vendor) Complexity of test	Reference	Comparator method(s)[a]	Specimen types	Total specimens	No. *vanA* (A), *vanB* (B), other *van* genotype(s) detected/present	Sensitivity/ Specificity[b] (%/%)	Result TAT[a]
LightCycler VRE kit	169	CV6	Rectal swab[d]	180	A: 4/4, B: 73/96	88/–	3.5 h
(LC-VRE, Roche)	186	BEAV6/8	Rectal swab	894	A: 22/21, B: 7/0, B2/3: 27/0	100/97	3.5 h
High	119	BEAV6, cPCR	Rectal swab	225	Only B: 41/24	100/93	1 h
	49	BEAV6, ET	Rectal swab	30,367	A: 353/330 (160 patients)	73.3/99.6	3.5 h
					B: 4,925/82 (43 patients)	85.4/83.9	3.5 h
IDI/GeneOhm VanR (VanR, BD) High	187	BEAV6	Rectal swab/stool	502	A: 149/144, B: 67/3	96.6/87	3.4 h
Xpert *vanA*/*vanB*	174	cIDV8	Rectal swab	804	A: 12/8, B: 115/3	100/85.4	1 h
(Xpert *A*/*B*, Cepheid)	182	BEAV6/8	Rectal swab[c]	184	A: 83/84, B: 25/15	96.4/93	1 h
Low	192	cID	Stool[d]	37	A: 11/13, B: 6/0	61.5/79.2	1 h
	189	LC-VRE	Isolates	126	A: 25/25; B: 30/30	100/100	1 h
	193	LC-VRE	Isolates	215	A: 133/133; B: 3/2	100/99.3	1 h
Seeplex (Seegene)		cIDV8	Rectal swab[c]	131	Only A: 38/37	92.5/98.9	48 h
High		cIDV8	Rectal swab[c]	8,815	A: 758/741 B: 3/0	98.2/99.6	36 h
Verigene		BA+ cID[e]	Blood	1,252	A: 26/26, B: 0/0	100/100	2.5 h
(Nanosphere)		BA+cID[e]	Blood	186	A: 24/23, B: 0/0	95.8/100	3.8 h
Medium							

[a]See Table 1 for acronyms for selective differential agars and methods used as comparisons and other abbreviations.
[b]Specificity refers to the ability of PCR assay to detect only culture-positive VRE, although *vanB*-positive PCR results may be specific for *vanB* operons in anaerobic bacteria.
[c]Pre-enrichment in enterococcosel broth with vancomycin.
[d]Pre-enrichment in brain heart infusion broth with 6 mg/l vancomycin.
[e]BA+cID, culture on 5% sheep blood agar plus conventional identification—positive catalase, leucine aminopeptidase, pyrrolidonyl arylamidase, and esculin agar tests and bioMérieux VITEK 2 GPI cards.

different geographic locations (49, 119). It is possible that in some geographic areas there are bidirectional reservoirs in the human gastrointestinal tract which may enable the further spread of the *vanB* operon.

The ongoing risk of *vanB* emergence means that, even in areas of current low prevalence such as the United States, phenotypic detection methods should be designed to identify low-level *vanB2*-type vancomycin-resistant isolates and verification collections should comply with *Cumitech 31A* verification guidelines (120) to include adequate numbers of *vanB* and other genotypes with low-level vancomycin MICs to ensure that methods used in clinical microbiology laboratories are capable of detecting these strains. Current automated susceptibility testing systems and commercially prepared screen agars have all been documented to fail in the detection of some *vanB1* and *vanB2* strains, especially when the vancomycin MIC falls within the 6 to 16 mg/l range (22, 116).

vanD

The first *vanD*-containing isolate was an *E. faecium* strain recovered from a patient hospitalized in New York in 1991. Subsequently, *vanD E. faecium*—and one *vanD E. avium* isolate (121)—have been identified from Boston (56), Canada (122), Brazil (123), France (121, 124–126), and Australia (127, 128), either as single isolates or in small outbreaks (56). *vanD*-encoding isolates have moderate-level vancomycin resistance and reduced susceptibility or low-level resistance to teicoplanin. The ligase of the first isolate demonstrated 69% homologies to *vanA* and *vanB* and 43% to *vanC*, and orientation of the $vanH_D$ dehydrogenase, the *vanD* ligase, and the $vanX_D$ D,D-dipeptidase genes is similar to that for *vanA* and *vanB*. Isolates have a two-component regulatory system, $vanR_D$ and $vanS_D$, but the resistance is nonetheless usually constitutive. Some isolates have chromosomal *ddl* impairment due to insertional inactivation. The small number of identified isolates have an intriguing array of mutational modifications or insertions in $vanR_D$ or $vanS_D$ genes with or without an impaired host *ddl* but have had similar phenotypes. An exploratory study to look for *van*-gene reservoirs in fecal flora of hospitalized patients found unexpectedly high *vanD* carriage rates of 43.8% in Montreal, Canada, and 26.7% in Boston, Massachusetts, and these investigators found a complete *vanD* operon in *Ruminococcus gauvreauii* (45, 118, 129). Thus, gut anaerobes may be a source for these clusters in VRE. However, how they are mobilized and acquired is unclear. Fortunately, isolates remain uncommon, and few have been associated with person-to-person transmission in hospitals.

vanM

vanM was the first mobile inducible operon to be identified after *vanA* that produced precursors terminating in D-Ala:D-Lac and that conferred high-level resistance to both vancomycin (MIC: >256 mg/l) and teicoplanin (MIC: 96 mg/l) (62). These operons were detected in multiple strains of *E. faecium* isolated from clinical infections in China in 2010. While sequencing of the *vanM* ligase demonstrated homology to *vanA* (79.9%), *vanF* (78.8%), *vanB*

(70.8%), and *vanD* (66.3%) genes, the overall gene organization most resembled the *vanD* operon. But in common with *vanA*, the *vanM* operon was readily transmitted by conjugative transfer in a 150-kb plasmid, which was made clear by its mobilization into *E. faecium* of diverse lineages as demonstrated by pulsed-field typing. This operon probably emerged some years earlier, as a 2008 Toronto isolate from a patient from the local Chinese community was found to share 100% homology across the operon. All *vanM* reported from China have been ampicillin-resistant *E. faecium* with high-level vancomycin resistance (MIC: >256 mg/l); however, only 35 (70%) exhibited high-level teicoplanin resistance (MIC: 32 to 128 mg/l). Most isolates were of the CC17 lineage.

ACQUIRED D-ALA:D-SER OPERONS

vanE

The chromosomally encoded five-gene *vanE* operon was first described in 1999 (57); the initial and all subsequent isolates have been found in *E. faecalis* in geographically distinct locales (i.e., Australia and Canada) with no significant evidence of clonal spread (130–133). The operon has organizational similarity and moderate sequence homologies (43 to 60%) to equivalent genes of *van*C-type clusters. Vancomycin resistance is constitutive in some isolates and inducible in others. In common with *van*C-type operons, all *vanE* operons produce peptidoglycan precursors terminating in D-serine, and these resulted in low-level vancomycin resistances (MIC: 6 to 16 mg/l) and teicoplanin susceptibility. *vanE*-mediated vancomycin resistance in clinical *E. faecalis* isolates tested by automated susceptibility systems may go undetected, and most VRE-selective agars do not reliably support the growth of this phenotype; thus, its prevalence may be underestimated. While attempts to mobilize the *vanE* operon have to date been unsuccessful so that the precise conditions and mechanism of acquisition and transfer remain unclear, Boyd et al. have demonstrated the *vanE* operon in *E. faecalis* N00-004 to be associated with the integrative conjugative element Tn*6202* (122).

vanG

In 1996, a cluster of *E. faecalis in* Australia with low-level vancomycin resistance (MIC: 12 to 16 mg/l) and susceptibility to teicoplanin (MIC: 0.5 mg/l) was found to have novel transferable chromosomally located seven-gene clusters (58). These encoded D-Ala:D-Ser peptidoglycan precursors similar to those synthesized by *van*C and were regulated from the 5′ end by a constitutive system encoded by three genes ($vanU_G$, $vanR_G$, and $vanS_G$), all transcribed from the P_{UG} promoter. Induction of the downstream five-gene cluster ($vanY_G$, $vanW_G$ [a gene with unknown function], *van*G, $vanXY_G$, and $vanT_G$) was initiated at the P_{YG} promoter (134, 135). Two additional *van*G *E. faecalis* isolates have been identified in Canada (136), one with only minor sequence divergence (>99% homology) and a second with 87% homology to the Australian *van*G. Investigations by Domingo et al. found a high prevalence of *vanG* in nonenterococcal species in human gut flora from patients in Quebec. After protracted incubation of an anaerobic culture, a vancomycin-resistant *R. gauvreauii* isolate with a partial *van*G-like cluster in its chromosome was found (45, 118, 129). An operon with gene organization and homology similar to *van*G was also found in about 85% of C. *difficile* clinical isolates. Although the operon was fully functional and produced precursors terminating in D-serine, the isolates remained vancomycin susceptible.

vanL

The *vanL* determinant was first identified in an *E. faecalis* isolate (vancomycin MIC: 8 mg/l) from a single rectal swab from a Canadian patient in 2006 (59). This cluster had a gene organization comparable to *van*C, with the exception that the *vanT* serine racemase was encoded by two separate genes. The *vanL* ligase gene demonstrated 57% homology with the *vanC1* ligase from *E. gallinarum*. No plasmids were demonstrated, and several attempts to transfer the operon were unsuccessful.

vanN

vanN was identified in an *E. faecium* isolate causing bacteremia in France in 2008 (60). The isolate produced low-level vancomycin resistance (MIC: 16 mg/l) as a result of D-Ala:D-Ser precursor production and was susceptible to teicoplanin (MIC: 1 mg/l). Sequencing identified that its closest relative was *vanL* (homologies 50 to 74%). It was constitutively expressed due to an amino acid substitution in the H domain of $vanS_N$ and was transferable by conjugation at low frequency by a 150-kb plasmid. *vanN* is unusual in that it was the first acquired operon producing D-Ala:D-Ser precursors to be found in *E. faecium* and in which the mechanism of transfer was plasmid-mediated. However, additional isolates have not been identified.

LABORATORY DETECTION OF ACQUIRED VANCOMYCIN RESISTANCE

Clinical microbiology laboratories must be able to detect vancomycin resistance in *E. faecium* and *E. faecalis* rapidly and accurately, as vancomycin is often the therapy of choice for susceptible isolates of these organisms and therapy will fail if resistance goes undetected. The detection of resistance by culture to selective screen agars from rectal swabs is also obviously critical for those hospitals/regions with transmission control programs for VRE (Table 1).

Enterococci with *vanA*, *vanB*, and *vanM* operons all have been shown to spread rapidly after introduction into new hospital settings, and rapid laboratory detection by molecular methods from rectal swab screens of high-risk patients when they are admitted can prevent further transmission (Table 2). Increasing medical tourism and global travel mean that introductions of novel VRE strains are an ongoing threat in all jurisdictions. All laboratories must have the capacity to detect newly introduced strains, as most strains appear to be able to cause both clinical infections and difficult-to-control outbreaks. This might seem daunting; however, if protocols sensitive to low-level vancomycin resistance phenotypes are implemented, then detection of other VRE regardless of genotype will be feasible.

Knowledge of VRE epidemiology in surrounding regions is important, as laboratories must be ready for the unexpected. The approach most likely to reduce major errors is to implement a combination of well-evaluated methods with overlapping detection capabilities that pay special attention to difficult-to-detect regional phenotypes and use molecular tests strategically. Clinical laboratories with the capacity to use both genotypic and phenotypic methods are more likely to detect new variants, an approach that will protect infected patients from inappropriate therapy

and hospitalized patients from avoidable exposure to VRE. The additional laboratory costs associated with this approach are likely to be more than balanced by reduced expenditures in infection control, housekeeping, and the costs of management of hospital-acquired infections (137–139).

To identify VRE accurately, laboratories must ensure (i) that their species-level identification of enterococci is accurate so as not to confuse intrinsic with acquired vancomycin resistance mechanisms, (ii) that routine susceptibility determination from clinical specimens is performed and is sufficiently sensitive to detect low-level glycopeptide resistance, and (iii) that agars for selective culture of stool, rectal, or environmental swabs are adequate to identify isolates with borderline resistance or those that are present in low densities. In some cases, a relevant genotype may fail to be expressed in culture but may emerge as resistant during therapy, such that direct genotypic testing may be a prerequisite to its detection and control. Each method's limitation should be understood to ensure that, when used together, the chosen methods will be sufficiently sensitive to cover all possibilities.

As already discussed, VRE are constantly evolving and notoriously prone to change, and the varied expression that is a result of gene mutation means that no single method will detect every isolate. Unusual phenotypes should be referred to reference centers for characterization, as they may represent novel strains or operons that may spread within hospitals. The characteristics of the novel genes will need to be incorporated into decisions about VRE identification and susceptibility testing in the future and in the evaluation of new test methodologies.

Antimicrobial Susceptibility Testing

While commercial susceptibility testing methods detect high or moderate levels of constitutive vancomycin resistance in enterococci with ease, all incur varying degrees of major errors due to low-level, heterogeneous, delayed or inducible phenotypes. Disk diffusion (140), agar gradient susceptibility (141), and commercial broth microdilution susceptibility panels for vancomycin resistance in enterococci should always be accompanied by parallel spot inoculation of enterococcal suspensions onto CLSI-recommended screening agar to reduce detection failures (69, 117, 142, 143).

Broth Microdilution

The reference method for glycopeptide susceptibility testing of enterococci is broth microdilution as per CLSI (103, 117) and EUCAST (144) guidelines. The standardized procedure uses cation-adjusted Mueller-Hinton broth and antibiotics prepared as per CLSI (117) and/or manufacturers' instructions, with inoculation with freshly prepared bacterial suspensions to obtain a final suspension of 10^4 CFU per well. For vancomycin, test wells are examined for any visible growth after 24 h at 35°C, and categorical interpretation is per CLSI M100-series (103) or EUCAST (144) breakpoints. However, use of these set breakpoints does not always correctly categorize isolates, since mutations producing small-colony variants (145), heterogeneity, inducibility, low-level expression, or vancomycin sensor inactivation (as previously discussed) may all affect growth in vancomycin, and molecular detection of resistance genes may be required to rule out or detect VRE (91). But broth microdilution testing requires significant technical expertise, and acquisition of all the necessary antibiotic powders may be costly; thus, testing in clinical laboratories is usually performed using commercially prepared panels. TREK Sensititre (Thermo-Fisher Scientific), MicroScan (Beckman Coulter) (146, 147), and Phoenix (BD) (148, 149) most closely resemble reference broth microdilution, but all deviate to some extent from the standard method by use of proprietary broth formulations or fluorescent chemicals to aid endpoint determinations. Because commercial panel constituents and software are regularly modified, only recent studies may accurately reflect current performance, especially for difficult-to-detect phenotypes.

The VITEK 2 (bioMérieux) system is another commonly used commercial system with bacterial suspension preparation as the only nonautomated step. In this system, broth microdilution susceptibility testing is conducted within miniaturized wells set in a plastic card format. The wells are electronically monitored for changes in turbidity over time (i.e., due to growth in broth with antibiotic versus without antibiotic). Because MIC endpoints are predicted algorithmically against a species-specific database in order to achieve earlier results, it is imperative that accompanying organism identification is accurate; otherwise, significant susceptibility errors will occur (23, 69, 150). More recently, VITEK 2 susceptibility testing problems related to suboptimal organism identification appear to have been solved by the transition in many clinical laboratories from the less accurate VITEK 2 to the VITEK MS Plus or similar matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) systems which, in contrast, perform highly accurate species-level identifications of enterococci (151, 152). Nonetheless, suboptimal sensitivity of VITEK 2 for *vanB*-containing isolates persists (116, 153–157). During an outbreak in a Rome hospital in 2007 (149), two VITEK 2 cards were compared with reference broth microdilution, Etest, disk diffusion, and conventional *vanA*/*vanB* PCR. Of 25 *vanA* isolates, all resistant to vancomycin by broth microdilution, the AST-P534 card reported 17 as vancomycin resistant and two as teicoplanin resistant, while the AST-P535 card reported 24 as vancomycin resistant and 4 as teicoplanin resistant. A 2014 multicenter Scandinavian study circulated a panel of 30 enterococci of *vanB1*, *vanB2*, *vanE*, and *vanG* genotypes, together with susceptible isolates to five laboratories; the overall VITEK 2 sensitivity was 87% (95% CI: 79 to 92%) with a major error rate of 7% (the maximum acceptable rate is 3%) (116, 120). Thus, laboratories using commercial broth microdilution systems should continue to use CLSI agar screening plates to detect VRE (103). Any vancomycin-susceptible/CLSI screen-positive should be tested by PCR for *vanA* and *vanB* genes, and if negative should be referred for testing to rule out unusual *van* genotypes.

Agar Gradient MIC Testing

The AB-Biodisk Etest (141) (now bioMérieux) is also widely used around the world; however, because of this method's single-vendor status, it was not standardized by CLSI or EUCAST. It is to be hoped that the availability of the M.I.C. Evaluator (Thermo-Fisher) and Liofilchem (Alere) strips will result in procedure standardization. When used in accordance with original AB Biodisk Macro-Etest VRE or GISA protocols (141), Etest gradient strips are highly sensitive for detecting glycopeptide resistance (111). The unconventional approach deviates substantially from CLSI methods, but modification of AB Biodisk

methods in an attempt to follow CLSI guidelines results in poor sensitivity for strains demonstrating heterogeneous or low-level resistance. The critical elements of the AB-Biodisk recommendations are the following: (i) use a heavy enterococcal suspension equivalent to a 2 MacFarland standard; (ii) use brain heart infusion agar containing 60 g/l casein; (iii) let the suspension dry for 15 min before vancomycin and teicoplanin Etest strips are placed on the agar surface; (iv) extend the incubation period to a full 48 h; (v) examine the two elliptical inhibition zones at both 24 h and 48 h; and (vi) read the endpoints at 100% inhibition to include all subpopulations present. It is important not to miss haze or pinpoint colonies within the elliptical zone; these colonies are often not visible on Mueller-Hinton or brain heart infusion agar without casein at 48 h. For detecting resistant subpopulations, it is advantageous to use PCR for genotype confirmation. If the AB-Biodisk methods are followed, the Etest agar gradient strip becomes one of the more reliable methods available for difficult-to-detect VRE phenotypes.

Disk Diffusion

Disk diffusion was removed as a testing option from the CLSI M100 guideline in 2014 (103), because it was considered too insensitive and subjective for detecting vancomycin resistance, especially for *vanB* strains with low-level MICs. However, EUCAST (144) continues to provide disk diffusion method guidelines for detecting VRE even though a 2014 VRE detection method comparison found a 7% major error rate for vancomycin susceptibility with disk diffusion testing. These authors stressed that if inhibition zones were interpreted by experienced readers, disk diffusion had a sensitivity equivalent to the CLSI agar screen method and was superior to the results of VITEK 2 testing (116). Furthermore, they concluded that the method would perform better if laboratories that use disk diffusion as their primary susceptibility testing method were trained in the differences between fuzzy-edged zones (typical of VRE) and sharp-edged zones (associated with vancomycin susceptibility) (116). Endpoint subjectivity of disk diffusion and agar gradient strip MIC methods might also be reduced by electronic imaging, which will allow for magnification that should make differences between fuzzy and sharp edges more evident.

CLSI Agar Screen

When VRE emerged, it was clear that not all strains were reliably detected by susceptibility testing methods available to most clinical laboratories. Thus, the vancomycin agar screen method was developed to provide an inexpensive and reliable means to prevent detection failures (142, 146). Standardization studies found that brain heart infusion agar with 6 mg/l vancomycin was the most sensitive and specific formulation, and the CLSI agar screen became recommended for parallel spot-testing of all enterococcal suspensions prepared for routine susceptibility testing (103, 143). For the most part, this method has retained its sensitivity and specificity, but as with any method using non-chemically defined media, quality may vary by manufacturer and should periodically be reassessed. A 2014 multicenter study designed to determine how well Scandinavian laboratories were able to detect enterococci with low-level vancomycin resistance found that the sensitivity of the CLSI agar screen varied between centers. In centers where unacceptably low detection sensitivities were identified, the poor performance was found to correlate with particular manufacturers of commercially prepared screen agars (e.g., Oxoid agar: 89%; 95% CI: 82 to 93%) (116) and the authors concluded that the overall sensitivity of 93% (95% CI: 92 to 95%) for the agar screen was acceptable because it was better than the overall performance of other methods tested in parallel (116). These data suggest that it would be prudent for this method to be periodically reassessed using contemporary hard-to-detect strains; alternatively, more stringent control of the agar should be implemented on a routine basis using a more appropriate quality standard.

CLSI recommends the *vanB E. faecalis* strain ATCC 51299 as a positive control (103), but the vancomycin MIC range of this strain is typically anywhere between 16 and 64 mg/l. Thus, it will not detect overly inhibitory agar preparations that suppress growth of isolates with vancomycin MIC in the 6 to 16 mg/l range. An additional problem introduced with the use of this VRE as a control strain was its repeated link to laboratory contamination events that resulted in false-positive clinical specimen reports (158, 159). In 1999, the Ontario Quality Management Program–Laboratory Services (160) recommended *E. gallinarum* ATCC 49573 to be used in lieu of the VRE *E. faecalis* ATCC 51299 because it has a stable vancomycin endpoint of 8 mg/l, which is more appropriately able to control the required vancomycin content of 6 mg/l. Furthermore, use of this benign alternative increased the confidence with which *vanB E. faecalis* results from this plate were received, ensuring that growth was the patient isolate and was not due to accidental splashing causing cross-contamination; this enabled VRE confirmation to be expedited by performing PCR directly from the growth spot on the agar (158, 160).

Primary Surveillance Screening

Soon after VRE was recognized, it was realized that, in most patient populations, the ratio of colonized to infected patients was as high as 20:1. Colonized patients form an important reservoir for VRE in hospitals, and detecting VRE-colonized patients is required to prevent and control hospital outbreaks (161). Thus, agar formulations developed for isolating enterococci in environmental laboratories were adapted by reference clinical laboratories to enhance their selection capabilities for VRE (Table 1) (114). The agar bases most commonly used were m*Enterococcus* (Difco) and Bile Esculin Azide (also known as Enterococcosel Agar); once supplemented with vancomycin, the former was sensitive but required incubation to be extended to 72 h, while the latter was specific but relatively insensitive, and both had difficulty with detection of low-level resistance (Table 1). Further, these agar media generally failed to inhibit enteric bacterial species with intrinsic resistance to vancomycin, such as the lactobacilli, pediococci, and leuconostocs, and these had a tendency to mask the growth of VRE or cause false-positive results due to mixes. While the development of chromogenic agars has significantly improved performance (Table 1), they are based on distinct formulations, most proprietary, and some incorporate substances inhibitory to species of *Enterococcus* not generally associated with VRE (85, 110, 162–164). In some cases, this resulted in a lack of sensitivity for isolates containing *vanC* or *vanB* operons with low-level vancomycin resistance (111, 165). Time to detection is significantly improved, although chromogenic agars are still unable to identify all VRE within a 24-h period, and breakthrough growth of

TABLE 3 Selected recent and/or comprehensive evaluations of antimicrobial test system for detecting vancomycin resistance in bacterial isolates

Detection method (acronym, standard/vendor, complexity of test)[a]	Reference	Comparator method(s)	Total specimens	No. *vanA* (A), *vanB* (B), other *van* genotype(s) detected/present	Sensitivity/ specificity (%/%)	Result TAT[b]
Broth microdilution (BMD[a], CLSI, high)	111	ET, VT2, cPCR	144	Only B: 129/111	86/–	24 h
Agar screen	116	BMD, ET, cPCR	540	Multicenter panel:	93/94	24 h
(AS, CLSI, low)			240	B: 3, B1: 4, B2: 18, E: 1, G: 1	96/100	24 h
Difco BHI agar			150		89/100	24 h
Oxoid BHI agar						
Disk diffusion	116	BMD, ET, cPCR	840	Multicenter panel:	93/98	24 h
(DD, EUCAST, High)			480	B: 3, B1: 4, B2: 18, E: 1, G: 1	97/100	24 h
MHA (Oxoid)			300		86/93	24 h
MHA (BD, BBL)						
VITEK 2 System	153	BMD, cPCR	121	A: 66/66, B: 14/14, C:40/0	98.3/–	<16 h
(VT2, bioMerieux, medium)	111	BMD, ET, cPCR	144	Only B: 129/113	87.6/–	~12 h
Version 4.1	116	BMD, ET, cPCR	150	Multicenter panel: B: 3, B1: 4, B2: 18, E: 1, G: 1	87/100	~12 h
Phoenix System (BD, medium)	148	Agar dilution	90	*van* not specified; 36/36	100/–	24 h
Etest on MHA (ET, bioMérieux, medium)	111	BMD, PCR	144	Only B: 129/129	83.7/–	24 h
MacroEtest on BHIA (MET, bioMérieux, medium)	111	BMD, PCR	144	Only B: 129/129	94.6/–	48 h

[a]Guidelines/institutes: CLSI, Clinical and Laboratory Standards Institute (103, 117); EUCAST, European Union Committee on Antimicrobial Susceptibility Testing (144).
[b]See Table 1 for acronyms for selective differential agars and methods used as comparisons and for other abbreviations.

E. faecalis or coagulase-negative staphylococci may still present minor problems (112, 113, 115, 162, 163, 165).

MOLECULAR DETECTION OF VRE

Usually, clinical microbiology laboratories undertake routine diagnostic testing including the isolation of VRE from surveillance and clinical cultures, with only a small proportion performing additional molecular detection of VRE (Table 3). While some laboratories with PCR capacity still perform one of the many possible in-house conventional PCR assays for VRE detection and/or confirmation (11, 21, 34, 88, 107, 166–172), for ease of use, many others now rely on commercially available kit-based assays (49, 119, 173–192). Referral laboratories are required to perform genotypic confirmation on isolates for diagnostic laboratories without PCR capacity and to characterize isolates that may represent novel genotypes or variant operons. As seen in Table 3, most commercial molecular assays only target the two common VRE genotypes, *vanA* and *vanB*, because these together account for >99% of clinically relevant isolates today.

In most parts of the world this is currently adequate, but as new epidemic genotypes emerge (i.e., *vanM* in China) (62), manufacturers will need to modify the assays to include new targets. Commercial PCR assays yield information regarding VRE genes more rapidly than culture and may be more sensitive than unenriched surveillance cultures, particularly for isolates exhibiting borderline vancomycin MIC values that are associated with some *vanB* genotypes. They remain, however, substantially more expensive than culture-based testing and thus are less commonly the primary method for VRE detection. However, there are circumstances in which molecular assays are a valuable addition to culture-based testing or may replace culture-based testing (171, 185). For instance, the substantially shortened time to results may make molecular testing a cost-effective choice for screening of high-risk patients or in the management of outbreaks. Similarly, molecular testing may be necessary in the management of outbreaks of "vancomycin variable" VRE (28–30). Other laboratories have used molecular testing to obtain rapid genotypic confirmation of suspected VRE isolated in culture (193); molecular testing can also be employed directly on blood cultures to shorten time to identification of VRE (175, 190).

Test accuracy related to primer design (21) and cost are critical in assessing the suitability of a commercial molecular test for clinical laboratory use. However, other important considerations are test complexity, whether controls are internally integrated or must be run separately in parallel, and whether the assay was designed for batch or random access testing. Technically complex assays are associated with the need for expertise, increasing personnel costs and introducing erratic personnel demands on the clinical laboratory during outbreaks. Requirements for batch testing delays reporting and may result in increased costs beyond the laboratory. On-demand commercial molecular testing options are available that obviate the need for batch testing.

One important limitation of all molecular assays is the fact that *vanB* operons are present in a variety of stool anaerobes, such that a positive test from stool or rectal swab specimens does not necessarily mean that a patient is colonized with VRE. As a result of this nonspecificity, commonly used commercial assays in the United States, such as BD GeneOhm VanR or the Cepheid Xpert *vanA*, were FDA cleared only for *vanA*. In Australia, Canada, and Europe these assays have been used to detect both *vanA* and *vanB* genotypes. Like other commercial assays, the Roche LightCycler VRE Detection kit detects *vanA* and *vanB*, but uniquely, it further distinguishes *vanB* as *vanB1* or *vanB2/3* using fluorescent resonance electron transfer probes combined with melting curve analyses. The newer Nanosphere Verigene assay detects *vanA* and *vanB* directly from positive blood cultures (175, 183, 184, 190, 194), as does the Seegene Seeplex assay (181), but these assays have undergone only limited verifications to date and thus have not yet been widely adopted.

Roche LightCycler VRE Detection Kit

The first commercial multiplex PCR assay, originally developed at the Mayo Clinic for detecting *van* genes, was the Roche LightCycler VRE Detection kit, a qualitative "research use only" assay run on the LightCycler instrument (Roche Diagnostics GmbH, Germany) (186). The assay amplifies *vanA*, *vanB1*, and *vanB2/3* genes, which are distinguished by the different temperatures at which the specific fluorescent resonance electron transfer hybridization probes "melt" from each amplicon during the final heating step. Early evaluations of this assay reported high inhibition rates; however, with appropriate extraction methods, this can be avoided. Thus, magnetic extraction using the Roche Total Nucleic Acid Isolation kit and the automated MagNA Pure instrument (Roche Applied Sciences)—which processes 32 samples in ~100 min—results in inhibitor-free reactions, while rapid DNA extraction by disruption in glass beads and saline followed by centrifugation risks failure due to inhibitors (188, 195). Both the extraction and PCR assays require technical expertise. Kits require—20°C for reagent storage, dedicated pipettes, and a PCR clean room for reagent preparation, and positive and reagent controls are run in separate tubes. The assay runs for 1 to 1.5 h depending on the number of samples, and performing the assay is intuitive but does require training. It has an advantage over other commercial assays in that it distinguishes *vanB1* from *vanB2/3*, which can be useful for patient management as *vanB2* operons are more commonly associated with anaerobes, while *vanB1* operons are more often in VRE (119, 180, 188, 191, 195). Both extraction approaches enable the swab to be cultured after it has been used for PCR. It is critically important to ensure that specimens positive for *vanB1* and *vanB2/3* genes are cultured on selective agars known to support the growth of enterococci with low-level vancomycin MICs (4 to 6 mg/l) or with inducible or heterogeneous expression of resistance. On the whole, this is a high-performance test, as long as it is understood that *vanB* may be present in anaerobic bacteria and *vanB2* genes may be present in anaerobes and in *Enterococcus* species (49, 119, 180).

BD GeneOhm (IDI) VanR Assay

The GeneOhm (IDI) VanR assay (BD Diagnostics) was developed in Quebec, Canada, to detect *vanA* and *vanB* genes from fecal surveillance specimens (187). It was the first VRE-related molecular test to gain FDA clearance for amplification of the *vanA* gene directly from rectal swabs. The GeneOhm VanR assay is a medium-complexity assay that performs DNA extraction and PCR amplification in the random-access modular Cepheid SmartCycler instrument. Although the assay comes in kit form, it requires a moderate degree of expertise because there are several precision pipetting and centrifugation steps in the protocol. The kit buffer enables the swab to be cultured after use for PCR. Each test (up to a maximum of six tests in an eight-module instrument) and a set of positive and negative controls are run in separate plastic PCR tubes that are placed into separate modules in the SmartCycler instrument for simultaneous amplification during the PCR run. To increase capacity when larger specimen batches require testing, multiple instruments may be linked together, in which case they are able to act as random access devices as long as every time the PCR run is initiated, it is done with an accompanying set of controls. If either the positive or negative control fails, all other tests will automatically fail regardless of their individual results.

The extraction comprises a basic spin column and is thus prone to inhibition, which may result in amplification failure. If inhibition occurs, the specimen is diluted and the test and controls are repeated. The VanR assay detects *vanA* and *vanB* genes, but does not distinguish between the *vanB1* and *VanB2* variants. The assay run takes ~1 h, and reporting of results is simple and direct. Overall the assay is less challenging than the Roche LightCycler assay, but with only marginally less hands-on time needed to set it up. The system is limited to six specimens in a batch unless multiple instruments are available, and time to result may be delayed by the wait for free modules in the instruments.

Cepheid Xpert *vanA/vanB* Assay

The Xpert *vanA/vanB* PCR assay is run on the GeneXpert instrument (Cepheid) (40, 173, 179, 182, 192). It was designed for direct testing of stool or rectal swab specimens, but it has since been validated for use on isolates. A clear advantage of this system compared to other commercial VRE assays is the simplicity of design, minimal hands-on time, and low degree of expertise required to run the assay. Other than the buffer, all extraction and amplification reagents, including the PCR tube itself, are built into the test cartridge. The cartridge contains an internal control of *Bacillus* spores to ensure that DNA extraction and PCR amplification are successful prior to reporting a negative result. Inhibition is generally preventable by avoiding overinoculation of fecal material. In the United States, the assay is FDA cleared only for *vanA* (*vanB* results are suppressed), but it is used widely for *vanA* and *vanB* in Australia, Europe (CE marked), and Canada (Health Canada approved) (189, 193). GeneXpert instruments are designed for random access testing, with modules that can hold 4, 8, 16, 48, or 80 cartridges, each of which can be a different assay.

A single Xpert *vanA/vanB* cartridge takes ~1 min to set up, and run time is less than 1 h. Results automatically print with crossing thresholds and clear interpretations for each detectable target. These may be reported directly via an interface to the laboratory information system, with hands-off finalized reporting of negatives if so desired. Kit storage is at room temperature, and setup does not require a clean room. However, single-package sterile plastic pipettes for buffer transfer are recommended, as are dedicated clean pipettes with filter tips for when direct testing

from liquid specimens is performed, because cross-contamination during handling can occur. Compared to other molecular VRE assays, the test cost is high, but this is offset by its low complexity, random access testing, and integrated positive and negative controls. Another advantage is that PCR may be performed directly from colonies selected from chromogenic agars or primary cultures without disrupting bench workflow and without incurring delays (189, 193). The assay does not distinguish *vanB1* from *vanB2* or detect other targets (*vanE* and *vanM*), which may be useful for patient management in some regions.

Other Molecular Tests

A number of other molecular tests are under development. These include the Seeplex VRE kit (Seegene, South Korea) (181, 185), the iNtRON kit (LG Life Sciences, South Korea) (196, 197), and the NanoCHIP Infection Control Panel (ICP) assay (Savyon Diagnostics, Israel) (198). At least one group has demonstrated loop-mediated isothermal amplification tests that may also be useful (167), and the Verigene (Nanosphere) is one of the first of several products likely to be available in the future that will permit the rapid detection of both species and *vanA*/*vanB* genes conferring resistance in blood cultures positive for Gram-positive cocci (175, 190). None of these tests has yet been validated by a sufficiently large and varied sample of VRE to adequately assess its performance characteristics. Many laboratories also continue to use a variety of different noncommercial PCR tests for the detection of vancomycin resistance genes (152, 176, 199, 200), a logical alternative as long as care is taken to ensure that regional variants of VRE are identified and that the culture methods used are able to grow the newly imported variants.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Adequate hospital-based transmission control programs for VRE strongly depend on the quality and efficiency of laboratory testing. Microbiology laboratories need to consider all factors including sensitivity of methods for detection of existing VRE, including their ability to detect newly introduced clones/genes, time to detection/reporting, and cost. Currently, in most laboratories, a mix of culture and molecular testing is likely to be most efficient, with careful attention paid to strategic use of molecular testing when rapid turnaround time is most needed, and to the extent to which detection of isolates with borderline resistance to vancomycin is needed. All laboratories need to be aware that low MICs and low fecal density of enterococci will continue to be a challenge for control programs (24).

A better understanding of the differences between genes associated with vancomycin resistance in enterococci as compared to those in other nonpathogenic organisms may assist the designers of molecular tests in developing more specific tests. However, the variability of gene sequences within enterococci, the close relationship between *van* genes in enterococci and other genera, and the plasticity and rapid evolution of enterococci means that the dual problem of lack of specificity of *van* gene detection for VRE and lack of sensitivity due to gene variants being missed is likely to persist. This should not be interpreted to mean that molecular tests are not useful; rather, it means that enterococci will continue to be challenging organisms, in which careful test interpretation is always needed, and tests must continue to coevolve with the microbes.

Not all newly proposed rapid tests for the detection of VRE are molecular; one recent attempt proposes an immune-chromatographic assay to detect *vanA* ligase-producing enterococci (201). However, the plasticity of enterococci makes maintaining sensitivity in new enterococcal variants an ongoing challenge. In this light, our best hope for better diagnostics may lie in developing methods that include sequencing and permit the identification of variability in the genome.

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section IV

Molecular Diagnostics and Public Health

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The Impact of Molecular Diagnostics on Surveillance of Foodborne Infections

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19

The Centers for Disease Control and Prevention (CDC) estimates that foodborne diseases cause illness in one in six Americans (or 48 million people) each year, leading to 128,000 hospitalizations and 3,000 deaths (1, 2). Among the known foodborne pathogens, bacteria proportionally cause the most severe illness, being associated with 64% of hospitalizations and 64% of the deaths. The foodborne bacteria associated with most hospitalizations and deaths are *Salmonella*, *Campylobacter*, Shiga toxin-producing *Escherichia coli* (STEC), and *Listeria monocytogenes*. These bacteria are all zoonotic; i.e., they may be found in the normal intestinal biota of animals without causing disease and may spread to the environment and contaminate the food we eat.

Public health surveillance is the continuous, systematic collection, analysis, and interpretation of health-related data (http://www.who.int/topics/public_health_surveillance/en/). In the area of foodborne disease, surveillance is used to detect outbreaks, count illnesses, determine foods and settings causing illness (attribution), track trends to monitor the efficacy of control efforts, and provide physicians with information for patient care. This chapter will primarily focus on outbreak detection and investigation.

Outbreaks of foodborne infections are common, with more than 800 reported in the United States in 2011 and 2012 (3). Outbreaks are investigated and controlled using a multipronged approach involving microbiologists and epidemiologists from the food producers, public health, and food and veterinary authorities. The role of the microbiology laboratory in outbreak investigation and the potential for enhancement by molecular-based subtyping depends both on how the outbreak is detected and the stage of the investigation. Foodborne disease outbreaks are detected by one or more of three basic mechanisms including (i) syndromic surveillance, (ii) notification (reporting of outbreaks recognized by the public or medical community), and (iii) pathogen-specific (laboratory-based) surveillance (4). Each of these mechanisms has strengths and limitations and functions to a greater or lesser extent in different jurisdictions. Although they are described here separately, more than one method may be used in individual investigations.

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Syndromic surveillance refers to the collection and analysis of preclinical and prediagnostic health information as an early warning system to detect trends of public health importance. Preclinical data are obtained from sources such as school absenteeism or sales of diarrhea medications. Examples of clinical prediagnostic data sources are emergency room admission complaints and diagnostic test orders (5). These data may be obtained before laboratory testing and therefore potentially trigger an investigation faster than laboratory-based surveillance. However, the lack of specific laboratory information creates substantial signal-to-noise problems, which requires standard investigations to establish that the outbreak is real and to identify populations at risk. The usefulness of syndromic surveillance for foodborne disease has not been established (4) and will not be discussed further in this chapter. An exception is the surveillance of rare and highly specific diagnosed syndromes such as postdiarrheal hemolytic uremic syndrome or botulism, which functions in much the same manner as pathogen-specific surveillance.

Notification is a method of outbreak detection by which illness clusters are directly reported to public health agencies. The connection between cases is made by the public or health care providers who notice a greater than normal occurrence of patients with symptoms typical of foodborne infections or intoxications. This is sometimes referred to as "event-based surveillance," with the typical scenario being a church, school, or restaurant event where participants notice that others are ill. Since laboratory identification of a specific pathogen is not necessary for initial detection of the outbreak, notification is one of the most effective tools available for identifying new disease-causing agents. The STEC strain O157:H7 was initially identified as a public health problem through investigation of a reported disease cluster at a fast-food restaurant (6). Event-associated outbreaks are usually, but not always, caused by errors in terminal food preparation, such as improper cooking, handling, or storage.

In pathogen-specific surveillance, laboratory-based diagnostics and subtyping are used to detect clusters of disease that may represent outbreaks. Cases are often linked through shared disease etiology, such as illnesses due to *Salmonella* spp. or *L. monocytogenes*, that are reported to public health agencies by physicians and clinical laboratories. This type of surveillance is greatly enhanced by increasing the specificity of the case definition through

molecular subtyping. With this approach, it is not necessary for cases to be in the same location for clusters to be recognized (as is needed for notification and usually when old, less discriminatory subtyping methods are used); pathogen-specific subtyping-based surveillance is our most effective tool for identifying widespread low-level outbreaks caused by problems in food production, processing, and distribution. While not as common as local "church supper" type outbreaks, typically identified through notification, the problems identified in this way are generally amenable to control since they involve systems that are often centralized and regulated. Investigation of these outbreaks is one of our best tools for discovering previously unrecognized unsafe food production practices and new vehicles of contamination.

Laboratory testing is required for initial recognition of outbreaks detected through the pathogen-specific surveillance process, but not necessarily for outbreaks detected by notification. The raw material for pathogen-specific surveillance is isolates, specimens, and disease data from patients diagnosed in hospitals and clinics. For rare conditions such as listeriosis, large outbreaks may be detected solely by species-level information. For example, a 2011 multistate outbreak of *L. monocytogenes* associated with contaminated cantaloupe was initially detected by an unusual number of cases in Colorado during a short time period (7). For detecting outbreaks when they are smaller, or detecting outbreaks due to more common pathogens such as *Salmonella*, more discriminating subtyping methods are necessary to distinguish trends from background noise. It is in the area of pathogen-specific surveillance that molecular epidemiological techniques have had their greatest impact to date. In the past, traditional methods such as biotyping, antibiograms, serotyping, and phage typing were the only subtyping methods available, but since the 1980s these methods have gradually been replaced by molecular subtyping methods for outbreak investigations because they provide superior discriminatory power and thereby provide better sensitivity and specificity for outbreak investigations. This increased discrimination adds specificity to the cluster definition: cases that are more likely to share an epidemiological association are grouped together and cases less likely to be associated are excluded, thereby increasing the chance that smaller trends will be identified and outbreaks will be solved. Although foodborne outbreaks may be caused by multiple different strains (polyclonal or polymicrobial outbreaks), focusing on a specific strain is useful in the initial phase to identify the outbreak (8). Cases that may have been overlooked in the preliminary case findings may be added during subsequent phases of the investigation. In general, as the laboratory tests and case definition become more specific, outbreaks become easier to detect and solve. The current "gold standard" for laboratory surveillance of outbreaks is pulsed-field gel electrophoresis (PFGE). This is a universal subtyping method, since it may be used to subtype almost any pathogen with just a few modifications. It will be described in more detail later.

PFGE is the preferred method used by PulseNet, the national molecular subtyping network for foodborne disease surveillance (9), and by its global counterpart, PulseNet International (10). Since 1996, PulseNet has revolutionized the response to foodborne outbreaks in the United States. In this network of more than 80 public health and food regulatory agency laboratories, the foodborne pathogens are subtyped as they are received in the laboratory, and the data are uploaded to the national databases at CDC. The PFGE profiles are analyzed in real time both locally and centrally at CDC, and every time clusters of cases with indistinguishable patterns are identified, this information is passed on to the epidemiologists for follow-up. This surveillance has proven extremely efficient for outbreak investigation, and at any one time on average 25 clusters are being investigated. The network has been critical for the successful investigation of almost all high-profile outbreaks that have occurred in the United States since its inception. These investigations have led to the identification of many new vehicles that likely would not have been identified without the molecular subtyping information provided by PulseNet. However, the issue of cluster definition is becoming more complicated with the implementation of whole-genome sequencing (WGS) because even highly epidemiologically related isolates vary at the genomic level.

Once outbreaks are detected, the role of laboratory testing and molecular diagnostics varies with the phase of investigation. The main components of outbreak investigations were described by Reingold (11). The laboratory is often involved in the case definition, the identification of new cases, hypothesis generation, culture confirmation of the source of the outbreak, and measuring the effect of control measures.

The first step in most outbreak investigations is to establish a case definition. In pathogen-specific surveillance, an outbreak is detected as a cluster of isolates of a pathogen or subtype of a pathogen that occurs more frequently than expected above the historical baseline in a particular location or set of locations; thus, the pathogen/subtype is used to detect the cluster and thereby also defines the cases included in it. For outbreaks detected through notification, cases are defined by disease (having a certain set of symptoms), place (e.g., having attended a particular event, belonging to an affected group), and onset during a particular time interval. Although descriptive epidemiology is sufficient in some circumstances to define the outbreak (12), the infectious etiology is often needed for the case definition.

Laboratory findings are used with associated epidemiological information to group cases into categories as "confirmed" (laboratory-demonstrated agent), "probable" (strong epidemiological association without laboratory confirmation), or "possible" (weaker epidemiological association). Since cases infected with the same strain are more likely than patients that have not been culture confirmed to have been infected from the same source, such confirmed cases are preferably included in analytical studies such as case control or cohort studies. These types of studies compare exposure information from cases and controls to determine the likely common source(s).

Sometimes cases also cluster with isolates from food, water, or environmental sampling programs; this may provide a hint about the source of an outbreak in the hypothesis generation phase. Food and environmental samples may also be collected and tested as part of a trace-back investigation. This is particularly useful when the epidemiological evidence is not clear and several sources are suspected. Identification of an outbreak strain in an implicated food or food production environment puts most doubt to rest and can be extremely helpful in root-cause analysis. Although a food source may be identified statistically based on epidemiological evidence alone, the isolation of the outbreak strain from that source will provide culture confirmation.

A highly specific and restrictive case definition is usually critical for identifying common sources of outbreaks detected through pathogen surveillance, but widening the case definition is useful to define the true scope of an outbreak to identify cases that may have been missed by a strict microbiological case definition. This phase of the outbreak investigation may require a combination of epidemiological and microbiological approaches. Sometimes patients that do not fit the microbiological case definition are included at this stage because they are ill contacts of cases or have been exposed to the vehicle causing the outbreak (13). In other instances, when an implicated product is tested, additional strains or even different pathogen species are detected. These can be matched with seemingly sporadic cases in the community. Standard epidemiological methods are then used to demonstrate linkage with the new cases in the same manner as for the initial cluster, and the use of molecular methods such as PFGE and WGS increases confidence in the findings. In 2007, an outbreak of *Salmonella* serotype Wandsworth was tracked to a snack food. Testing of the product revealed the presence of another *Salmonella* strain of serotype Typhimurium; the PFGE pattern of this strain did not occur significantly over the historical background in the PulseNet database, but follow-up with interviews nevertheless confirmed 18 additional cases infected with this serotype (14). In the cantaloupe-associated listeriosis described above, patients infected with isolates displaying one of five PFGE patterns were also ultimately associated with exposure through product testing (7).

Finally, the effect of control measures to determine when an outbreak is over is done by monitoring of the incidence of infections caused by the outbreak strain until it has decreased to the level of the historical background.

In summary, investigation of foodborne disease outbreaks provides a unique opportunity for finding and correcting problems in the food supply that might not be otherwise recognized. Advances in molecular diagnostics should accelerate our understanding of the causes of foodborne disease in our communities, thus increasing opportunities for prevention.

METHODS USED FOR OUTBREAK DETECTION/INVESTIGATION AND CONTROL

Pulsed-Field Gel Electrophoresis

PFGE has been extensively discussed in a number of reviews (15, 16) but will briefly be discussed here because since the 1990s it has been the gold standard for discriminatory subtyping of foodborne bacterial pathogens and therefore is the standard against which new subtyping methods are compared.

During the 1970s, advances in molecular biology underscored the fact that the bacterial chromosome is the most fundamental molecule of identity in the cell. This understanding had enormous influence on approaches to epidemiological analysis, leading to the development of a variety of molecular methods for bacterial strain typing. Many of these methods were based at least in part on analysis of DNA restriction fragment patterns by agarose gel electrophoresis but suffered from the limitation that fragments larger than ca. 50 kb in size could not be reproducibly separated. This was remedied in 1984 with the discovery by Schwartz and Cantor (17) that PFGE could reliably separate megabase-sized DNA molecules. While the equipment required for PFGE was somewhat complex, the underlying electrophoretic principle was quite simple. Electrophoretic pulses at equidistant left and right angles from center could force the continuous reorientation of DNA molecules as they move toward the cathode. The larger the molecule, the longer the pulse or "switch time" required for reorientation. Thus, moving from shorter to longer pulses over time "windowed" the separation of smaller to larger DNA fragments ranging from ca. 20 kb to >1 Mb in size. Two additional discoveries were essential to the use of PFGE for epidemiological analysis: (i) the ability to isolate intact chromosomal DNA by *in situ* lysis of bacterial cells encased in agarose blocks and (ii) restriction enzymes with rare chromosomal recognition sites resulting in a manageable number of DNA fragments (e.g., 10 to 30) for post-electrophoresis visual and computer-assisted analysis.

PFGE quickly became the *de facto* standard for high discriminatory subtyping of most bacterial pathogens because (i) the same basic method could be used to subtype virtually all pathogens, a degree of universality that few other molecular methods possess; (ii) with proper standardization and quality control, results were reproducible between different laboratories; (iii) the output was provided in a "bar-code" like representation of >90% of the bacterial chromosome (16) that was easy to interpret; and (iv) semi-automatic computerized analysis was possible using specialized software, e.g., BioNumerics (Applied Maths, Sint-Martens-Latem, Belgium) and FPQuest (Bio-Rad Laboratories, Hercules, CA), enabling comparisons of profiles run on different gels and making it suitable for library type (definitive) subtyping. For these reasons, PFGE has since 1996 been the staple for all PulseNet surveillance as described in the previous section.

However, PFGE also has drawbacks: it is a fairly labor-intensive method requiring at least 2 days from start to finish with a pure culture; specialized equipment and well-trained personnel are a must; not all strains are typeable with PFGE; not all restriction fragments can be separated, making interpretation somewhat subjective; and the method carries no phylogenetic information, since fragments of similar size in different strains may originate at different places in the genome: i.e., patterns that appear similar may only be distantly related phylogenetically. This is the reason why PulseNet routinely only accepts indistinguishable isolates in their case definitions (16). Finally, PFGE does not always provide optimal discrimination for outbreak investigations: some PFGE patterns are more common than others, and it can be difficult to detect and delineate outbreaks caused by very common patterns; e.g., the most common PFGE profile among *Salmonella* serotype Enteritidis makes up almost 50% of the patterns belonging to that serotype in the database (http://www.cdc.gov/SALMONELLA/ENTERITIDIS/) (18).

Whole-Genome Sequencing

PFGE has served PulseNet well for almost 20 years, but because of the drawbacks mentioned in the previous section, the network has constantly been exploring new methods to supplement or even replace PFGE. Until recently no methods were identified that are as universally applicable and have sufficient discriminatory power to be used without PFGE. However, next-generation sequencing (NGS) technologies have become available that enable fairly easy and cost-efficient determination of whole-genome bacterial sequences in 1 to 2 days. Since the genomes of all

strains can be sequenced, WGS is a universal subtyping method; it may provide phylogenetically relevant information, and theoretically it has unsurpassable discriminatory power.

The details of the process of NGS are beyond the scope of this chapter and may be found elsewhere (19, 20). The different sequencing platforms use their own proprietary technology; in common, they all sequence the genome in short random fragments by a shotgun sequencing technique, generating fragment sizes or reads ranging from less than 100 bp to several thousand bp. The shotgun sequencing is repeated multiple times in the same process (massively parallel sequencing), whereby virtually the whole genome is sequenced a number of times. The sequencing coverage is the number of times the sequencing process is repeated across the genome. The reads are cleaned and assembled electronically after they have been generated; the assembly is either performed by aligning the fragments to a well-annotated genome of a closely related strain (reference-guided assembly) or *de novo*, where sequences are assembled based on overlapping regions between different fragments. This procedure is very computer resource-intensive and rarely results in a fully closed genome with the current technologies. Depending on the complexity of the genomes and the read lengths provided by the sequencing platforms, genomes are usually assembled into 5 to 200 pieces of contiguous sequences, or contigs. The notion of "whole"-genome sequencing thus is somewhat of an overstatement since the genomes are rarely fully assembled into one contig.

For surveillance purposes it is useful to retain backward compatibility with previously used subtyping methods. Theoretically, WGS is backward compatible with most molecular subtyping methods, but in practical terms this is rarely the case with the current NGS technology. Restriction fragment length polymorphisms, like PFGE patterns, cannot be reliably predicted from a whole-genome sequence, but this may be possible if the genome is closed and methylated bases are identified, since methylation blocks the action of the most commonly used restriction enzymes. Most sequencing platforms do not generate read lengths that are long enough to bridge regions with many repeats, and NGS is not an efficient way to resolve variable-number tandem repeats (VNTRs), which are the foundation for another molecular subtyping method, multilocus VNTR analysis (MLVA), that has been used increasingly in recent years (21–23). Thus, MLVA profiles can usually not be predicted from shotgun sequencing, either. The newest NGS platform, the PacBio RS II (Pacific Biosciences, Menlo Park, CA), provides information about methylation and generates the longest read lengths so far, enabling the closing of many genomes, which could solve the problem of the lack of WGS compatibility with PFGE and MLVA.

WGS data have been used to identify the phylogenetic diversity within and between bacterial species and are increasingly applied to the investigation of outbreaks detected by other means (24–30). Several analytical approaches may be used, the most common of which are kmer-based approaches, single-nucleotide polymorphism (SNP) analyses, and whole-genome multilocus sequence typing (wgMLST). The principle of the different approaches is illustrated in Fig. 1 and in the text below. Their strengths and weaknesses are outlined in Table 1.

Kmer-based approaches use a computer algorithm to chop the short raw reads or assemblies into smaller pieces of a defined length k ($k = 10$ in Fig. 1A). The optimal length of the kmer is determined by finding which length gives the most specificity and sensitivity when comparing kmers between isolates, and this length may vary by organism (31). Unlike restriction enzymes that only cut at specific sequences, the computer algorithm that generates kmers uses a sliding window to move down the length of the sequence reads or assemblies, 1 bp at a time, until

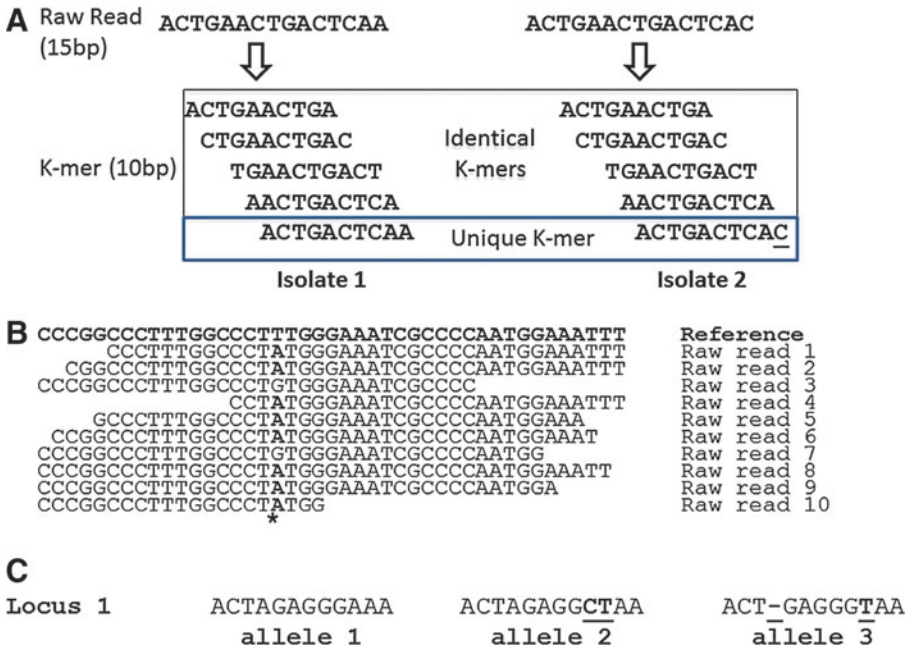

FIGURE 1 Schematic analytical principle of three approaches to whole-genome sequence analysis. (A) kmer, (B) hqSNP, (C) wgMLST.

TABLE 1 Key characteristics of different whole-genome sequencing analysis techniques used in outbreak investigations

Characteristic	kmers	hqSNPs	WgMLST
Speed of initial detection of targets	Intermediate	Slow	Slow
Speed of subsequent analysis	Intermediate	Intermediate	Fast
Requires reference genome	No	Yes	Yes
Identifies position of difference in whole genome	No	Yes	Yes
Leads to strain type nomenclature	No	No	Yes
Can extract virulence, antibiotic resistance, or serotype information from analysis	No	No	Yes
Resolution (for cluster detection)	Coarse initial analysis	High discriminatory subtyping	High discriminatory subtyping

kmers of the specific length *k* can no longer be generated (Fig. 1A). The resulting kmers are then compared between isolates, and the similarities and differences identified are used to approximate relatedness. A variant of the kmer approach is kSNPs, which looks at variation between kmers at the central nucleotide to determine SNP differences between isolate genomes (31). One of the main advantages of kmer-based approaches is that they do not require alignment to a reference genome, as opposed to some SNP-based approaches, and are less computationally intensive than other WGS comparison approaches. Kmers work especially well for coarse initial analysis; for finer-grain analysis the two analytical approaches described below are better suited. A disadvantage of using kmers is that they depend on data quality and do not provide positional information; i.e., kmers in different strains may not be located in the same genetic region, so like PFGE, kmer analysis may not reflect true phylogeny. Since the kmer content changes as isolates are added or removed from the comparison, the kmer profile of an isolate cannot be named unambiguously; i.e., the kmer approaches are comparative by nature.

Another genome comparison approach is the high-quality SNP (hqSNP) approach, which in general is more discriminatory than kmer-based approaches. In the hqSNP approach, the reads from each isolate are aligned against a closed or high-quality draft reference genome ("Reference" in Fig. 1B), generating what is referred to as a pile-up, and SNPs are "called" at positions where the base pair in the pile-up differs from the base pair in the reference genome (A in the pile-up of 10 raw reads in Fig. 1B). Often a SNP is not called until it reaches or exceeds a quality threshold for the number of reads that are aligned at that position (coverage) and the frequency at which that SNP call is found in the reads. These high-quality thresholds vary depending on the clonality of the organism. For rapidly evolving bacteria such as *E. coli*, higher thresholds are used to filter out SNPs in reads that may be due to noise or errors generated by the sequencing procedure; infrequent variants, i.e., rare SNPs, are often also filtered. For *E. coli* the threshold of detection may be set high, at 20 times coverage and 95% frequency. Alternatively, for clonal organisms like *Mycobacterium tuberculosis* the goal of an hqSNP analysis would be to pick up any variation while filtering out errors and noise due to the sequencing process. The threshold of detection of hqSNPs may thus be set lower, e.g., at 5 times coverage and 40% frequency (32). If the organism in Fig. 1B was *M. tuberculosis*, the A SNP would be called because the coverage was 10 times and the frequency 8 out of 10 (80%).

Another consideration when calling hqSNPs is where in the genome the SNP is located. Depending on the organism and the epidemiologic context, it may be more informative to consider only SNPs that are found in coding regions, or even core genes, rather than the entire genome. It may also be advantageous to filter out SNPs from genes involved in horizontal gene transfer, e.g., phages or transposons, which may create noise in comparisons between phylogenetically and epidemiologically closely related isolates. Once the thresholds and SNP positions have been determined, a phylogenetically relevant comparison may be performed. The drawbacks of hqSNP analysis are that it is computationally intensive and, like kmers, is by nature a comparative subtyping method since the hqSNPs are only called if they are present among the isolates being compared.

The wgMLST approach compares genes between isolates rather than individual base-pair positions as is done in hqSNP analysis. In contrast to a traditional MLST approach that compares a limited number of conserved housekeeping genes, typically seven, to determine the sequence type or clonal complex, wgMLST extends the analysis to almost all genes in a genome (33). The different genes or loci included in a wgMLST scheme are determined from a collection of well-annotated reference genomes of isolates that represent the full genetic diversity of the species, pathotype, or serotype being investigated. After the loci are defined, the reads or assemblies of test isolates are compared against these loci and an allele number is assigned if the locus is found. If a test isolate does not contain a locus, it is registered as missing. For each allele, any change in the gene sequence, including insertions, deletions, and SNPs, is used to define a new allele number.

In Fig. 1C, three alleles from a locus named locus 1 are shown differing from the first at the underlined positions. In a seven-gene MLST scheme variations in seven genes or loci are assessed, whereas in wgMLST variations in all genes, typically a thousand or more, are assessed. Often a set of parameters are defined to decide whether the differences between the potential new allele and locus definition are within the variation that is expected in that

coding region. Once all the alleles have been called, these allele numbers are used to compare the genotype of the isolates. As opposed to kmer or hqSNP comparisons that require a new computer-intensive analysis every time new isolate genomes are being added to a comparison, once allele numbers are assigned, just those numbers, rather than the entire WGS, are compared. This greatly cuts down on computational demand for doing new comparisons. Additionally, wgMLST types are definitive and may be named, thereby greatly easing communication between the partners in an epidemiological investigation at variance with kmers and hqSNPs. wgMLST seems to be as discriminatory as hqSNP comparisons in its most inclusive form, and the discrimination may be tailored to the clonality of the organism and the epidemiological context; e.g., in an outbreak situation all loci may be considered, whereas that may not be necessary for microbiological attribution studies. For these reasons, the wgMLST approach seems to be most useful for surveillance networks like PulseNet. A drawback of the wgMLST approach is that allele curation is required.

WGS is now increasingly used to supplement PFGE in the investigation of outbreaks that have been detected by other means. In 2013 it was used as a supplement to PFGE in the case definition of a listeriosis outbreak traced back to artisan cheese produced in Wisconsin (http://www.cdc.gov/listeria/outbreaks/cheese-07-13/). Initially, only PFGE was used to define the cases. However, the outbreak pattern was fairly common, and for two potential case patients, the relation to the outbreak was uncertain: one patient denied consumption of any cheese and another patient who lived in an area where the cheese was sold was lost for follow-up. WGS was performed on isolates from all known patients related to the outbreak, a product isolate, and a number of current and historical clinical isolates displaying the outbreak PFGE pattern; by hqSNP analysis, all isolates and the product isolate formed a tight cluster separate from the historical isolates (Fig. 2). The isolate from the patient who denied cheese consumption did not cluster with the outbreak isolates, whereas the isolate from the patient who had been lost for follow-up did; the case counts of the outbreak were adjusted accordingly.

Months after the closure of the outbreak investigation, additional cases of listeriosis were identified with the outbreak PFGE pattern. There was concern that the outbreak had not been controlled, and the isolates were sequenced. The data was analyzed by all three approaches—hqSNPs, kmers, and wgMLST—and it was shown that these new cases clustered separately from the outbreak isolates. Some of the isolates formed a separate cluster, and a new cluster investigation was started; unfortunately, no common source was identified (CDC, unpublished).

WGS was recently introduced for the surveillance of infections caused by *Salmonella* serovar Enteritidis in the state of New York (18). The utility of the method using hqSNP comparison was first confirmed on historical isolates including a well-defined local outbreak. In this way two additional small clusters that had not been recognized through PFGE-based PulseNet surveillance were identified. Then, the method was implemented for prospective surveillance in 2012. Isolates with an unusual PFGE pattern that was part of a multistate outbreak associated with ground beef also clustered by WGS along with additional isolates with different PFGE patterns not recognized to be part of the outbreak. Unfortunately, the relationship to the outbreak was not epidemiologically confirmed for these isolates. Additionally, a number of clusters were identified among isolates with common PFGE patterns. However, no outbreak vehicle was identified for any of these. This indicates that WGS may provide more specificity to an outbreak investigation than PFGE and at the same time recognize relationships between isolates not apparent by PFGE. The utility of WGS to differentiate between isolates of *Salmonella* serovar Enteritidis displaying the most common PFGE pattern (PulseNet pattern JEGX01.0004) in the United States was also established in a retrospective study by Allard et al. (34).

It is clear that it is feasible to use WGS for the detection and investigation of foodborne outbreaks and that its use adds power to the investigation, but a number of open questions need to be addressed before it may be used for routine surveillance replacing PFGE.

When PFGE is used for cluster/outbreak detection, only isolates with indistinguishable patterns are routinely considered (35). However, with WGS hardly any isolates are identical, and differences between isolates represent a continuum, with outbreak-related isolates showing fewer differences than epidemiologically unrelated isolates. It would be desirable to establish criteria to define an outbreak by WGS. However, few data are currently available to establish such criteria, and currently only data on SNP differences are available. For example, in a comparison of *Salmonella* serovar Javiana isolates from two confirmed outbreaks associated with three PFGE patterns, two of which were related (CDC, unpublished), isolates from the first outbreak (A) differed by 16 to 45 hqSNPs, whereas 6 to 28 hqSNP differences were found between isolates in the other outbreak (B). The median difference between these two outbreaks was 1154.5 hqSNPs. Outbreak A was local, associated with a common meal provider, and B was a multistate outbreak associated with a sandwich chain, with cases identified in five states. These data taken together could indicate that fewer than 50 hqSNPs might be used to define an outbreak. However, in other instances this definition may be too stringent, failing to identify outbreak-related cases, or be the opposite, too relaxed, thereby falsely linking isolates that are not related.

For example, in the study of den Bakker et al. (18) isolates of the highly clonal *Salmonella* serotype Enteritidis in confirmed outbreaks differed by fewer than 3 hqSNPs, whereas isolates from different outbreaks sometimes differed by fewer than 50 hqSNPs. The isolates linked to the artisan cheese listeriosis outbreak mentioned above also displayed very few hqSNP differences (Fig. 2). Thus, at present it is not possible define hard criteria to delineate all outbreaks reliably. This definition will depend on the epidemiological context, the type of outbreak (point source versus ongoing), the clonality of the organism, and the analytical pipeline used.

WGS also shows promise for replacing a number of phenotypic and molecular methods currently being used to identify and characterize foodborne pathogens. For many years, targeted sequence of 16S rDNA and other housekeeping genes has been used to identify the genus and species of bacteria (36). Likewise, virulence genes, serotype encoding genes, and antimicrobial resistance genes are routinely detected by PCR and/or hybridization to characterize foodborne pathogens in reference laboratories. Information about these genes may also be extracted from WGS (37), and such applications are already available on the Web, e.g., ResFinder, VirulenceFinder, and CARD (38, 39). However, to be truly useful for surveillance, these characteristics

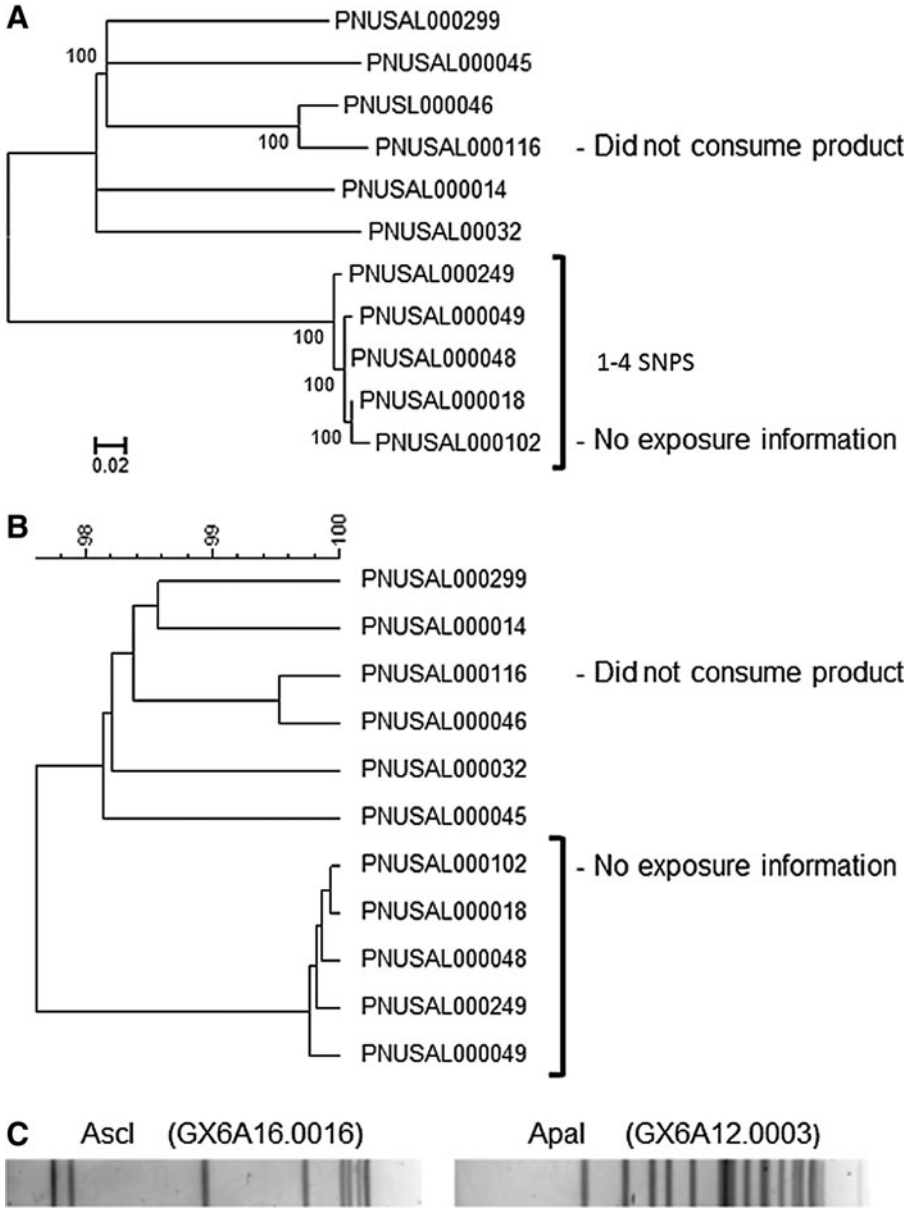

FIGURE 2 hqSNP (A) and wgMLST (B) trees of isolates of *Listeria monocytogenes* that are indistinguishable by PFGE (C). Isolates associated with an outbreak related to consumption of artisan cheese are highlighted with brackets and shown along with contemporary and historical isolates with no relation to the outbreak. The isolates from the patient who did not consume the artisan cheese product or did not have exposure information are denoted. The hqSNP and wgMLST analyses produced equivalent results.The hqSNPs were defined by consensus ≥75% and coverage ≥×10. The wgMLST analysis and UPGMA tree was generated using BioNumerics 7.5 (Applied Maths).

should all be built into the same workflow, thus eliminating the need to manually query multiple websites.

Culture-Independent Diagnostics and Foodborne Disease Surveillance

Molecular diagnostics is changing both clinical medicine and public health practice. An emerging generation of culture-independent diagnostic tests (CIDTs) for rapid diagnosis of patients is in the process of revolutionizing clinical microbiology practice, especially in the area of gastrointestinal disease. These syndrome-based panels not only provide results in a clinically useful timeframe (which is not always the case with culture), but make it possible to efficiently consolidate bacterial, viral, and parasitic pathogens into a single laboratory workflow. Some panels include agents for which practical clinical laboratory tests have not been available, such as enterotoxigenic *E. coli*, enteropathogenic *E. coli*, and norovirus. Despite their obvious benefits for patients, physicians, and clinical laboratories, the emergence of these tests poses a variety of challenges for clinicians and especially for public health (40, 41).

Often, multiple pathogens will be detected in the same specimen, raising the question of whether all are responsible for the patient's symptoms. In some instances, one pathogen is more likely to be causing the patient's symptoms than the other; for example, if *Clostridium difficile* is found along with *Salmonella* in an outpatient that has not recently been treated with antibiotics and that has acute diarrhea, the *Salmonella* is the likely cause of the patient's symptoms. But what if the same pathogens are found in a patient that has been hospitalized for a week? Systematic epidemiological studies are needed to answer questions like this. Of particular public health importance, CIDTs do not result in production of an isolate as part of the testing process. Current subtyping methods used for strain characterization, surveillance, and outbreak detection such as PFGE and WGS require isolates.

Therefore, it will be necessary in the short term to maintain the flow of isolates through reflexive (i.e., automatic) culturing of CIDT positives by clinical or public health laboratories to sustain surveillance. The optimal long-term approach for public health is to develop strain characterization tests that are themselves culture-independent. However, the development challenges are not trivial. As with clinical diagnostic tests, the targets must be conserved within the pathogen group of interest and not be present in the normal enteric biota. Additionally, targets must yield epidemiologically meaningful subtype, virulence, and susceptibility information, and a mechanism must be developed to link targets to a single organism. This is necessary since many virulence and susceptibility mechanisms are not unique to specific enteric pathogens. For example, many STEC strains require specific adherence factors such as intimin to be virulent. Intimin may be present in other gut biota (42) but only functions as an STEC covirulence factor when it is present on the same cell as the Shiga toxin gene.

Metagenomics

Metagenomics is the study of genetic material recovered directly from defined environments. The principal environments of interest regarding foodborne disease are the human gastrointestinal tract; the plants, animals, and complex foods that humans consume; and the environments where food is grown and processed. As will be described below, metagenomics is only partially practical at this time, but it is anticipated that this emerging field will play an increasingly important role in surveillance of foodborne infections as NGS technology, bioinformatics, and computer technology continue to advance. Metagenomics is by nature culture-independent, which is important because (i) only a very small percentage of gut microbes are known to be culturable (43), (ii) the etiology of approximately 80% of cases of foodborne disease is unspecified and mostly unknown (1), (iii) public health pathogen characterization methods that do not require culture will be needed to keep public health monitoring programs viable as clinical diagnostics moves toward culture-independent diagnostics, and (iv) culture-independent characterization methods have the potential for being faster and more efficient than culture. Metagenomics has the same drawback as CIDTs for clinical diagnostics concerning the problem of determining the cause of a patient's symptoms if multiple pathogens are detected. The range of potential applications for metagenomics in foodborne disease surveillance is vast and largely untapped, including characterization of pathogens for disease monitoring programs such as PulseNet, pathogen discovery in outbreaks of undetermined etiology, improving understanding of microbial communities in illness and health, food identification pre- or postconsumption as part of outbreak investigations, and examination of host factors. We anticipate that at some time in the next 15 years clinical diagnostics and public health testing will merge into a single testing paradigm that includes metagenomics and perhaps one or more of the other "omics" such as metabolomics or metaproteomics.

The human gut is a complex environment with over 10^{14} microorganisms, 1,200 described species and 10,000 to 40,000 predicted total species. Each individual is thought to typically harbor 1,000 bacterial species, and 2% of one's microbial species are shared by at least 50% of the population (43). The microbial load includes bacteria, viruses, fungi, and eukaryotic parasites. In addition, human DNA is present, presumably at higher levels during an inflammatory response to pathogens such as *Salmonella*, *Shigella*, and pathogenic amoeba. DNA is also present from plant and animals consumed as food. On the surface, it would seem that applying metagenomic analysis to such a mixture would be a fool's errand, that signal-to-noise and data management obstacles would be overwhelming. However, as both sequencing and computing technology rapidly advance, this approach should become increasingly practical.

Two basic approaches to metagenomics exist: amplicon sequencing and shotgun metagenomics (44). Both have potential applications in foodborne disease surveillance.

The most widely used amplicon sequencing approach uses 16S and 18S rRNA loci for characterization of bacterial and eukaryotic populations, respectively. Since this approach involves amplification, signal-to-noise issues are greatly reduced. Assays based on 16S are used routinely in research and are becoming increasingly common for diagnostic applications such as fever of unknown origin (45) and for broad characterization of microbial populations in the environment. Molecular markers including 18S have been used in the identification of seafood to control mislabeling (46) and could presumably be used to identify complex food components in an outbreak setting. Both 16S and 18S were used by Ottesen et al. to characterize the microbial biota of tomato plants in an effort to understand root causes of tomato-associated salmonellosis outbreaks (47). Unfortunately, 16S does not provide sufficient differentiation of most foodborne pathogens and would not be applicable for subtyping or further characterization. Alternate targets for use in a fecal or food matrix may prove useful for foodborne disease. Such targets would need to be bounded by loci that are conserved within and unique to the pathogen of interest. The region between the PCR binding sites would need sufficient heterogeneity to answer epidemiological questions as needed (e.g., species, serotype, subtype, virulence, and antibiotic susceptibility), and markers would need to be linked by quantitative or other means to assure that they are on the same cell.

Shotgun sequencing ("deep sequencing") represents the other major metagenomics approach. In this process, total nucleic acids are sheared, sequenced, and analyzed. Raw reads are either identified directly by comparison to reference genomes or reads are assembled and contigs identified in the same manner as when assembling genomes from pure cultures. Shotgun sequencing theoretically allows any organism present to be sequenced with no *a priori* knowledge. Maximum information about the sample is obtained, and it should be possible to tease apart complex microbial

interactions. This could be especially useful for foodborne outbreaks of undetermined etiology. A process that made it practical to assemble pathogen genomes directly in specimens without the need for culture could replace current surveillance methods. Some success was achieved by Loman et al. with sequencing of the *E. coli* O104:H4 genome from the massive 2011 outbreak in Germany (48) using this approach. However, the authors have highlighted important limitations with current technology including high cost, lack of speed, and poor diagnostic sensitivity. With fecal samples, sensitivity may be the largest barrier to overcome because target pathogens may be present at frequencies many orders of magnitude below that of the background biota. For example, to obtain 30× coverage for a pathogen present at 10^5 genomes/ml in a background of 10^{11} genomes/ml, 3.0 $\times 10^7$ coverage would be required—a staggering amount of sequencing. Computational resources are also limiting with current algorithms for assembly and analysis (44). However, several likely advances may alter this adverse equation including (i) better physical mitigation methods (removing nucleic acids that are not of interest), (ii) sequencing technology that produces longer and higher-fidelity reads, which require less coverage, (iii) cheaper sequencing technology, (iv) more efficient pipelines and improved analysis software, and (v) greater computing and data storage infrastructure. Active research and development are occurring in all of these areas.

The change in molecular epidemiological methods used by public health agencies from PFGE to WGS and ultimately to metagenomics will likely continue for at least a decade. The technological knowledge and the insight into the genomics of pathogens we gain from culture-based WGS are critical to the development of culture-independent metagenomic methods to meet the food safety challenges of the 21st century. In the interim period the challenge will be to retain cultures for public health surveillance until the sequencing technology and bioinformatics have evolved to a state that makes real-time metagenomics feasible and cost-effective.

The findings and conclusions in this chapter are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Use of trade names is for identification only and does not imply endorsement by the Centers for Disease Control and Prevention or by the U.S. Department of Health and Human Services.

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Role of Molecular Methods in Improving Public Health Surveillance of Infections Caused by Antimicrobial-Resistant Bacteria in Health Care and Community Settings

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20

The first multidrug-resistant isolates of *Enterobacteriaceae* that were recognized as clinically significant were strains of *Shigella* spp. isolated in Japan in the late 1950s. These strains demonstrated resistance to chloramphenicol, streptomycin, sulfonamides, and tetracycline (1). By 1960, Watanabe and Fukasawa had demonstrated that all four resistance markers were transmissible from *Shigella* donor strains to recipient strains of *Escherichia coli* and *Salmonella enterica* serovar Typhimurium via R-factors (2). Resistance to these and other first-line antimicrobial agents, however, remained rare among other genera of the *Enterobacteriaceae*. Resistance was seen as an anomaly. The discovery of the TEM beta-lactamase (3), which would become the most common cause of ampicillin and first-generation cephalosporin resistance in contemporary *E. coli* isolates, was still 6 years away. In the 1970s, reports of multidrug-resistant bacteria began to appear more frequently, and susceptibility to beta-lactams, aminoglycosides, and sulfonamides began to wane (4). Fast-forward to the 21st century when pan-susceptible clinical isolates of bacteria are now rare and multiple drug-resistant organisms (MDROs) representing dozens of bacterial species have been recognized and are spreading worldwide (5). Multidrug-resistant strains of staphylococci, enterococci, pseudomonads, acinetobacters, and members of the *Enterobacteriaceae* family are isolated with increasing frequency in clinical microbiology laboratories around the world, and controlling their spread has become a public health priority (6). Globally, we have now moved into the era of antimicrobial resistance.

SURVEILLANCE DEFINITION

Surveillance is a key component of public health practice for both health care-associated and community-associated infections. The World Health Organization (WHO) defines public health surveillance as "the continuous, systematic collection, analysis, and interpretation of health-related data needed for the planning, implementation, and evaluation of public health practice" (http://www.who.int/topics/public_health_surveillance/en/). Antimicrobial resistance surveillance is a crucial public health activity, which must also fulfill all of these roles. It must not only provide epidemiologic data but monitor the effectiveness of interventions and inform public health policy. Ideally, to accomplish this broad mission, a truly global and highly integrated surveillance system is needed since resistant bacteria do not honor national borders. But, as the 2014 WHO surveillance report makes abundantly clear, there is a critical absence of harmonization among the many local, regional, and national surveillance systems around the world (5). This chapter will explore multiple aspects of antimicrobial resistance surveillance and the role that molecular methods play.

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SURVEILLANCE SYSTEMS

European Centre for Disease Prevention and Control (ECDC)

EARS-NET

The European Antimicrobial Resistance Surveillance Network (EARS-NET), a program of the ECDC, tracks 35 specific organism-resistance combinations (some of which include just resistant levels and others in which resistant and intermediate values are combined) and makes the data available on the ECDC website. These can be viewed at http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx. This is a valuable tool for following trends in antimicrobial resistance longitudinally among multiple organism-drug combinations in Europe and exploring or ascertaining the effects of natural bacterial evolution and various public health and health care-based interventions. The data are collected from multiple laboratories in up to 31 countries. The EARS-NET website notes the following limitation: "The number of isolates reported per country to EARS-NET can vary substantially due to large variations in the number of inhabitants or reporting laboratories. To avoid extreme values, country data are only shown on maps if they are based on at least 10 isolates." Even given this limitation, the data provide tremendous insights into the changing epidemiology of antimicrobial resistance across Europe.

Regional MRSA Control Efforts in Europe

The decline of methicillin-resistant *Staphylococcus aureus* (MRSA) rates in a number of countries across Europe over the last decade has been well documented in the EARS-Net system (Fig. 1). Regional surveillance efforts, such as those in Germany, have also demonstrated the success of the region-wide "search and follow" strategy (slightly different from the "search and destroy" policy used in The Netherlands [7]) for reducing MRSA rates, a program that has been implemented in 40 German hospitals (8). These are important data because they demonstrate the impact of specific efforts to stem the decrease in MRSA incidence in a region, not just in a single hospital, and emphasize the importance of monitoring the effectiveness of the intervention with continued surveillance.

Coupled with the surveillance efforts focused on defining MRSA rates of infection are the efforts to track specific strain types of MRSA across Europe. Data on the staphylococcal protein A (spa) types of MRSA circulating in Europe have been instrumental in delineating the local and regional evolution of MRSA in Europe (9). Strain typing is a valuable public health tool because it indicates fluctuations in, and evolution of, strains in regions over time. It is intriguing to compare the spa types circulating in Europe with those in the United States (9, 10). Of the top five spa types in Europe in 2010, only two are commonly seen in the Unites States (i.e., t002 and t008), showing the importance of local selective pressures on the evolution of strains. Local evolution of strains of MRSA was also described by Li et al. (11). As MRSA strains change, so do virulence characteristics, such as the acquisition of the Panton-Valentine Leucocidin (PVL) toxin, as well as antimicrobial resistance patterns, which typically adapt to antimicrobial use patterns in a region. It is critical from both a public health and a medical standpoint that antimicrobial resistance patterns be monitored so that interventions to halt the spread of resistant microorganisms can be monitored and so empiric therapy for bloodstream and other infections can be optimized. Molecular assays, such as PCR-based tests for MRSA and methicillin-susceptible *S. aureus* (MSSA) that can be performed directly on clinical samples (12), can guide therapy in a hospital or emergency rooms to decrease unnecessary use of MRSA-active drugs (13). Molecular assays that track the spread of clindamycin resistance determinants, such as *ermA*, and erythromycin resistance determinants, such as *msrA*, which does

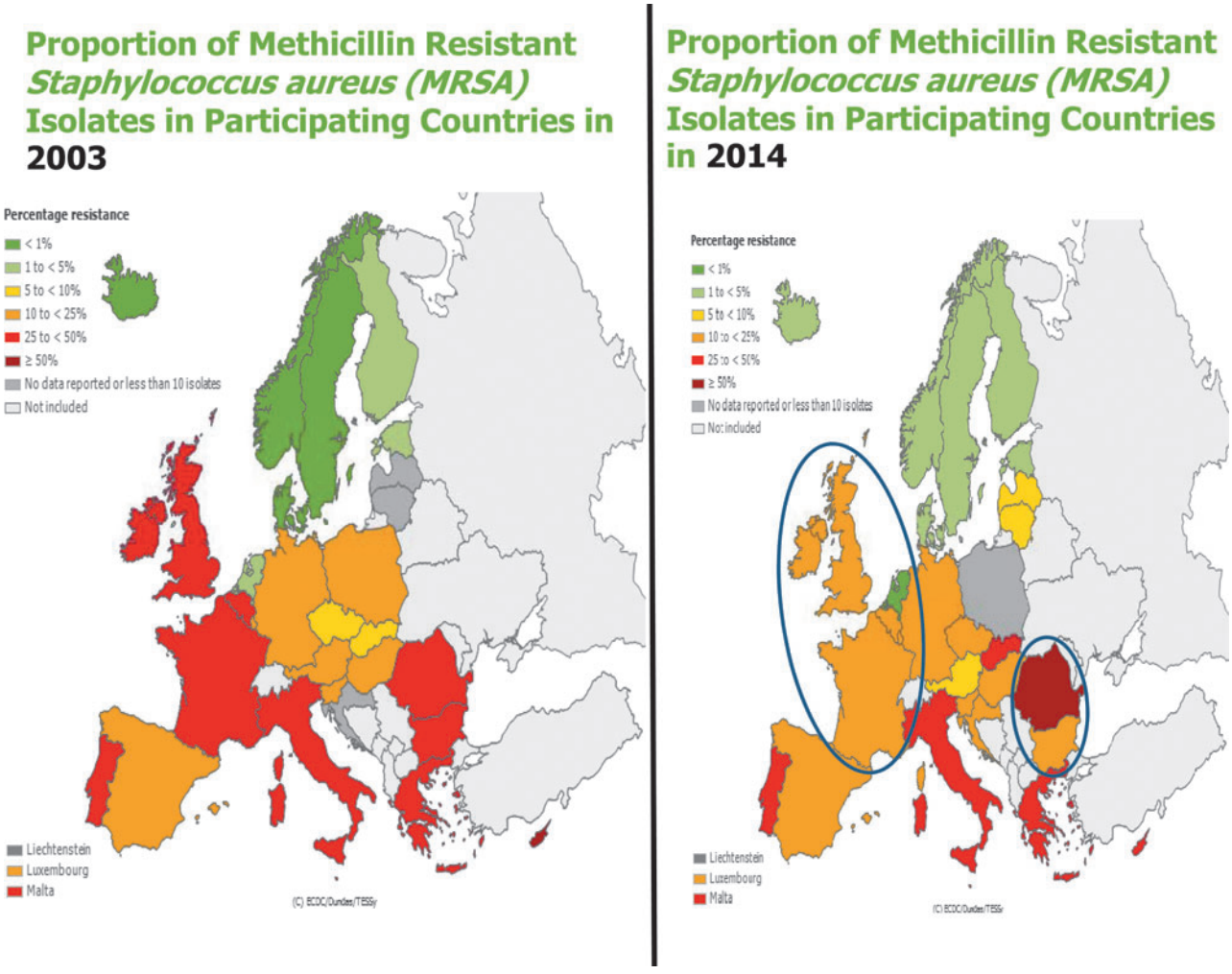

FIGURE 1 Data on prevalence of MRSA in Europe in 2003 and 2014 from EARS-NET (http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx). Significant changes are indicated in the circled areas.

TABLE 1 Susceptibility of *Klebsiella pneumoniae* isolates to carbapenems in France, Greece, Italy, United Kingdom, and Germany in 2013[a]

Country	No. susceptible	No. intermediate	No. resistant	Total (*n*)	%S[b]	%I[b]	%R[b]
France	1,821	9	12	1,842	98.9	0.5	0.7
Germany	737	1	5	743	99.2	0.1	0.7
Greece	479	12	718	1,209	39.6	1.0	59.4
Italy	930	24	499	1,453	64.0	1.7	34.3
United Kingdom	1,044	2	5	1,051	99.3	0.2	0.5

[a]Data from EARS-NET (http://www.ecdc.europa.eu/en/healthtopics/antimicrobial_resistance/database/Pages/map_reports.aspx).
[b]S, susceptible; I, intermediate; R, resistant.

not mediate clindamycin resistance (14), could potentially be of value in defining empiric therapy for wound infections when clindamycin is a consideration (15). However, such programs are not currently in place.

Tracking Carbapenem Resistance

Carbapenem resistance in *Klebsiella pneumoniae* and *E. coli* is also tracked by EARS-NET. Table 1 shows an example of data for carbapenem resistance in *K. pneumoniae* in five countries. Phenotypic data are important for understanding changes in resistance patterns over time, but molecular analysis of the mechanisms of carbapenem resistance is critical for understanding the epidemiologic spread of resistance. Data from several studies, including surveillance studies in the United Kingdom (16), Belgium (17), and France (18), provide important insights into how carbapenem resistance is evolving in Europe, including the emergence of OXA-48-containing strains. This is important because such strains may not grow well on first-generation chromogenic agars, especially those that contain third-generation cephalosporins as the selective agents (19, 20).

North American Surveillance Systems

CDC

National Antimicrobial Resistance Monitoring System (NARMS)

The CDC has several surveillance systems that collect and analyze data on antimicrobial resistance. NARMS is a collaboration among state and local public health departments, the CDC, the U.S. Food and Drug Administration (FDA), and the U.S. Department of Agriculture (USDA) to track changes in the antimicrobial susceptibility patterns of key enteric bacteria that (i) cause human infections (CDC), (ii) are present in retail meats (FDA), and (iii) are recovered from food animals (USDA) in the United States. Public health laboratories submit *Salmonella*, *Campylobacter*, *Shigella*, *E. coli* O157, and *Vibrio* (other than *Vibrio cholerae)* spp. isolated from clinical specimens from humans to a central laboratory for antimicrobial susceptibility testing (21). Retail meat sampling is carried out in 14 states, which includes sampling of chickens, ground turkey, ground beef, and pork chops. Isolates also undergo strain typing. The USDA performs similar testing on *Salmonella*, *Campylobacter*, *Enterococcus*, and *E. coli* obtained from food-producing animal specimens at slaughter houses and processing plants throughout the United States. There is only limited use of molecular testing for mechanisms of resistance in these surveys.

National Healthcare Safety Network (NHSN)

NHSN (http://www.cdc.gov/nhsn/dataStat.html) monitors health care-associated infections (HAIs) in the United States among almost 13,000 facilities and generates data by state, by region, and nationally. The system, which began as the National Nosocomial Infection Surveillance System, was developed to help hospitals identify potential outbreaks of HAIs in their institutions, measure the effectiveness of infection prevention efforts, and ultimately control the spread of HAIs. The data that were collected, initially from approximately 300 hospitals, were used to establish benchmark rates of key device-associated infections that hospitals could use to judge the effectiveness of their infection control programs against those of other hospitals of similar size or case mix. Data gathered included central line-associated bloodstream infections, catheter-associated urinary tract infections, surgical site infections, and ventilator-associated pneumonia (22). The system also tracks rates of antimicrobial resistance by organism for key organism-drug combinations (23), providing trending data over time. These data have shown the rise and eventual decline of central line-associated bloodstream infection MRSA rates in the United States over the last decade (24). Both the National Nosocomial Infection Surveillance System and NHSN have also served as an early warning system for the emergence of vancomycin-resistant *Enterococcus* infections, vancomycin-resistant *S. aureus* (25), and most recently, carbapenem-resistant *K. pneumoniae*.

These systems have also documented the positive impact of a variety of public health interventions, such as antibiotic stewardship and implementation of contact precaution strategies, to halt the spread of MDROs, such as those detailed in the CDC's latest report on HAIs (26). The report documents the progress that has been made over the last several years with regard to the "National Action Plan to Prevent Healthcare-Associated Infections: Road Map to Elimination" set by the U.S. Department of Health and Human Services. NHSN has played a central role in monitoring the epidemiology of HAI problems, and the data have guided the CDC and Department of Health and Human Services in developing public health policy. Among recent developments, NHSN data indicate that healthcare-associated *Clostridium difficile* infections are now more common than health care-associated MRSA infections in the United States (27)—likely a consequence of the way antimicrobial agents are used. Since organisms are not collected by the NHSN system, characterization of resistance mechanisms using molecular methods and strain typing data is not generated on a routine basis.

Gonococcal Isolate Surveillance Program (GISP)

Another CDC-based antimicrobial-resistance surveillance program is GISP, which tracks the development of resistance in *Neisseria gonorrhoeae* from a modest number of public health clinics where antimicrobial susceptibility testing is performed on the first 25 isolates recovered each month from men who seek health care at the clinic. Sexually transmitted infection clinics from 29 state or city health departments contributed 5,495 gonococcal isolates to GISP in 2012 (http://www.cdc.gov/std/gisp2012/gisp_2012_text_figures_tables_web.pdf). Fortunately, decreased susceptibility to ceftriaxone (defined as MIC ≥ 0.5 μg/ml) remained low at 0.3%. The data on fluoroquinolone resistance, however, are not as encouraging. In 2012, 27.1% of *N. gonorrhoeae* isolates from men who had sex with men demonstrated ciprofloxacin resistance (defined as MIC ≥ 1 μg/ml), while 8.7% of isolates from men who had sex with women showed resistance. Thus, ciprofloxacin is rarely used during first-line treatment. Strain typing is not routinely undertaken by GISP. The availability of rapid molecular methods to test for antimicrobial resistance to fluoroquinolones and extended-spectrum cephalosporins directly in clinical samples would be of tremendous value. Methods to identify wild-type strains, i.e., fluoroquinolone-susceptible isolates, have been described but never implemented as part of the surveillance program (28).

Canadian Antimicrobial Resistance Surveillance System

Canada also has an excellent surveillance system for antimicrobial-resistant bacterial pathogens. The Canadian Antimicrobial Resistance Alliance launched a website in early 2007 (www.can-r.ca). The site is described as "an online research portal designed to aid and educate Canadian health care providers on the escalating issue of antimicrobial resistance in Canada." The Canadian Hospital Ward Antibiotic Resistance Surveillance study was "the first national, ongoing, prospective, surveillance study assessing antimicrobial activity against pathogens from Canadian hospitals, including hospital clinics, emergency rooms, medical and surgical wards and ICUs." Unlike many other surveillance systems, this study included the molecular characterization of key pathogens such as MRSA in their methods. Pulsed-field gel electrophoresis (PFGE) typing of MRSA isolates as well as staphylococcal cassette chromosome *mec* (SCC*mec*) typing and detection of PVL toxin genes by PCR are part of the analyses. In addition, molecular characterization of extended-spectrum beta-lactamase (ESBL) genes is undertaken to better understand the epidemiology of resistance genes in Canada. Thus, the understanding of antimicrobial resistance determinants in the Canadian system is more comprehensive than in many other surveillance systems. The increase in the diversity of carbapenem resistance determinants in Canada is shown in Fig. 2.

Australian Group on Antimicrobial Resistance (AGAR)

The AGAR system in Australia supports a survey of antimicrobial resistance results for *S. aureus*, *Streptococcus pneumoniae*, enterococci, and Gram-negative bacilli. The resistance data are supplemented with molecular testing to delineate the mechanisms of resistance that are spreading across the continent (http://www.agargroup.org/surveys). AGAR collects data from 30 microbiology laboratories in Australia, including 4 private laboratories. AGAR has also conducted a number of strain typing studies to delineate changes in the epidemiology of MRSA and other pathogens in Australia.

WHONET

WHONET is not a surveillance system *per se*; rather, it is a freely available software tool that facilitates analysis of

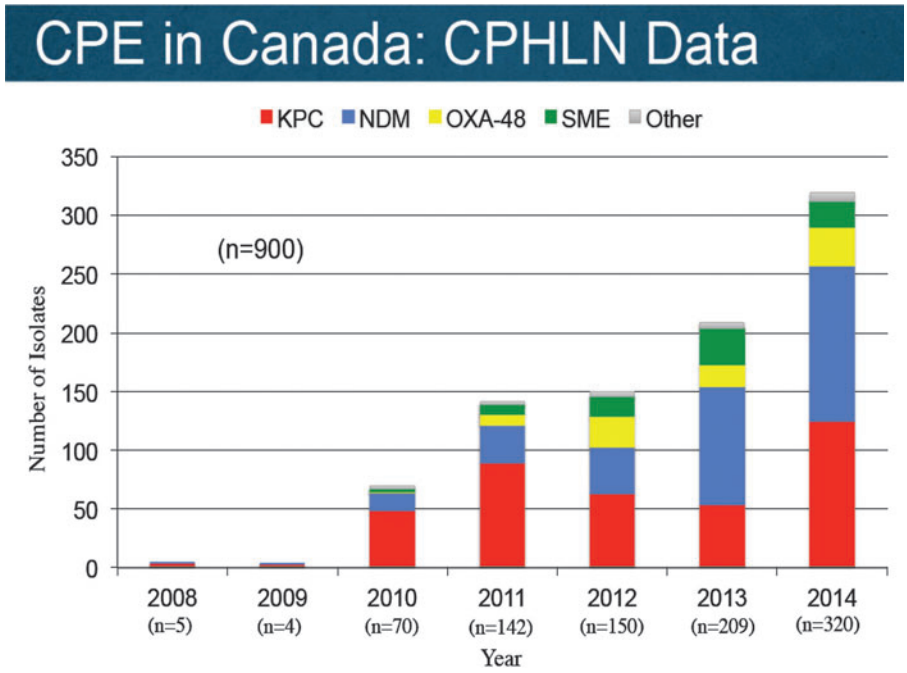

FIGURE 2 Types of carbapenemase-producing *Enterobacteriaceae* in Canada. Data from the Canadian Public Health Laboratory Network. Used with permission from Michael Mulvey.

antimicrobial resistance data at the laboratory level (described in detail in chapter 48). While national surveillance data are critical for monitoring the development and spread of antimicrobial resistance, local monitoring is also important. The WHONET software can be used to amalgamate resistance data from a single hospital, or the scope of the surveillance can easily be expanded to include data from multiple laboratories within a region. It can generate antibiograms from automated antimicrobial susceptibility testing systems in hospitals, indicate potential outbreaks, and monitor trends of antimicrobial resistance by bacterial species. It can also be used regionally, as was done in laboratories in Argentina to detect outbreaks of *Shigella* spp. (29). WHONET has the potential to be used globally to provide an unprecedented view of antimicrobial resistance trends by linking the routine antimicrobial susceptibility data generated daily from thousands of microbiology laboratories around the world into a single database (30). The quantitative data (both MICs and zone diameters) are an untapped resource that can be compiled irrespective of the breakpoints used for monitoring resistance trends. Of course, ensuring the quality of the data when they are drawn from a very diverse sampling of laboratories internationally is always the challenge. It would be necessary to make sure that the data imported to the database had undergone rigorous quality control and were generated using standardized laboratory protocols. This is more likely now than a decade ago, given the broad adoption internationally of both the European Union Committee on Antimicrobial Susceptibility Testing (EUCAST) and Clinical Laboratory and Standards Institute (CLSI) guidelines. The WHONET system has tremendous potential for informing public health agencies about the evolution of antimicrobial resistance globally and may also provide options to integrate typing data, e.g., those freely available after diagnostic mass spectrometry or those developed during specific molecular typing projects.

MOLECULAR TOOLS FOR SURVEILLANCE OF ANTIMICROBIAL RESISTANCE

Molecular Methods for Detection and Characterization of Antimicrobial Resistance Genes

Public health laboratories often collect antimicrobial-resistant isolates for further characterization. PCR assays are frequently used to identify specific antimicrobial resistance genes within resistant strains both to understand the mechanisms of resistance present as well as for epidemiologic purposes. Microarrays with multiple resistance genes have also been used to characterize large collections of isolates to understand the diversity of resistance mechanisms present in geographic regions (31). The ever-expanding list of extended-spectrum beta-lactamases and carbapenemases has been a challenge for molecular surveillance tools. Findlay et al. recently reviewed several commercial methods that can detect carbapenem resistance genes directly in clinical specimens, such as rectal swab samples, and that can be used to detect patients who are colonized with carbapenemase-producing organisms (32). These included the CheckPoints microarray, EasyPlex, and Xpert Carba-R. The study focused on what the authors called "the big 5" carbapenemase families, namely KPC, NDM, OXA-48, VIM, and IMP. The authors noted in their study that both EasyPlex and Xpert Carba-R detected the genes encoding OXA-48 but not the subset containing OXA-181. OXA-181 is becoming more common in Europe (33), so being able to detect strains containing this carbapenem resistance gene was deemed important. A footnote in the article noted that newer versions of both assays that include OXA-181 were tested subsequent to the original study, and those determinants are now detected. Updating commercial assays will be a continuing challenge for manufacturers, especially given the regional differences in the epidemiologic spread of specific resistance determinants. Thus, more generic methods including those based on targeted DNA sequencing may need to be developed.

Epidemiologic studies conducted by Lascols et al. have been undertaken using commercial arrays of beta-lactamase genes (34). These data have been helpful for clarifying the distribution of both ESBL and carbapenem resistance genes in *K. pneumoniae*, *Klebsiella oxytoca*, *E. coli*, and *Proteus mirabilis* in the United States. While these snapshots (i.e., point prevalence studies) provide very helpful information, such surveys are not conducted in a systematic fashion, so trending information, which would be very useful from a public health perspective, is not readily available.

Tuberculosis Detection Methods

Approximately one-third of the world's population continues to be infected with tuberculosis. WHO estimates that in 2013, 9 million people developed tuberculosis and 1.5 million died from the disease (35). Human immunodeficiency virus infection contributes to the spread and severity of tuberculosis. WHO estimates that of those who died from tuberculosis in 2013, 360,000 were HIV-positive. A major factor that contributes to our inability to control tuberculosis is the development and spread of multidrug-resistant (MDR) strains. Approximately 3.5% of all new tuberculosis cases were MDR in 2013 (35). This translates into an estimated 480,000 people having developed MDR-TB in 2013, which is an astonishingly high number. Unfortunately, the treatment success rate for MDR-TB is typically <50%.

Laboratory diagnosis of tuberculosis has traditionally relied on a combination of acid-fast smears and agar- and broth-based culture methods. However, molecular detection and identification methods, including the Gen-Probe (now Hologics) Amplified Molecular Test (36), the Hain line probe assay (37), and the Cepheid Xpert MTB/RIF test (38), have transformed the diagnosis of tuberculosis from a 4- to 6-week time frame to same-day diagnosis, and ultimately, to less than 2-hour diagnosis. Line probe tests are also available to detect resistance to second-line antituberculous drugs (39). Confirmation of the presence of *Mycobacterium tuberculosis* in a clinical specimen and detection of drug resistance markers, such as rifampin resistance, which is a surrogate marker for multidrug resistance, are critical for treating patients effectively (40). According to WHO, in 2013 only "58% of the 4.9 million pulmonary TB patients notified globally were bacteriologically confirmed via a WHO recommended test, including rapid tests such as Xpert MTB/RIF" (35). This means that although there are over 100 high-burden developing countries that have access to rapid, PCR-based diagnostics for tuberculosis, there is still considerable room for improvement in the diagnosis and treatment of tuberculosis globally. In South Africa, the laboratory data from approximately 300 GeneXpert PCR platforms is captured in real time in a cloud-based system, which makes monitoring

the data on tuberculosis (e.g., number of tests performed, number positive for *M. tuberculosis*, percentage of rifampin-resistant strains) feasible for the National Health Laboratory System to optimize the tracking of tuberculosis nationwide and make the best use of health care resources (see chapter 49).

In the United States, the Xpert MTB/RIF test has been cleared by the FDA for releasing patients from respiratory isolation (41). This follows the publication of several articles indicating the utility and cost effectiveness of using rapid diagnostic tests for optimizing the use of respiratory isolation rooms in hospitals (42, 43).

Use of Molecular Strain Typing Methods To Track Specific Clones of MDROs

Strain typing is critical to public health efforts to track and control bacterial diseases. Typing methods have evolved over time from simple phenotypic methods, such as biochemical analyses, serotyping, antimicrobial susceptibility testing pattern analysis (antibiograms), and bacteriophage typing, to molecular methods including plasmid analysis, randomly amplified polymorphic DNA, repetitive element PCR, PFGE, multilocus variable number tandem repeat analysis, and multilocus sequence typing (MLST), to whole-genome sequencing. Several attempts to develop interpretive criteria for typing results and harmonize typing methods internationally have been undertaken (44–46), and databases to facilitate the analysis of strain typing information, such as for MLST (e.g., http://www.mlst.net/) and spa types (http://www.spa.ridom.de/) are now available.

MSSA and MRSA Investigations

One of the first pathogenic clones to come to the attention of public health agencies was the MSSA strain designated bacteriophage type 80/81 (47). This organism, which was once the scourge of Australian, American, Canadian, and European hospitals, causing devastating outbreaks of staphylococcal disease, especially in nurseries and neonatal units, was the focus of intensive public health efforts for over a decade. Widespread use of hexachlorophine, streptomycin, and intensive infection control programs finally brought outbreaks under control. In fact, the strain type seemed to fade away in the 1960s, only to reemerge a decade later as a novel MRSA strain, after acquisition of a SCC*mec* element type IV (which contains the methicillin resistance gene, *mecA*). It was positioned in the lineage called the Southwestern Pacific Clone, which by multilocus sequence typing, was in clonal complex 30 (48). Interestingly, this lineage is one of the few that contains the PVL toxin, a potent virulence factor (49), which likely contributed to its disease-inducing potential. The importance of PVL toxin as a virulence factor was to achieve broader public health significance with the emergence of PVL-positive strains of MRSA in France (50) and community-associated MRSA strains in the United States, especially pulsed-field types USA300 and USA400 (51). Such strains, especially USA300, have become the predominant cause of skin infections in the United States (52) and cause devastating cases of staphylococcal pneumonia (53).

Controlling the spread of MRSA has been a challenge for public health agencies for nearly five decades (54). The rise of MRSA strain USA300 is a clear example of the important role molecular typing data play in tracing not only the emergence and spread of microorganisms but also the evolution of pathogens. USA300 is a PFGE type (44), originally defined by McDougal and colleagues at the CDC in the United States (55). The "USA type" PFGE definitions were developed in response to the problem of trying to track the movement of the international MRSA strains in the United States when those strains had multiple designations in the medical literature (55). The multiplicity of names for the same MRSA strain was particularly confusing when trying to understand whether MRSA outbreaks in the United States were being caused by endemic strains or were due to the influx of novel MRSA strains from Europe, Australia, and the Far East (56). The USA type designations attempted to consolidate and clarify the multitude of strain designations that were appearing in various publications. The USA typing scheme served its purpose well, especially during the period in the 1980s and 1990s when PFGE was the predominant typing method for *S. aureus* for public health laboratories.

Strain typing of MRSA isolates now focuses more on MLST, spa typing, and SCC*mec* typing results than PFGE data, yet PFGE data still have considerable utility for tracking hospital outbreaks. Translating one strain type to another is still a problem. For example, Tenover et al. typed a large series of MRSA isolates obtained from nasal colonization studies and bloodstream infections from patients in the United States by both spa and PFGE typing (57). They reported that isolates of PFGE type USA300 could be subdivided into at least 14 spa types. USA300 has emerged not only as the major cause of skin infections and bacteremia in the United States, but has spread globally as well (58). The USA300 lineage has continued to evolve, adding multiple antimicrobial resistance determinants (59), including those mediating resistance to gentamicin, tetracycline, and fluoroquinolones.

Tracking Multiresistant Gram-Negative Infections

In addition to MRSA, public health laboratories also track the spread of resistant Gram-negative organisms, such as extended-spectrum beta-lactamase-producing strains of *E. coli* (60) and carbapenem-resistant *K. pneumoniae* (61). bla_{KPC} (*K. pneumoniae* carbapenemase), which is a class A serine-based beta-lactamase (class 2f in the scheme of Bush and Jacoby [62]), was first described by Yigit et al. in 2001 (63). However, the *K. pneumoniae* isolate that harbored bla_{KPC} was first isolated in North Carolina in 1996, which raised the question of whether there were many other carbapenemase-producing isolates that simply escaped laboratory detection during that 5-year period. Molecular epidemiologic studies using MLST to trace the spread of bla_{KPC}-containing *K. pneumoniae* isolates revealed that although there was a multiplicity of MLST types represented among the strains collected from across the United States, the majority were ST 258. Examination of international strains of carbapenem-resistant *K. pneumoniae* revealed an epidemiologic link between the United States and Israel, which also experienced widespread dissemination of bla_{KPC}-containing *K. pneumoniae* during this time (64). Subsequent studies have shown the global spread of multiple clones of highly virulent bla_{KPC}-containing *K. pneumoniae* isolates (65), many of which can be divided into two distinct clades by molecular typing (66). Tracing the epidemiologic spread of all the major carbapenem resistance genes is a continuing challenge for public health laboratories but is important for updating guidance for national control policies (67).

RECOMMENDATIONS FOR SURVEILLANCE AND CONTROL OF MDROS

ESCMID Guidelines

The "ESCMID guidelines for the management of the infection control measures to reduce transmission of multidrug-resistant Gram-negative bacteria in hospitalized patients" represent a comprehensive guide to infection control practitioners (68). The guidelines contain several key observations regarding the transmission of Gram-negative bacteria. First, published strain typing data demonstrate that extraintestinal MDR *E. coli* strains are not readily spread in health care environments. This is in contrast to MDR strains of *K. pneumoniae*, which are frequently spread in hospitals, often via the hands of health care workers (68). *Serratia marcesens* and *Enterobacter* spp. are also capable of causing outbreaks in hospitals. Recommendations supported by strong evidence include hand hygiene and the use of contact precautions, but it is emphasized that there is no consensus on when contact precautions can be discontinued. For patients colonized or infected with MRSA, three negative cultures are often required to release a patient from contact precautions. However, a recent study by Shenoy et al. demonstrated that one negative PCR test had a negative predictive value of 96.6% when compared to the results of three negative cultures (69). This resulted in a cost avoidance of >$1.5 million by removing patients from contact precautions prior to the results of three negative cultures. Such data are not available for patients colonized with ESBLs or carbapenem-resistant organisms, but this may be a consideration for future studies. Given the number of MDROs encountered in hospitals these days, effective management of isolation precautions has become a major challenge.

CDC

In December 2013, the CDC issued its antimicrobial resistance report (70). The activities described focused on (i) preventing infections and preventing the spread of resistance (i.e., the importance of infection control and infection prevention activities), (ii) tracking (i.e., upgrading surveillance activities), (iii) improving antibiotic prescribing and stewardship (i.e., better utilizing the antimicrobial agents available and reducing the selective pressure that facilitates the development and spread of resistant bacteria, fungi, parasites, and viruses), and (iv) developing new antimicrobial agents and diagnostic tests. The CDC defined three groups of antimicrobial-resistant organisms based on their threat level (Table 2). Urgent threats included *C. difficile*, carbapenem-resistant *Enterobacteriaceae*, and ceftriaxone-resistant *N. gonorrhoeae*. *C. difficile* may seem an odd choice to include in the list since these organisms are not MDROs *per se*, although some strains can be resistant to multiple antimicrobial agents, such as the PCR-ribotype 027 strains, which are typically fluoroquinolone-resistant in addition to showing resistance to clindamycin, rifampin, and other drugs (71). These organisms made the urgent list because their spread is a direct result of antimicrobial use, which is a key risk factor for developing *C. difficile* infection. In fact, in the United States, *C. difficile* is now a more common cause of health care-associated infections than MRSA (27). In its recommendations, the CDC called for the use of nucleic acid amplification tests for rapid diagnosis of *C. difficile* and stressed the need for effective infection control interventions.

As the other urgent threat (besides carbapenem-resistant *Enterobacteriaceae*, which is discussed above), the CDC noted the global spread of ceftriaxone-resistant *N. gonorrhoeae*, which due to multidrug resistance has become untreatable in some parts of the world (72). While over 36 countries have reported ceftriaxone-resistant *N. gonorrhoeae* (5), surveillance for these organisms is essentially lacking on a global scale.

The CDC's list of "concerning threats" contains organism-drug resistance combinations that have already emerged, but are rare, yet have the potential to cause serious medical and public health issues should they emerge in large numbers. This includes vancomycin-resistant *S. aureus*, of which only a little over a dozen infections have been reported (73), erythromycin-resistant group A streptococci (also known as *Streptococcus pyogenes*), which is a major cause of skin and throat infections, and clindamycin-resistant group B streptococci (also known as *Streptococcus agalactiae*), which can colonize the vaginal vaults of women, leading to serious and often fatal neonatal infections if the mother is not treated with antimicrobial agents prior to labor and delivery.

TABLE 2 CDC antibiotic resistance threats[a]

Urgent threats	Serious threats	Concerning threats
Clostridium difficile	Multidrug-resistant *Acinetobacter*	Vancomycin-resistant *Staphylococcus aureus*
Carbapenem-resistant *Enterobacteriaceae*	Drug-resistant *Campylobacter*	Erythromycin-resistant group A *Streptococcus*
Drug-resistant *Neisseria gonorrhoeae*	Fluconazole-resistant *Candida*	Clindamycin-resistant group B *Streptococcus*
	Extended-spectrum beta-lactamase-producing *Enterobacteriaceae*	
	Vancomycin-resistant *Enterococcus*	
	Multidrug-resistant *Pseudomonas aeruginosa*	
	Drug-resistant nontyphoidal *Salmonella*	
	Drug-resistant *Salmonella typhi*	
	Drug-resistant *Shigella*	
	Methicillin-resistant *S. aureus*	
	Drug-resistant *Streptococcus pneumoniae*	
	Drug-resistant tuberculosis	

[a]From CDC report issued December 2013 (70).

The third category of threats includes a variety of resistant organisms that are already widespread globally and need to be monitored and, if possible, held in check by medical and public health interventions (Table 2). Such resistant infections are contained by infection prevention programs in hospitals, including hand washing, surveillance, and decolonization strategies, and community-based public health programs, such as directly observed therapy for patients with tuberculosis infections.

Barriers to Harmonizing Global Surveillance Programs

One critical problem with pooling antimicrobial susceptibility testing data for bacterial pathogens from around the world is the interpretive criteria (also known as breakpoints) used to define antimicrobial susceptibility and resistance. While most European countries as well as a number of laboratories outside of Europe use the EUCAST interpretive criteria (http://www.eucast.org/clinical_breakpoints), the United States and a variety of other countries, especially in Latin America, use CLSI criteria (http://clsi.org/standards/micro/sub-ast/), which have critical differences in breakpoints for susceptibility, particularly for cephalosporins and carbapenems. This can complicate interpretation of resistance data for those trying to understand the global movement of resistant strains. If quantitative data, such as MICs or disk diffusion zone diameters, were collected in lieu of the interpretations (i.e., susceptible, intermediate, or resistant) for the data, it might be possible to analyze the data using any set of interpretive criteria. Unfortunately, this is rarely the case. The interpretive criteria that are used in a clinical laboratory also dictate the MIC ranges of antimicrobial agents tested in commercial susceptibility testing systems. Thus, laboratories that use CLSI criteria may not have the range of dilutions in MIC panels that span both the CLSI and EUCAST breakpoints (e.g., from 0.25 μg/ml to 16 μg/ml for carbapenems). So reinterpreting the data after the fact may not be feasible.

In the United States, the FDA sets the interpretive criteria for MIC and disk diffusion testing when an antimicrobial agent is first approved for use. These criteria may be accepted as-is or modified by the Antimicrobial Susceptibility Testing Subcommittee of the CLSI when they publish their annual review of interpretive criteria (the M100 document series), which lists breakpoints for both MIC and disk diffusion testing (74). Fortunately, the discrepancies between FDA and CLSI interpretive criteria continue to decrease as both groups work to resolve them. Yet there is tension in the clinical laboratory because diagnostic instrument manufacturers are obliged to use FDA interpretive criteria in automated testing instruments, although the clinical laboratory may choose to reprogram its instruments to follow CLSI guidelines. The key surveillance issue is that some fraction of U.S. laboratories (which is essentially unknown) still uses older CLSI interpretive criteria for cephalosporins and carbapenems, while others have made the switch to the newer (lower) breakpoints, which are more sensitive for detecting resistance. Thus, U.S. surveillance data taken directly from clinical laboratories may represent a mix of interpretive criteria, especially if the data collected include only the "susceptible, intermediate, or resistant" interpretations and not the actual quantitative data (such as zone of inhibition diameters or MICs), which could be reanalyzed using consistent breakpoints. This may underestimate the true resistance rates in the United States and other regions in the world.

SUMMARY

Molecular methods inform surveillance activities and have made vital contributions to our understanding of the epidemiology of many antimicrobial-resistant organisms, but there is considerable room for growth and improvement of methods and data management.

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Molecular Diagnostics: Huge Impact on the Improvement of Public Health in China

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21

China has undergone rapid demographic and epidemiological changes over the past 30 years. One of the most exciting changes is the health improvement with significantly increased life expectancy (1). Infectious diseases, which used to be the primary threat in public health, have been greatly reduced (2). However, infectious diseases continue to exact a substantial toll on public health resources in China. Attention to and action against emerging infections and infectious diseases greatly increased after the outbreak of severe acute respiratory syndrome (SARS) in southern China in 2003. In addition, endemic diseases, including H5N1, *Streptococcus suis*, human granulocytic anaplasmosis, EV17, H1N1, H7N9, H10N8, and some other zoonotic infections, affect millions of individuals, further heightening public health concerns.

An important priority in meeting these challenges is to ensure that advanced molecular diagnostics for disease detection and surveillance are well integrated into the public health system (3). Molecular diagnostic tests have been playing a critical role in the management of infectious diseases in clinical settings, including rapid detection and characterization of specific organisms, optimization of antimicrobial use, monitoring of responses to therapy, discovery of novel pathogens, and disease surveillance. Rapid and accurate diagnosis of infectious disease is critical not only for early clinical interventions, but also for controlling the spread of pathogens and avoiding wasting resources on ineffective treatments. More importantly, molecular diagnostics such as PCR can be made affordable for the developing world (4).

In the past few years, more clinical tests based on molecular methods have been available in China for rapid detection of common, toxin-producing, or antimicrobial-resistant pathogens. In this chapter, we discuss the molecular-based methods, including PCR, multiplex PCR, real-time PCR, nested PCR, biochip assay, and loop-mediated isothermal amplification (LAMP), that have been used in Chinese hospitals and how these methods influence public health in China.

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CURRENT IMPACT OF MOLECULAR DIAGNOSTICS ON BACTERIAL INFECTIONS

Culture methods and microscopy remain the predominant approaches in clinical laboratories in China. Although these methods are adequate for detecting and characterizing a wide range of bacterial species, they are time-consuming and not suitable for many infectious agents, which limits their application. Thus, molecular diagnostics are particularly useful for identifying these difficult to culture pathogens.

Mycobacterium tuberculosis

Tuberculosis (TB) remains one of the major causes of death worldwide. Recent data indicate that all-form tuberculosis incidence was 14.6 million and number of deaths was 2.7 million in 2013 worldwide (5). With 1.4 million cases of TB, China has the second highest burden of TB in the world (6). Since the early 1990s, the Chinese government has appreciated, in particular, the importance of controlling the spread of TB, and great commitment has been made. Recommended by the World Health Organization (WHO), directly observed treatment, short-course therapy has been recognized and applied as a nationwide TB control strategy. As a result, the prevalence of TB has been greatly reduced. From 1990 to 2010, the prevalence of smear-positive TB decreased from 170 to 59 cases per 100,000 population, and disability-adjusted life-years caused by TB dropped by about 71% (1, 7).

Molecular Diagnostics of *M. tuberculosis*

In China, TB patients are diagnosed mostly by clinical manifestations, acid-fast bacillus smear, sputum culture, or chest X ray. However, the conventional methods prolong the diagnosis time. Symptoms of *M. tuberculosis* and non-TB *Mycobacterium* infections are clinically similar, and diagnostic delay can significantly affect disease management. To better prevent the transmission of TB and decrease its prevalence, rapid detection and diagnosis are critical. In the past two decades, a number of molecular diagnostics for TB have been developed, resulting in improved sensitivity and specificity. Technologies such as multiplex PCR, real-time PCR, LAMP, the multiplex SNaPshot technique, and biochip assay have been modified and applied in direct de-

tection and identification of M. *tuberculosis* complex in China (8–13). A novel two-step multiple displacement amplification-PCR assay, which combines multiple displacement amplification with IS*6110*-specific PCR, can detect M. *tuberculosis* complex in sputum specimens with low copy numbers (14). This method greatly improves the sensitivity and specificity of TB diagnosis by sputum specimens. Many individuals infected with HIV died of co-infections with M. *tuberculosis* or *Pneumocystis carinii* pneumonia in China. With the use of a microfluidic isothermal amplification chip incorporating LAMP and a microfluidic technique, HIV, M. *tuberculosis*, and *P. carinii* pneumonia infections can be detected simultaneously (15). This system has considerable significance for providing a rapid, practical, multiple-target-point, and low-cost diagnostic method in clinical practice in China (15).

Molecular Genotyping of *M. tuberculosis*

Molecular epidemiology is a valuable tool for understanding the current TB pandemic by investigating clinical features, tracking transmission dynamics, detecting suspected outbreaks, and distinguishing relapse and reinfection. Based on advances in PCR techniques, some genotyping methods have been developed to compensate for the limitations of restriction fragment length polymorphism (RFLP). Spacer oligonucleotide typing (spoligotyping) is a good alternative to traditional IS*6110* RFLP due to its convenience and reliability. Another widely used genotyping method is mycobacterial interspersed repetitive unit and variable-number tandem repeats (MIRU-VNTR). The combination of the two genotyping methods facilitates the understanding of M. *tuberculosis* transmission with discriminatory power, economical cost, and reproducibility (16). In addition, several studies have established the standard MIRU-VNTR locus set for investigating molecular epidemiology (16–20).

With the assistance of these genotyping methods, many studies have focused on the molecular typing of M. *tuberculosis* isolates in various regions in China, including Jilin, Tibet, Heilongjiang, Shanghai, Jiangsu, and Inner Mongolia (16, 18–20). These reports showed that the Beijing family is the most prevalent isolate in most provinces of China. The distribution of the Beijing genotype in China showed regional characteristics, with northern China more prevalent than southern China (18). Data also showed that in Fujian province, the proportion of rural to urban migrant workers with the Beijing genotype was significantly higher than those of permanent residents (21). These epidemiological data are important for understanding the genotype and transmission patterns of M. *tuberculosis* and the development of effective control strategies.

Molecular Detection and Surveillance of Drug-Resistant *M. tuberculosis*

Although there have been substantial decreases in TB incidence and deaths in China, drug-resistant TB is still a substantial challenge. Among 3,037 new TB cases and 892 previously treated cases, 5.7% and 25.6% were multidrug-resistant TB (defined as disease that was resistant to at least isoniazid and rifampin) (22). Duo et al. reported that PCR-based identification of M. *tuberculosis* in cerebrospinal fluid was not only an alternative diagnostic approach for tuberculous meningitis, but was also useful for the detection of drug resistance when combined with the MTBDRplus assay (23). Molecular diagnostics on resistance genes can facilitate the rapid detection of drug-resistant TB, e.g., the fluoroquinolone-resistance-determining region of the *gyrase A* (*gyrA*) gene and the *gyrase B* (*gyr B*) gene, the rifampin-resistance-determining region of the *rpoB* gene, and isoniazid resistance of the *inhA* gene and *katG* gene (24–26). The development of Xpert MTB/RIF is considered a significant breakthrough in the fight against TB and was endorsed by WHO in 2010 (27). The GeneXpert system and Xpert MTB/RIF assay remain the only automated cartridge-based DNA testing method for simultaneous detection of TB and rifampicin resistance directly from sputum in less than 2 hours (27). The Chinese Center for Disease Control and Prevention has introduced the 774 GeneXpert machine with support from the Global Fund to Fight Tuberculosis and implemented the systems into the National Tuberculosis Control Program, covering a wide range of areas in China. These systems are not only practical for decentralizing facilities in low-resource settings, but also play a critical role in reducing the incidence and mortality of multidrug-resistant M. *tuberculosis* in China.

Streptococcus suis

S. *suis* is an important pathogen associated with a wide range of diseases and can be transmitted to humans by direct contact. In July 2005, the third outbreak of human S. *suis* infections occurred in Sichuan province, China, with two earlier outbreaks occurring in 1998 and 1999 (28). The repeated and intensive outbreaks have raised public concern regarding S. *suis* as an emerging zoonotic agent (28).

Various methods for identifying S. *suis* strains have been developed, including serological, molecular, and other diagnostic techniques. PCR is a rapid method used to detect serotypes or strains of S. *suis* in animal carriers and to identify strains obtained from infected or healthy pigs or from sick patients for clinical diagnosis or epidemiology studies (28). PCR-based S. *suis*–specific 16S rRNA region and a species-specific probe targeting 16S rRNA can be used to identify S. *suis* strains (28). Serotyping is one of the most valuable methods to understand the epidemiological patterns of S. *suis*. Recently, a multiplex PCR assay was developed as a relatively systematic typing tool for S. *suis* (29). It provides a rapid, specific, and cost-effective way to identify all except for two pairs of serotypes (29). Other molecular methods such as RFLP, pulsed-field gel electrophoresis, and multilocus sequence typing have also been used to study the genetic diversity of S. *suis* strains, the colonial relations between the strains, and the virulence of particular clones (30–32).

Neisseria meningitidis

N. *meningitidis* is a leading cause of bacterial meningitis and sepsis in China. Nationwide epidemics previously occurred every 8 to 10 years (33). Historically, epidemics of meningococcal disease were caused by pandemic waves from serogroup A (33). However, since 2003, a substantial increase in serogroup C meningococci infections has been observed in China (33).

Efficient methods for detection and genotyping of N. *meningitidis* isolates are needed to monitor the prevalent serogroups as an important method of disease control and prevention and vaccination strategy (34). Molecular typing with pulsed-field gel electrophoresis enables critical examination of the pathogen change at the molecular level (33). Multilocus sequence typing is a commonly used tool for tracking the national spread of meningococcal clones

and for identifying lineages that have an increased risk of causing infections (35). Based on molecular analysis, infections caused by *N. meningitidis* serotype W135 ST11, which belongs to a hyperinvasive lineage, were reported in Anhui and other provinces in China (36, 37). In addition, a multiplex PCR using 12 individual serogroup-specific and species-specific primers was developed for *N. meningitidis* detection and genogrouping and may be applied in clinical diagnosis and epidemiological surveillance (34). In combination with available epidemiological data, information on serogroups has the potential to significantly enhance our knowledge of molecular epidemiology of *N. meningitidis*, thereby increasing our ability to respond to disease outbreaks appropriately and effectively (35).

Yersinia pestis

Plague, caused by *Y. pestis*, remains an endemic threat and public health concern in many regions widely distributed in China. Since the 1990s, the incidence of plague in China has slightly increased, with a total of 631 cases between 1995 and 2004 (38). The Ministry of Health of China reported a cluster outbreak of primary pneumonic plague in Qinghai province in July 2009 (39).

Y. pestis in China was traditionally identified by a four-step protocol consisting of bacterial isolation and microscopy observation, the phage lysis assay, and animal experiments (40). However, this is time-consuming and laborious. Nucleic acid–based detection techniques could be an excellent alternative for detection of *Y. pestis*.

Recent progress in molecular typing methods enables large-scale outbreak investigations and provides information about its evolution patterns. Regions of deletion analysis, clustered regularly interspaced short palindromic repeat analysis, whole-genome sequencing, SNP analysis, and multilocus variable number tandem repeat analysis (MLVA) have been widely used in genotyping and evolutionary research of *Y. pestis* (41–44). MLVA is a promising technique to track the source of the outbreak (39). As indicated by MLVA, an outbreak of plague in 2009 was begun by an infected dead dog (39). Understanding the infection source and dynamics further aids the epidemiological analyses by setting up the basis for an accurate and robust typing system for plague surveillance and promotes diagnostic development and control measures.

Novel Pathogens

Molecular diagnostic methods can be used for identification of novel pathogens. One of the major techniques is broad-spectrum DNA amplification targeting the bacterial 16S rRNA gene with universal primers. 16S rRNA is present in almost all bacteria and can provide genetic classified information. This approach, especially combined with DNA sequencing, has facilitated the identification of new pathogens and novel strains of bacteria. Besides the sequencing analysis of the 16S-23S rRNA gene, other housekeeping genes, such as *rpoB*, *tuf*, *recA*, *gyrA*, *yrB*, *sodA*, *groEL*, and *cpn60*, are also commonly used to identify and describe the molecular characteristics of species.

Application of broad-spectrum PCR amplification and direct sequencing has had a considerable impact on the detection of clinically infrequent and special bacteria, such as slow-growing, fastidious, or uncultured bacteria (e.g., *Campylobacter fetus*, *Acinetobacter parvus*, *Mycobacterium phocaicum*, *Roseomonas mucosa*, and *Halomonas johnsoniae*) (45). Since the initiation of widespread use of 16S rRNA based on PCR, many new and clinically significant *Mycobacterium* species have been identified in China (46, 47).

CURRENT IMPACT OF MOLECULAR DIAGNOSTICS IN VIRAL INFECTIONS

SARS Coronavirus (SARS-CoV)

A serious "plague" called atypical pneumonia, or SARS, broke out in China starting in November 2002 and lasting to July 2003 (the major epidemic areas are shown in Fig. 1). SARS was caused by a novel animal coronavirus (SARS-CoV) that exploited opportunities to adapt to the palm civet and human, and certain mutated lineages became readily transmissible between humans. It spread quickly and affected more than 8,000 people in 25 countries and regions across five continents within weeks (WHO, http://www.who.int/csr/sars/country/table2004_04_21/en/index.html). By the end of the global outbreak on July 5, 2003, SARS-CoV had killed 774 people, with a global economic loss of $59 billion (48).

Molecular Diagnostics and Impact of SARS-CoV

The clinical syndrome and viral etiological agent of SARS-CoV were quickly described and identified (49, 50). Some diagnostic tests targeting different regions of the virus were described (49, 51), and the genome of the virus was sequenced completely (52). Drosten et al. obtained a 300-nucleotide-length sequence using a PCR-based random-amplification procedure, which showed that SARS-CoV is only distantly related to known coronaviruses. Specific and sensitive detection of the novel virus was accomplished by conventional and real-time PCR assays, and high concentrations of viral RNA of up to 100 million molecules per milliliter were found in sputum of patients with SARS (49). Similarly, Poon et al. used SYBR Green to detect samples with sequence variations (mutations) in the virus. A 182-bp region within the RNA-dependent RNA polymerase-encoding sequence of the virus was amplified and confirmed by sequencing. This approach (e.g., RNA extraction, reverse transcription, and real-time PCR) can be completed in 3 to 4 h, allowing rapid identification of SARS-CoV (51).

The Chinese SARS Molecular Epidemiology Consortium analyzed 61 SARS-CoV genomic sequences derived from the early, ongoing, and late phases of the epidemic together with two viral sequences from palm civets (53). Most variations observed between human and civet SARS-CoV genomes were located at the orf8 region and the spike genes (S). Genotypes characteristic of each phase were somewhat different: the earliest genotypes were similar to the animal SARS-like coronaviruses, while the earliest and latest genotypes showed major deletions in the orf8 region. The neutral mutation rate of the viral genome was stable, but the amino acid substitution rate of the coding sequences showed decreases during the epidemic.

West Nile Virus (WNV)

WNV is a spherical, enveloped virion containing 11 kb of single-stranded, positive-sense RNA. It is the most widely distributed flavivirus in the world, including at least two major lineages, I and II (54). Infection with WNV leads to a wide range of diseases from mild fever to severe neurologic symptoms; however, asymptomatic infections occur most frequently (55). From August 5 to September 3,

FIGURE 1 The major epidemic areas of SARS-CoV (red), West Nile virus (blue), and H7N9 avian influenza virus (yellow) in China.

2004, an epidemic of fever and meningitis/encephalitis broke out in several villages in Xinjiang province, China (56). Among 80 cases, 10 people died (the major epidemic areas are shown in Fig. 1).

Molecular Diagnostics and Impact of WNV

In the study mentioned above, cerebrospinal fluid and serum samples were collected, and WNV and six other arboviruses were detected by immunofluorescence assay, enzyme-linked immunosorbent assay (ELISA), and plaque reduction neutralization assay for the existence of immunoglobulin M antibody or neutralization antibody (56). However, the accuracy of laboratory diagnosis of viral encephalitis caused by WNV still needs to be improved.

In 2011, arbovirus surveillance was performed in Kashi Region, Xinjiang province (57). Acute-phase serum samples obtained from patients with fever or viral encephalitis were performed for immunoglobulin M against WNV. A total of 23/38 patients were positive. Of the 23 positive samples, 11 had a 4-fold increase in titer of WNV-neutralizing antibody (57).

Enterovirus 71 (EV71)

Hand, foot, and mouth disease (HFMD) is a common illness of children younger than 5 years. HFMD has emerged as a significant public health issue in China in recent years (58, 59). The Chinese Centers for Disease Control and Prevention surveillance system reported 7,200,092 suspected cases of HFMD from 2008 to 2012, of which 2,457 (0.03%) were fatal (60). EV71, the major causative agent of HFMD, was first identified in 1969 in California and has become more predominant across the Asia-Pacific region in the past decades (60). Because there is no effective treatment or reliable vaccine for EV71, prevention and early diagnosis are critical.

Molecular Diagnostics and Impact of EV71

The standard diagnosis for EV71 is cell culture following neutralization tests with serotype-specific antisera, which is time-consuming and not suitable for on-site testing (61, 62). Nucleic acid diagnosis methods have been used widely in early diagnosis. The highly conserved and specific *VP1* gene is employed in detecting EV71 with various kinds of nucleic acid assays, such as nested reverse-transcriptase PCR (RT-PCR), real-time RT-PCR, and microarray (63–65).

In 2010, when HFMD was quite active in Hong Kong, real-time RT-PCR was performed routinely for enterovirus (EV) infection detection (66). Compared to conventional methods, EV RT-PCR significantly improved the efficiency of laboratory diagnosis of EV infection. However, these methods need to be modified before they are used in local medical institutions because expensive equipment and highly trained staff are required.

Asymmetric capillary convective PCR and one-step nucleic acid dipstick assay could complete the amplification and detection process within 30 minutes (67, 68). With this method, the bottom of the reaction tube is heated by the dry bath while stable continuous bottom-up temperature gradients are established and continual convection currents are formed. Then the reagents in tubes circulate spontaneously among the three steps of the PCR cycle

when they flow through the corresponding temperature zones in typical convection. When PCR is finished, the products can be directly applied onto the dipstick and detected visually without the addition of probes, incubation, and dilution.

Because there are still some shortcomings to asymmetric capillary convective PCR and one-step nucleic acid dipstick assay in on-site tests, appropriate improvements have been integrated into the convenient and integrated nucleic acid test (69). The results can be observed with the naked eye after 3 min. The convenient and integrated nucleic acid test EV71 assay showed a clinical sensitivity and specificity of 98.5% and 100%, respectively, and could serve as an on-site diagnosis tool.

Recently, a real-time simultaneous amplification and testing technology was developed to detect EV71 (SAT-EV71) (70). The RNA of EV71 and an internal control were amplified and analyzed simultaneously using routine real-time PCR by isothermal amplification and real-time detection of fluorescence. Afterward, clinical specimens were detected by this SAT-EV71 method and confirmed by the virus-cell culture method. The results showed that SAT-EV71 has a high sensitivity and specificity: it detected the VP1 with a minimum of 10 copies per reaction at an optimal concentration of internal control (5,000 copies per reaction); the use of internal control effectively prevented false-negative results by monitoring the processes of nucleic acid extraction and amplification. Thus, this easy-to-perform SAT-EV71 might be used in the molecular diagnosis of EV71.

Hepatitis B Virus (HBV)

HBV has infected more than 2 billion people worldwide, and of these, more than 350 million people suffer from chronic infection (71, 72). About 130 million carriers and 30 million chronically infected people live in China. Since the National Center for Clinical Laboratories in China performed a 10-year external quality assessment program in 2003, the false-positive and false-negative results decreased significantly in qualitative tests, and the accuracy and precision of quantitative analysis have been increasing (73).

Molecular Diagnostics and Impact of HBV

Currently, serologic immunity and nucleic acid testing are commonly used in clinical diagnosis of HBV (74). The serological tests such as ELISA assays detect a series of serological markers of HBV with low sensitivity and accuracy. Nucleic acid detection based on PCR amplification showed higher sensitivity, accuracy, and specificity. One of the most popular methods used for detection of HBV DNA is the TaqMan technique, which uses a TaqMan probe containing a fluorophor and a quencher at both ends, respectively. In the single-stranded form, fluorescence of the probe is not detected. When the PCR extension progresses, a DNA polymerase with the activity of 5′-3′ exonuclease leads to the fluorophor-modified DNA probe being degraded, and the close proximity between the fluorophore and quencher is broken, thus allowing fluorescence of the fluorophore. Fluorescence detected in the quantitative PCR thermal cycler is directly proportional to the fluorophore released and the amount of DNA template (75). However, the modified fluorogenic oligonucleotide probes in TaqMan methods are expensive, and sophisticated equipment is required, which limits access to this technology for many laboratories.

Researchers reported a novel approach for both qualitative and quantitative analysis of HBV DNA (76). During PCR amplification, Taq DNA polymerases cleave the probe and release a DNAzyme sequence that has been embedded in the probe. After PCR reaction, the DNAzyme formed G-quadruplex and bound with hemin, catalyzing the oxidation of different substrates by H_2O_2 to generate colorimetric or fluorometric signals. This method could detect HBV DNA with high specificity, sensitivity, and accuracy.

In addition, the peptidomic profiling in the process of HBV infection was investigated (77). Serum from HBV-infected (asymptomatic carrier and chronic hepatitis), HBV-immunized, and normal subjects was incubated with MB-WCX (weak cation exchange-based magnetic beads) kits and analyzed by the Clinprot/matrix-assisted laser desorption ionization–time of flight mass spectrometry. Purified serous proteins were subjected to Fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry analysis and further confirmed by Western blot. Using the WCX magnetic bead-based MALDI-TOF-MS, specific serum peptide profilings for host responses to HBV infection were investigated. Results showed that m/z 2882.89 and 4476.12 could be valuable prognostic tools for HBV infection.

Influenza A Virus

Influenza A virus, belonging to the family *Orthomyxoviridae*, can be classified into subtypes H1-H17 and N1-N10 according to the antigenic specificity of the hemagglutinin (HA) gene and neuraminidase (NA) genes, respectively (78, 79). These viruses could infect humans and a wide range of animal species, causing yearly epidemics that threaten public health, and some species can even jump the species barrier from poultry to humans (80, 81).

Molecular Diagnostics and Impact of Influenza A Virus

Virus isolation is time-consuming and requires complicated technology. Molecular methods such as RT-PCR, real-time RT-PCR, microsphere-based duplexed immunoassay, and padlock probes combined with DNA microarray were developed for influenza viruses detection (82–85).

Wu et al. reported a multiplex RT-PCR (mRT-PCR) assay for the rapid diagnosis of influenza A virus in clinical samples (86). The HA subtypes of H1, H3, H5, and H9 were detected in a single reaction mixture with specific primer sets. The sensitivity was the same with conventional RT-PCR, but 10 times less sensitive than SYBR Green real-time RT-PCR. However, the mRT-PCR assay was a rapid, convenient, and relatively inexpensive method.

Some newly emerged variants (for example, H1N1 pdm09 virus and H3N2 virus that occurred in Taiwan between 2012 and 2013) were not detected in clinical specimens via a common real-time RT-PCR assay that targeted the conserved regions of the viral matrix genes, because the viral matrix gene sequence was located in regions where the primers or probes of the real-time RT-PCR assay bind. Yang et al. established a real-time RT-PCR assay using degenerate nucleotide bases in both primers and probes. The sensitivity in detecting influenza A viruses was increased in this method (87). Therefore, the importance of the simultaneous use of different gene-targeting real-time RT-PCR assays for the clinical diagnosis of influenza was highlighted.

Subtypes of Influenza A Virus

Highly Pathogenic Avian Influenza Virus Subtype H5N1 (H5N1)

H5N1 was first reported in Guangdong province, China, in 1996, resulting in widespread poultry outbreaks and 18 cases of human infections in 1997 in Hong Kong (88, 89). In early 2004, H5N1 spread to 16 provinces in China, and multiple distinguishable sublineages were identified (90). Subsequently, H5N1 disseminated continuously from Asia to Europe, the Middle East, and Africa (91). By August 2013, WHO reported 637 laboratory-confirmed human cases, including 378 fatalities, from 15 countries (http://www.who.int/en/).

ResPlex III was employed in H5N1 diagnosis (92). In brief, the ResPlex system incorporated the target-enriched multiplex PCR to simultaneously amplify multiple molecular targets in one reaction. The ResPlex III assay targeted the H1, H2, H3, H5, H7, H9, N1, and N2 genes of influenza A virus, and the NS genes of influenza A (NSA) and B (NSB) viruses. The clinical sensitivity and specificity for detecting H5N1 were 93.3% and 100%, respectively, indicating that the ResPlex III genotyping approach could quickly and correctly identify different subtypes of influenza A virus.

In another study, H5N1 detection was based on the fluorescence resonance energy transfer from quantum dots (QDs) to carbon nanotubes (CNTs) in a QDs-ssDNA/oxCNTs system, in which the QDs (CdTe) modified with ssDNA were used as donors (93). Initially, QD fluorescence was effectively quenched with strong interactions between ssDNA and oxCNTs. The interactions between the QDs-ssDNA and oxCNTs decreased upon recognition of the target, and QDs fluorescence was recovered and shown to be linearly proportional to the concentration of the target in the range of 0.01 to 20 μM, with a detection limit of 9.39 nM. Thus, this simple, sensitive, and quantitative method could have a wide range of applications in molecular diagnosis.

Influenza A/H1N1/2009 Virus (H1N1/2009)

The influenza A/H1N1/2009 virus (H1N1/2009) was first reported in Mexico in mid-April 2009 and spread to over 160 countries within a short time. It had killed 816 people among 134,500 cases by July 27, 2009. H1N1/2009 was characterized by a unique combination of gene segments that had not been reported previously (94). Seasonal influenza A and B viruses often generate new genetic variations and cause symptoms similar to H1N1/2009. Thus, rapid diagnostic methods for detecting H1N1/2009 are required to control the disease.

The HA gene sequences of H1N1/2009 and all formerly published seasonal influenza A (H1N1) viruses were aligned, and specific primers and probe sets were designed (95). Compared with the WHO assay, the new assay with the specific primers and probes showed a much greater sensitivity and could be employed as a rapid, alternative method in detecting H1N1/2009.

H7N9 Avian Influenza Virus (H7N9)

In February 2013, a serious disease manifesting as an influenza-like illness and severe pneumonia was attributed to a novel avian influenza A (H7N9) virus that emerged in eastern China (the major epidemic areas are shown in Fig. 1) (96). One month later, it killed 39 people among 131 or more laboratory-confirmed cases. It cost more than $6.5 billion and threatened the public health system (97); it was likely that another pandemic would occur if the virus acquired the necessary mutations to enable effective human-to-human transmission (98, 99).

In clinical detection, the immunological colloidal-gold test method has low sensitivity (100), while the sensitive and specific quantitative real-time RT-PCR of HA and NA genes of H7N9 requires expensive equipment and is not suitable for on-site detection in developing countries (101, 102).

Researchers developed two sets of one-step reverse transcription loop-mediated isothermal amplification (RT-LAMP) detection methods with hydroxynaphthol blue dye, one specific for the HA gene and the other for the NA gene (103). Compared with real-time RT-PCR (WHO recommended), RT-LAMP has the same sensitivity in the HA gene and 100 times greater sensitivity in the NA gene. Because no special equipment is needed in RT-LAMP, it could play a role in prevention of influenza pandemics.

CURRENT IMPACT OF MOLECULAR METHODS IN DETECTION OF PARASITIC INFECTION

In China, malaria, schistosomiasis, kala-azar, filariasis, and ancylostomiasis are regarded as five important parasitic diseases that call for intensive prevention and treatment. The diagnosis of parasites includes etiological diagnosis, immunodiagnosis, molecular testing, and clinical manifestation.

As the most common method in etiological diagnosis, microscopic detection is cost-effective, simple, and rapid. Microscopic detection is the "gold standard" of diagnosis of parasitic diseases. However, it is of limited value for the detection of cases with low parasite density, latent infection, early infection, or undistinguished parasitic morphology.

Immunodiagnosis plays an important role in parasitic disease detection. ELISA, benefiting from varieties of serological tests, has practical value in parasitic disease screening. However, immunodiagnosis is a nondiagnostic method but an assisted diagnostic process; because the specificity is low, cross-reaction exists and can be influenced by changing antigen or antibody levels.

Nucleic acid testing methods have been implemented widely and have drawn more and more attention over the past several years, since they are more reliable and stable than immunoserology. Nucleic acid tests use specific base pairs to detect and identify DNA or RNA of pathogenic agents directly, without the influence of host or antigen variation at each stage of development. Coupled with the conventional PCR principle, several variations of molecular techniques were developed, including nested PCR, multiplex PCR, real-time PCR, and LAMP (104). Genetic microarrays also provide a reliable way to diagnose parasitic diseases. In China, molecular diagnostics have been successfully applied to the detection of several genera of parasites including *Plasmodium*, *Schistosoma*, *Leishmania*, *Toxoplasma*, and *Filaria*.

Plasmodium

Malaria is one of the most important parasitic diseases threatening human health worldwide. In 2010, an estimated 219 million people were infected with *Plasmodium* species, with 660,000 deaths (105). In China, malaria infection rates are still high in some regions. What is worse, people from areas of malaria endemicity continually feed

into low epidemic areas, and they cannot receive treatment promptly. Thus, rapid molecular diagnostics are still valuable in malaria prevention and treatment.

PCR can be used to detect *Plasmodium vivax*, *Plasmodium falciparum*, and *Plasmodium ovale* in 2 hours with low threshold values. In addition, patients with low levels of parasitemia or asymptomatic infection can be detected as well. Compared with nested PCR, LAMP is less expensive and easier to perform, thus providing a promising platform for the molecular detection of malaria parasites in areas of endemicity of China.

Schistosoma

Schistosomiasis is caused by blood-dwelling flukes of the genus *Schistosoma*. About 200 million people from 70 countries are infected by schistosomiasis (106). This communicable disease, together with HIV/AIDS, TB, and hepatitis B, has been assigned high priority for control in China (107).

A number of serologic tests have emerged to diagnose patients who have or had schistosomiasis japonica (108, 109). Indirect hemagglutination assay, an important antibody detection approach with high sensitivity, is extensively used for clinical screening in regions of endemicity. However, the low specificity and high false-positive results limit its application, because antibodies do not wane immediately after clearance of parasites (110, 111). Commercial kits utilizing colloidal dye immunofiltration assay have been developed for antibody detection. This method has shown high sensitivity and specificity, but it cannot distinguish current infection from recent or past infection (112).

In addition to traditional methods, conventional PCR, PCR-coupled methods, LAMP, and mass spectrometry–based methods are gradually used in molecular diagnosis (104). These molecular diagnostic methods showed superiority in sensitivity, specificity and efficiency.

Leishmania

Leishmania species are the pathogen of leishmaniasis, a neglected tropical disease. In the past 5 years, 400 new cases occurred annually in the western provinces of China. At present, desert and hill regions are the primary epidemic areas.

Leishmaniasis caused by different species or subspecies needs different diagnosis and treatment. It is critical to identify species rapidly and accurately. Different types of PCR have been applied for detecting *Leishmania* DNA with high sensitivity.

Toxoplasma gondii

T. gondii is an obligate intracellular protozoan of clinical importance, especially in immunocompromised patients. Due to a lack of specific clinical signs, the diagnosis of toxoplasmosis can be challenging. Diagnosis of toxoplasmosis can be achieved by biological, serological, histological, or molecular methods. Detection of antibody and antigen in serum is the most routinely used method in clinical practice. The rapidly developed molecular techniques provide an important basis for the diagnosis of toxoplasmosis. PCR can detect infective toxoplasmosis by detecting single-genome DNA of tachyzoite.

CONCLUSIONS

During the past 30 years, great accomplishments have been achieved in controlling the spread of infectious diseases in China. However, China remains a major contributor to the global infectious disease burden. The emergence of novel and reemerging infectious diseases and the increased prevalence of antibiotic-resistant bacterial infections further complicate prevention and control strategies, indicating a clear need for faster, more accurate, and effective molecular-based tests. Since the first application of molecular diagnostic tests in clinical laboratories in China, a striking improvement in public health has been achieved, including reduced initial period of treatment, clinical management, and improved public health control. The recently FDA-approved tests with high sensitivity and specificity can be used directly for clinical samples, offering a more sensitive and specific result with a more rapid turnaround time than has ever before been possible. The newly developed molecular diagnostics are changing the way we practice clinical microbiology. Undoubtedly, we are entering the era of molecular diagnostics. However, for each of these methods, the additional costs must be calculated against the potential advantages of diagnostic value. In addition, the conventional methods for clinical microbiology cannot be replaced, and more well-controlled studies are still needed to evaluate the efficacy of these methods.

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Surveillance and Epidemiology of Norovirus Infections

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22

Norovirus is recognized as the leading cause of diarrheal disease in many countries. A study of the published literature shows that in the United Kingdom it is estimated to cause around 3 million cases each year (1). In the Netherlands, it has been estimated that norovirus is responsible for 11% of the 4.5 million diarrhea cases annually (2). In Canada, the estimated number of cases of gastrointestinal disease attributed to norovirus overall was a little over 3.3 million (3), accounting for 42% of gastrointestinal disease. A review by Hall et al. suggests that the number of people infected with norovirus in the United States is between 19 and 21 million each year (4). In the Southern Hemisphere, norovirus is the most common cause of gastrointestinal disease; one study from New Zealand suggests that norovirus was responsible for 26% of outbreaks, and in Australia norovirus causes the highest number of cases of gastrointestinal disease (5). A recent systematic review suggests that globally, norovirus is responsible for 20% of the burden of gastrointestinal disease (6).

The estimates of the burden of illness in some of the countries noted above are the result of modeling based on assumptions about the relative contribution of pathogens from studies elsewhere. By applying estimates from these studies to a mathematical model, an estimate of the likely burden in their own country is made. For example, in one of the first papers to estimate the incidence of norovirus in the United States, the figure was derived by applying the proportion of norovirus estimated from the Sensor study in the Netherlands to the incidence of gastrointestinal disease incidence from national hospital discharge data in the United States (4). This is a pattern repeated in later studies; the study in Canada used the incidence estimates for norovirus from the second Infectious Intestinal Diseases (IID2) study in the U.K. (3).

SURVEILLANCE

The utility of surveillance is to provide information for action, that is, to obtain data, in this case about disease, and provide insight for policy makers and public health officials. The objective is to provide information so decisions can be made about where to prioritize efforts in health policy and design interventions with an aim to reduce illness. It is important to acknowledge that surveillance is not a survey and that the notion that surveillance can be carried out for a short period of time ignores the importance of feedback. Surveillance can both identify the problem and assist in recognizing how interventions or policies have fared. For example, do the levels of disease decrease after the intervention or continue to rise?

Surveillance is described as being either active or passive. Active surveillance entails making an effort to gather information and is often limited in scope because of resource issues. For example, actively obtaining data by regular contact with clinicians or others at regular intervals, daily or weekly, ensures the completeness of data. Because of the intense nature of this approach, it is normally restricted to specific sentinel sites. These will have agreed at the outset of setting up the surveillance scheme to be data providers and will therefore likely be compliant. A rare disease might be amenable to active surveillance on a national scale, but this would need to be justified on the basis of its clinical importance, for example, if the disease in question had a high mortality rate. For common diseases it is not practical to conduct active surveillance on a national scale. Thus, for the most part, surveillance of disease is passive. Epidemiologists rely upon data collated from laboratory tests carried out in response to people becoming ill. When people seek medical attention, the clinician might request a sample, and for diarrheal diseases this normally would be a stool sample. This will be tested for the presence of pathogens, and the results will be recorded. These data can then be used to monitor the trend of an illness. Although not an end in itself, the results of testing may alert a public health agency to increases in incidence and detection of outbreaks, which can lead to further investigation to determine the cause.

CHALLENGES FOR SURVEILLANCE OF NOROVIRUS

The surveillance of any infectious disease is largely affected by two variables, first, the reservoir of infection and, second, the severity of the illness experienced. In the case of norovirus, the reservoir is the human gastrointestinal tract. This means the infection is mostly spread between people, i.e., person-to-person transmission via the fecal-oral route, with additional vectors such as food, water, and the environment. For gastrointestinal infections that have an animal reservoir (such as bacterial infections), foodborne

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transmission will predominate, with a small component of person-to-person transmission through secondary cases.

The severity of illness will influence whether those who contract an illness contact medical services. Infection with norovirus is characterized by a short incubation period followed by a sudden onset of symptoms, a short duration of illness, and recovery with no long-term effects. This provides a major challenge for diagnosis. The nature of these symptoms means the vast majority of people affected in the community will not have contact with medical services. In the IID2 study, conducted in the U.K. between 2008 and 2009, only 2% of those who contracted a gastrointestinal infection visited their family doctor, which equates to around 1 million consultations a year (1, 7). The IID2 study also found that the incidence of gastrointestinal infections had increased by 43% since the previous study (conducted in the mid-1990s) and that the proportion of cases presenting to their family doctor had declined by 50%. So more people were becoming ill, yet fewer were consulting their doctor as a result of their illness (7). These factors can have an impact both on the surveillance of norovirus, determining how many infections are occurring, what strains are circulating, and the optimal approaches to control of infection. Figure 1 shows a reporting pyramid for norovirus (adapted from reference 8). This illustration shows the pyramid as representing the total number of norovirus cases, with the top fraction being that which is reported to national surveillance (in England) and the rest being unreported. The number of laboratory reports for norovirus in England is merely the tip of this pyramid. In the second IID2 study carried out in the U.K. (1, 7), there were around 280 cases of norovirus in the community for each specimen in which norovirus was detected and reported to national surveillance in England.

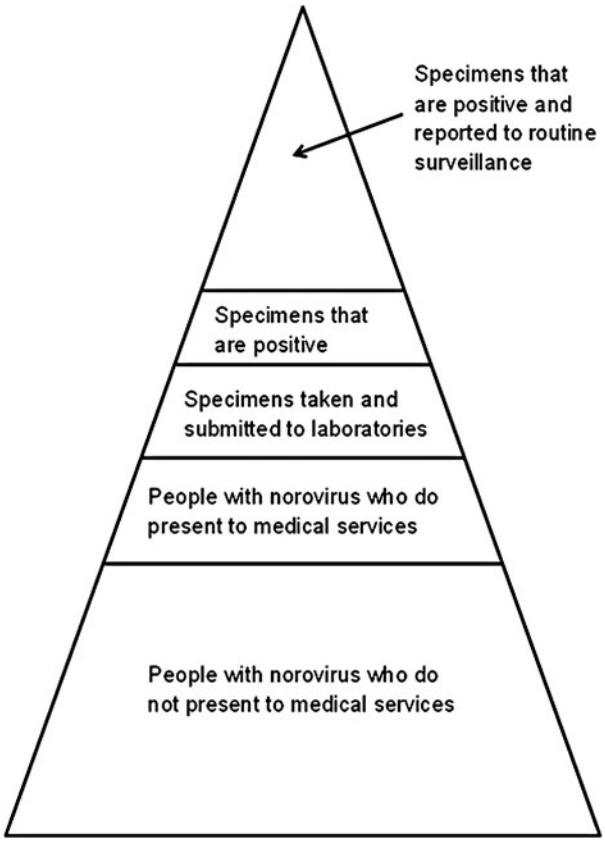

FIGURE 1 Reporting pyramid for norovirus. Adapted from Wall et al. (8).

For a mild, self-limiting disease such as norovirus, while there is less visibility within the general population (i.e., community illness), there is a more obvious burden in other settings. Norovirus is highly contagious and has a low infectious dose and a lack of long-term immunity to infection in the population. These factors provide the ideal ingredients for outbreaks to occur when people are in close proximity to one another. Outbreaks in such a setting are readily noticeable and can trigger actions needed to contain and record the outbreak. The lack of contact with medical services when people are infected with norovirus not only makes it extremely difficult to estimate the number of individuals affected, but it also makes ascertainment of disseminated outbreaks within the community extremely difficult.

OUTBREAK RECORDING IN ENGLAND

In 1992, surveillance of general outbreaks of gastrointestinal disease (involving two or more people in different households) was established following a recommendation of a U.K. government committee (8). The first review of data (1992–1994) from reported outbreaks suggested that norovirus (then called SRSV) accounted for 27% of outbreaks (9). In this review norovirus was responsible for only 6% of outbreaks in which food was the suspect vehicle, but 54% of outbreaks in which person-to-person spread was considered to be the route of transmission. The most common setting of an outbreak was in residential homes (i.e., long-term care facilities) and hospitals, accounting for 37% of reported outbreaks. A later review of outbreaks to the reporting scheme in 2003 provided stronger evidence of the importance of norovirus as a cause of gastrointestinal disease in England (10). This showed that over a quarter of all outbreaks occurred in hospitals, and 80% of these were attributed to norovirus. In the period for which this reporting scheme ran there were over 1,800 reported outbreaks of norovirus in hospitals (11).

The surveillance system in this study entailed sending a two-sided questionnaire to the lead person investigating the outbreaks (usually the Consultant in Communicable Disease Control). On receipt of the completed questionnaire, the data were then entered into a database. This method has the potential for data being lost if the questionnaire is never returned or at the very least could lead to a considerable reporting delay.

Changes to the requirements for mandatory reporting to the European Food Safety Authority led to Public Health England (formerly the Health Protection Agency) altering the way in which information on outbreaks of both foodborne and non-foodborne outbreaks is captured. Electronic reporting was introduced to improve the timeliness and at the same time enhance the information captured during outbreaks (12). This created a stand-alone reporting system, which now excluded person-to-person outbreaks of norovirus in hospital or community settings. From 1992 to 2013 Public Health England's surveillance scheme for foodborne outbreaks recorded 306 outbreaks associated with norovirus in which food was reported to be a vehicle of exposure, an average of only 14 outbreaks a year. Some outbreaks in which foods are a vector can be quite large; the largest outbreak of norovirus in a restaurant

occurred in 2009 when over 500 diners were affected (13). One outbreak in the United States, which is estimated to have affected around 3,000 people, occurred because a food handler contaminated a food coating used on cakes (14). This large outbreak is a good example of how foods can act as a net reproductive number amplifier, whereby large outbreaks occur that start with one infected person. In the outbreak reports in England in which food was believed to be involved, oysters were frequently reported as being consumed prior to illness. Oysters can become contaminated with sewage, and a recent survey of oyster beds in England suggested that over 70% were contaminated with norovirus (15). Despite such a high proportion of contaminated oyster beds, relatively few outbreaks of norovirus were reported associated with the consumption of oysters.

The redesign of outbreak reporting in England led to a reduction in both the number and quality of reports of outbreaks in hospitals. The U.K. Department of Health recognized the need to collect more detailed and systematic information on norovirus epidemiology in hospitals (11). This information was needed to develop rigorous evidence to inform infection control policies at local and national levels. In January 2009, the Hospital Outbreak Reporting Scheme was launched (11). The system was designed as an online reporting tool with only a few essential data items requested. The definitions within the dataset were agreed on by consensus with infection prevention staff. The system is designed to capture information on the start of the outbreak, when it ends, how many patients and staff are affected, whether the outbreak led to the closure of a ward or part of a ward, and how many bed days were lost as a result. At the launch of the system, all of the directors of infection prevention were invited to provide data. Data are entered directly by infection prevention and control staff working in the hospitals.

The first detailed analysis of the HNORS data (11) showed that there were over 3,900 outbreaks reported from January 2009 to December 2011. These outbreaks affected over 40,000 patients and over 10,000 staff (medical and nursing staff) and led to a reported 46,500 bed days lost. Furthermore, while the number of outbreaks reported varied between the two seasons, there was little variation in the impact of outbreaks. Over 50% more outbreaks were reported in the winter season of 2009/2010 compared to the following winter, yet the median number of patients or staff affected and outbreak duration remained the same (11). Reporting outbreaks of norovirus in hospitals through HNORS is still voluntary and therefore is likely to lead to under-reporting. An estimate of this likely under-reporting was carried out using a form of capture-recapture analysis. This suggested that the under-reporting was around 20% for the two norovirus seasons. Even with that limitation, the new Web-based surveillance scheme provided much greater insight into the burden of norovirus outbreaks in NHS hospitals. In the first year alone of the new scheme, more outbreaks were reported than during the 17 years in which the previous outbreak reporting system was used (1,884 episodes compared to 1,817).

Use of electronic surveillance via the Internet is well established, and the increase in reporting and the improved quality of the information included in these outbreak reports are in some part due to the introduction of an online reporting scheme. Online reporting is amenable to on-the-spot guidance in definitions, such as "case" and "outbreaks." Furthermore, because the reporting scheme is targeted at collecting well-defined information, any data will conform to greater epidemiological rigor. Previously, the reporting scheme (GSURV) was limited because definitions had to be broad to encompass outbreaks from various settings, and these are different from outbreaks in hospitals (11). This is likely to have manifested itself by reporters varying in their interpretation of what constituted an outbreak; i.e., did an outbreak on more than one ward of a hospital constitute a single outbreak (presumably from a single source) or was this several outbreaks? (This question is revisited later.) The other major benefit of introducing an electronic surveillance scheme is the increase in the timeliness of outbreak reporting. Reporters are able to enter the details of an outbreak at any time during the course of the outbreak. Data can be updated at any time during the outbreak or once it has run its course.

LABORATORY SURVEILLANCE

Public Health England and its predecessor organizations (PHLS, HPA) have routinely collected and published data on laboratory-diagnosed pathogens for more than 50 years (8). These reports have been collated on a database at the Center for Infectious Disease Surveillance and Control since 1975 (8). For much of the early years, norovirus (or SRSV, as it was known then) did not feature in routine laboratory reporting. As methods for identifying norovirus improved, and as part of outbreak reporting, norovirus began to be reported more often. The number of laboratory reports increased (Fig. 2). Laboratory reporting increased throughout the 1980s and 1990s.

In 2007, reverse-transcriptase PCR (RT-PCR) for norovirus testing was rolled out to all laboratories that formed part of the Public Health England regional network. The increased sensitivity of this test led to greater numbers of norovirus detections in stool samples. The introduction of new, more sensitive laboratory techniques can lead to misinterpretation of the data. Uncritical analysis of the pattern of laboratory reports would present as a sudden increase in the level of norovirus infection. Figure 2 shows a time series of laboratory reports of norovirus infections from Public Health England from 2000 to 2014. The laboratory reports appear to indicate an increasing trend of laboratory-confirmed cases. However, this would not provide a good indication of the true pattern of norovirus infections without consideration of changes in laboratory techniques introduced in the mid 2000s. What is clear is the seasonality of norovirus. The peak number of laboratory reports occurs in the winter months each year. However, in some years this peak is sharper, and in others it is more prolonged and less intense. The winter season of 2013/2014 in England was comparable to that in the years before the routine use of PCR for diagnosis of norovirus in England and compares in intensity to 2004. Norovirus does not have a predictable seasonality, and as will be discussed later there are clearly other determinants of why this might be.

The seasonality of norovirus laboratory reporting has been a useful tool in estimating the burden of disease due to norovirus, particularly in the elderly. Previous research had shown that although norovirus is considered a mild and self-limiting disease, some populations seem to fare worse than others (16). The seasonal pattern of laboratory reports modeled against mortality data suggested that norovirus is associated with 20% of the deaths caused by infectious gastrointestinal disease in the elderly (17). Fur-

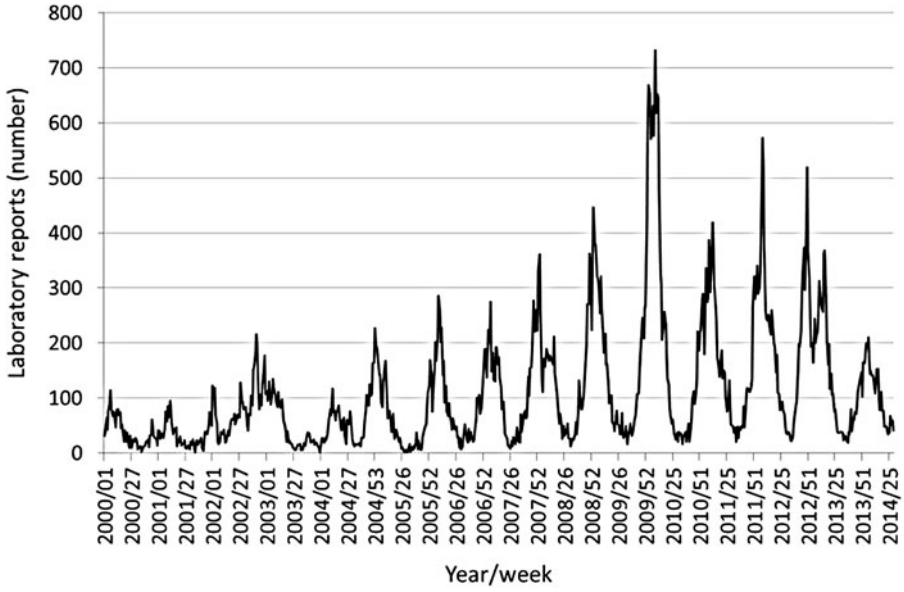

FIGURE 2 Public Health England laboratory reports of norovirus 2000–2014.

thermore, it was associated with 13% of noninfectious gastrointestinal disease deaths in the same population (17). A study in the Netherlands also found a significant association with norovirus activity and excess mortality in the elderly (18).

METHOD OF VIRUS DETECTION

Norovirus was only discovered in 1972. Stool samples from an outbreak in Norwalk, Ohio, were tested using immune electron microscopy (19). Serum samples from people recovering from illness were dropped onto fecal samples from the Norwalk outbreak, causing virus particles to clump together and enhancing the likelihood of detecting them under an electron microscope (19). Electron microscopy became the mainstay of diagnostic testing for norovirus, and classification of the virus was based on viral morphology (20). The unraveling of the norovirus genome facilitated the development of PCR-based assays (21, 22). In addition, the production of viral antigen particles and development of enzyme-linked immunosorbent assay tests also greatly increased the sensitivity of norovirus diagnostics (23, 24). While the enzyme-linked immunosorbent assay tests are shown to be reasonably sensitive, a large European study showed variability in their performance and concluded that they were not as sensitive as RT-PCR (25).

GENETIC DIVERSITY

The application of these detection methods has also led to a greater understanding of the diversity of norovirus genetics. Currently, noroviruses can be classified into five genogroups (there is putatively a sixth group identified in a small study in Europe [26]); three of these (I, II, IV) affect humans, and groups III and V affect animals. Each of the genogroups is further subdivided into genotypes (27, 28). Figure 3A illustrates the diversity of noroviruses; genogroup I (blue) contains the reference strain (Norwalk), and genogroup II contains the strain that causes most outbreaks (genogroup II.4, in red). This genotype is particularly noted in outbreaks in health care settings. Figure 3B illustrates the continued diversification of this genotype; the bars indicate how closely related each of the strains is to one another. Where the lines bifurcate indicates a common ancestor, so although these are all GII.4 genotypes they are clearly continuing to diversify.

LABORATORY TECHNIQUES

The introduction of newer technologies for detecting norovirus has led to more complexity in interpretation of the results. For example, RT-PCR is sensitive enough to detect the RNA of virus in samples from people who have not reported any gastrointestinal symptoms in the last several months. When archived stool samples from the first IID study (carried out in the mid-1990s) were retested using PCR, 16% of the samples from healthy controls contained norovirus RNA (29). Reanalysis of these data led to the recognition that around 12% of the population are likely to have an asymptomatic infection (30). Furthermore, prevalence increased in children below the age of 5, a group that already showed the highest prevalence (30). This raises the issue of the clinical significance of detecting norovirus in stool samples, especially when using highly sensitive assays. One method to assess whether detecting norovirus in stools is associated with illness is to use the cycle threshold (Ct) value as a measure of viral load (31). One complication of this study was the lack of calibration between Ct value and estimated viral load for all genotypes. For example, amplification of rarer genotypes, such as GII.7 and GII.8, was not as efficient as that for noroviruses of other genotypes (31). Genogroup I noroviruses were not included in the study, in part due to the variability of the efficiency of the assays for this genogroup (31). Clarifying both epidemiologic and clinical issues will require collecting sufficient specimens containing noroviruses of other genotypes to assess the potential Ct cut-off values that could be implemented as predictors for other genotypes of fecal viral loads (31).

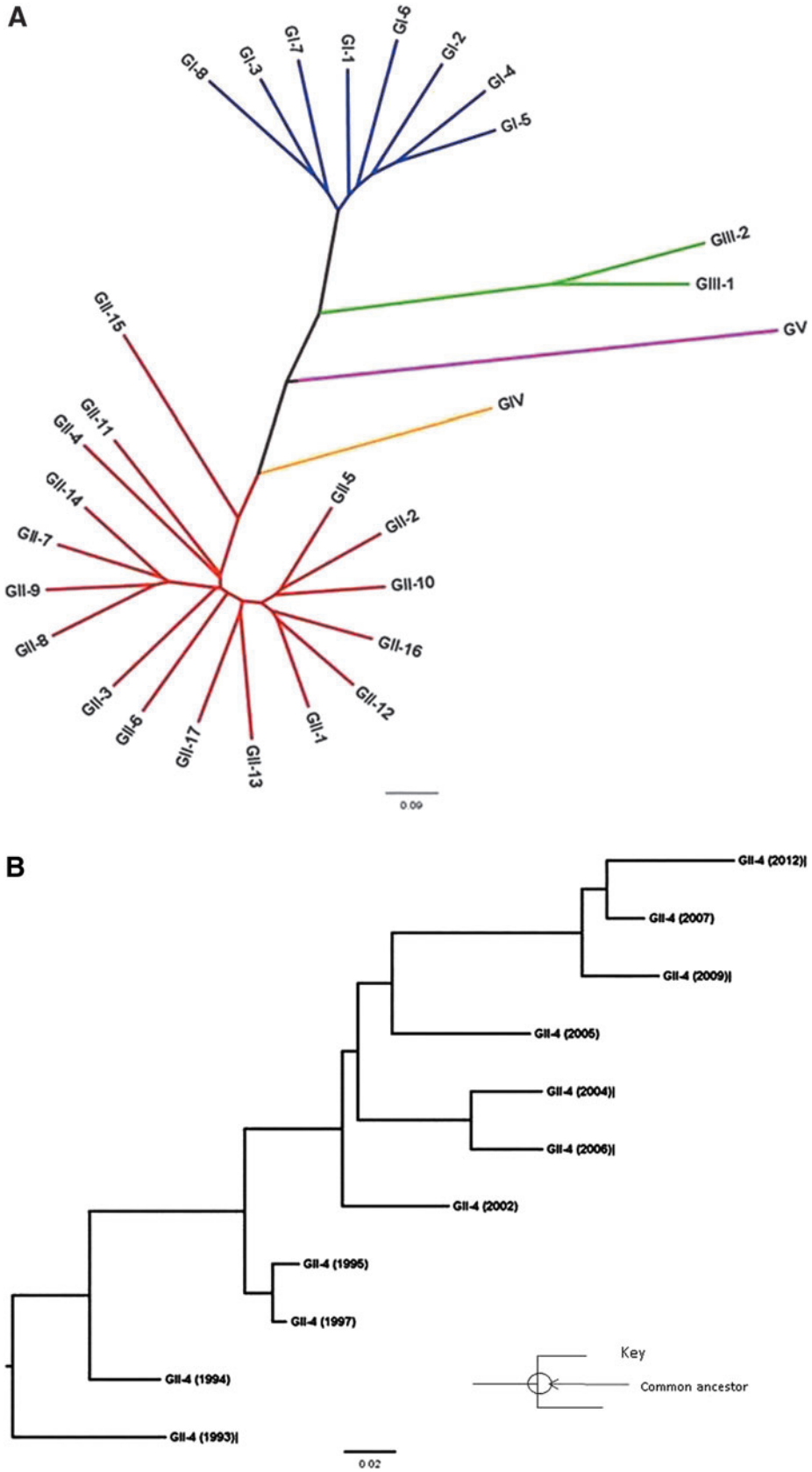

FIGURE 3 (A) Phylogram showing distribution of norovirus genogroups. (B) Diversity of GII.4 genotypes. (Courtesy of David James Allen, Virus Reference Department, Public Health England.)

Laboratory testing during outbreaks does not necessarily provide evidence of a source of infection or indicate whether a single outbreak is occurring or multiple outbreaks are occurring simultaneously. For example, norovirus outbreaks in which food is a source of infection do not always have a single genogroup or genotype of norovirus identified from patient specimens (32–35). When multiple genotypes of norovirus are detected in an outbreak investigation, one cannot use "norovirus detected" alone as part of a case definition (as is typically used for investigations of bacterial gastrointestinal foodborne infections) without being more specific as to the type or group. If laboratory analysis and reporting is separate from epidemiological reporting, problems will likely occur if only "norovirus detected" is used due to lack of specificity.

REPORTING PATTERN/SEASONALITY

Some winters have had noticeably increased numbers of laboratory reports of norovirus infections. These have coincided with increased reporting of norovirus around the world. For example, in 2002, it was widely reported around Europe that there was an increase in norovirus activity associated with the emergence of a new variant of the genotype II.4 strain (36). This increased norovirus activity was also noted because of the increase in infections occurring outside of the normal winter season, with a noticeable increase in the spring and summer (36). In 2006, there was yet another emergent strain of the genotype II.4 globally (37). This variant was associated with a number of outbreaks on cruise ships, and most of these happened in the early summer (38, 39). The emergence of new strains can lead to epidemic years with much greater numbers of norovirus infections, but this association is not straightforward. In 2012, it was widely reported that norovirus activity had begun earlier than usual in several countries (40). This appeared to be associated with the emergence of a new genotype II.4 strain, labeled Sydney 2012 (40, 41). This led to predictions of another season of unusually high activity; however, this did not materialize. Laboratory reporting was higher in the early part of the season, but there was low activity later on in the winter and fewer laboratory reports for the winter overall (42). The emergence of a new strain does not necessarily imply a season of higher activity, and it is likely that differences in seasonal norovirus activity are linked to other factors which are not fully understood.

ALLYING EPIDEMIOLOGICAL SURVEILLANCE WITH LABORATORY ANALYSIS OF STRAINS

The future of surveillance will depend largely upon the development of new technologies in the sphere of diagnostic microbiology. Analysis of strains circulating in real time can provide clearer information on what strains are causing illness and if some are causing larger outbreaks than others. This means a closer integration of both laboratory and surveillance data. Linking epidemiological data gathered on outbreaks with laboratory results and analysis will provide a clearer picture of any differing impact that circulating strains have on outbreaks (assuming one exists). At present, this is very unclear.

As mentioned earlier, the question often arises as to whether a number of cases in an outbreak are linked. Outbreaks occur on more than one ward in a hospital or in several rooms in a nursing home. The approach to dealing with outbreaks in health care settings is typically to take specimens only from a few individuals; often these are the first few symptomatic cases recognized. This approach is a holdover from the days of using electron microscopy, a relatively insensitive, time-consuming, and costly method of detecting norovirus. The advent of molecular techniques, i.e., RT-PCR, has increased both the speed and sensitivity of detecting norovirus in patient samples.

One technological development that has considerable potential as a typing and epidemiologic tool is sequence analysis of a small hypervariable region of norovirus genome. Studies have analyzed the P2 domain to assess whether infections are the result of clonal transmission or are the result of multiple sources (43–45). This would provide useful evidence of infection control breakdowns where transmission events are shown to occur from one individual to another. In outbreaks in which the vector is likely to be food, this would be useful in pinpointing failures of hygiene, such as an infected food handler who has contaminated food during preparation. These methods, which are still in development, have yet to be used routinely and are likely to be expensive.

The best strategy for sampling and laboratory testing during outbreaks is largely dependent upon the nature of the investigation. For example, most physicians are probably not interested in which genotype of norovirus the patient has, just that the patient has a norovirus infection. This can impact the scope of testing. Does a hospital test a few patients just to establish that there is an outbreak, or do you test broadly to ascertain whose diarrheal illness is caused by a specific norovirus and exclude patients that may have diarrhea due to other pathogens (e.g., *Clostridium difficile*, sapovirus, or rotavirus). For infection control professionals, testing broadly and providing subtyping information that clarifies the number of norovirus variants present is helpful for ascertaining whether patients are part of one or more outbreaks. Having information on genogroup/genotype also will provide supporting evidence for any planned interventions and help to assess the success of those interventions once they are put in place. Unfortunately, such testing is costly and often is not undertaken, simply for financial reasons.

In an increasingly litigious world where holidays are said to be ruined by outbreaks, the expense of taking samples and testing to a higher resolution, such as analysis of the P2 domain, might be justified since the data generated could potentially prove or disprove whether someone was part of an outbreak. The major question is who bears the cost of that testing. Given the scope of some norovirus outbreaks, the capacity for laboratories to deal with the increasing workload associated with more specimens being taken for analysis can quickly be overwhelmed. The availability of newer commercial methods that simplify testing may impact this dilemma in the future.

An alternative approach to testing that has been suggested is to apply the use of metagenomics to identify noroviruses in fecal samples. In general, the advantage of such techniques for dealing with bacterial infections is that the time required for culturing and isolating the bacterium can cause a delay in identifying the etiologic agent of the illness, especially if there isn't an easy and reliable test for a particular organism, such as for enteroaggregative *Escherichia coli* or other diarrheagenic varieties of *E. coli* (46). Metagenomics provides a mechanism for identifying an organism by sequencing the nucleic acid extracted directly

from samples (46). However, for diagnosing norovirus infections, RT-PCR is straightforward and is widely becoming the standard of care, so for simple diagnosis metagenomics does not provide any advantage, particularly since metagenomics is still quite expensive. Where metagenomics may be of value is in identifying other microbial factors associated with susceptibility to disease. For example, a recent article suggests that the bacterium *Enterobacter cloacae* facilitates norovirus binding to human cells (47). Metagenomics might be useful for identifying carriage of commensal bacteria, which, if removed, could help prevent norovirus infection in susceptible populations. However, at present this is speculative.

FUTURE TECHNOLOGIES/DEVELOPMENTS

The difficulty of relying on molecular methods for diagnosis, as highlighted above with detecting the presence of norovirus in patient stool samples, is that it leaves the question of causality unanswered. Development of methods to show viability of virus in samples would be useful in determining whether norovirus is the cause of the illness. As mentioned above, norovirus contamination of oysters in U.K. waters, particularly during the winter months, is common. Despite the detection of norovirus in oysters, outbreaks or illnesses associated with the consumption of oysters are seldom reported; outbreaks are under-ascertained, and only larger outbreaks where parties of people who are known to each other are more likely to be reported. Techniques are now being developed to help distinguish between virus in samples that are infectious or noninfectious. The idea is that it is possible to distinguish between intact virus particles, which are more likely to cause illness, and virus particles that are not intact (48). This may provide a very different view of norovirus epidemiology.

CONCLUSIONS

The reservoir for norovirus infection is the human gastrointestinal tract. This means that any sporadic illness must inevitably be part of a chain of transmission from one person to another. Norovirus does not grow on foods and therefore cannot be truly foodborne in the same way that zoonotic bacteria cause gastrointestinal illness. Nonetheless, foods can be effective vehicles for infection whereby one person, through lack of diligence to hand hygiene during food preparation, can infect a large number of others with norovirus. Similarly, the contamination of food with norovirus at the source, such as either through contact with sewage or through irrigation, has the potential to infect many consumers, sometimes over large distances, when the food is consumed raw. Once a large number of people have become infected, person-to-person transmission can result in further amplification in large community outbreaks.

The principal challenge for surveillance of norovirus infections is the lack of contact ill people typically have with medical services, therefore evading a definitive diagnosis. This lack of direct measurement of illness means our current knowledge of the burden of disease from norovirus infections is often based on mathematical modeling to estimate the burden of norovirus illness in a population. This problem is not unique to norovirus. For example, *Campylobacter* spp. are the most common cause of bacterial gastroenteritis identified in England, yet the number of reported outbreaks remains low (49, 50). Even though campylobacteriosis is a more severe disease with a longer duration of illness, it is subject to considerable under-reporting. Electronic reporting can speed up the recognition of outbreaks and can improve the determination of the ultimate impact on public health, particularly in terms of the number of people affected and the duration of outbreaks. However, not all facilities use electronic reporting. The collection of data in the community, particularly the occurrence of sporadic norovirus illness, is likely to remain a challenge given the lack of contact of patients with medical services to get a definitive diagnosis.

What is clear is that to obtain a greater understanding of the epidemiology of norovirus infections requires a close integration between microbiology and epidemiology resources. The possibility of a norovirus vaccine in the near future (51) will clearly require greater understanding of the epidemiology of the disease to determine which populations would benefit most from vaccination and what the ultimate impact of a vaccine could be. The introduction of PCR testing has increased awareness of the prevalence of asymptomatic carriage of norovirus, yet the implications of such asymptomatic carriage are not fully understood, especially in controlling spread of disease. Unraveling this question may require novel methods, such as those being developed to ascertain if virus in samples is intact and therefore likely to cause illness. Norovirus will likely be a challenge for years to come.

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Molecular Diagnostic Assays for the Detection and Control of Zoonotic Diseases

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23

Zoonotic pathogens, those that are naturally transmissible from animals to humans, are important causes of morbidity and mortality in humans, with outcomes ranging from mild to fatal and occurrences from sporadic disease to pandemics. These pathogens have had profound impacts on human health throughout recorded history and will continue to pose challenges, as the majority of emerging pathogens are zoonotic (1). Continued encroachment of humans into previously undeveloped regions, concentration of human and animal populations from urban sprawl, expanded animal and vector ranges resulting from climate change, increasingly mobile animal populations, and rapid international movement of animal-based foods facilitate the emergence and rapid dissemination of zoonotic pathogens. Recent years have been accompanied by dramatic instances of newly identified zoonotic pathogens (e.g., severe acute respiratory syndrome coronavirus [2], Middle East respiratory syndrome coronavirus [3, 4], Hendra virus [5]), marked range changes in known pathogens (e.g., West Nile virus in North America [6]), and new variants of well-known pathogens (e.g., H1N1 pandemic influenza A [7]). Potential new clinical roles for known pathogens have also been identified, such as the recent association of *Bartonella henselae* with some neurocognitive disorders (8). The close relationship between animals and humans has also been reflected in the identification of interspecies transmission of important pathogens such as methicillin-resistant *Staphylococcus aureus* (MRSA) (9) and extended-spectrum beta-lactamase-producing *Enterobacteriaceae* (10, 11). At the same time, "traditional" zoonotic pathogens such as rabies virus, influenza A, *Salmonella*, *Campylobacter*, and *Coxiella burnetii* continue to pose tremendous challenges. While the overall impact of zoonotic diseases is not known (and virtually impossible to quantify), it is undeniable that diseases spread directly from animals, from animal products, and from animal contamination of the environment have a profound impact on human health and require substantial efforts for diagnosis, treatment, prevention, and control. All of those have a foundation in accurate and timely diagnostic testing, so it should be no surprise that molecular diagnostic testing is increasingly being used to identify zoonotic pathogens.

The use of molecular diagnostic testing for zoonotic diseases encompasses a wide range of potential activities, each with different objectives and challenges (Table 1). These include:

- Clinical diagnostic testing of sick animals to detect zoonotic pathogens
- Routine surveillance of healthy animals, targeting a zoonotic pathogen
- Routine surveillance of food or water for zoonotic pathogens
- Testing of animals in response to a zoonotic disease case or outbreak
- Testing of food or water in response to a suspected zoonotic disease case or outbreak
- Clinical diagnostic testing of humans that identifies a zoonotic pathogen
- Testing of animals, humans, food, and water in response to an unknown disease problem
- Distinguishing vaccination antigen from natural infection

In some ways, a separate discussion of the use of molecular diagnostic assays for zoonotic pathogens (versus other pathogens) is redundant since the concepts of testing apply across all pathogens, regardless of whether or not they belong to the large percentage of infectious diseases that may have a zoonotic origin. The ultimate goal is the same: to identify (and potentially characterize) a pathogen. Yet differences in pathogen biology, sampling challenges, test development, laboratory availability, regulatory scrutiny, quality control, cost, and related factors indicate a need to consider the non-human side of the zoonotic pathogen continuum.

Thus, while the overall objectives of the different areas listed above are similar, approaches taken for animals, food, and water can vary greatly from approaches taken for humans. Further, there can be marked differences between animal populations, which could encompass food-producing animals, household pets, and wildlife. Widespread national and international movement of some domestic animals (e.g., horses, dogs) and migration of vast numbers of wildlife create further challenges in zoonotic disease investigation, prevention, and control, all of which may involve molecular diagnostic testing.

POTENTIAL BENEFITS AND COSTS

As with any application of a diagnostic test, the potential costs and benefits of molecular diagnostic testing for

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TABLE 1 Examples of molecular diagnostic approaches for zoonotic diseases

Category	Pathogen example	Comments
Food	Shiga toxin–producing *Escherichia coli* (STEC)	Immediate differentiation of STEC from other *E. coli*. Short turnaround time reduces holding delays while awaiting results or release of food before results are available.
Food animals	*Coxiella burnetii*	Much faster and easier for this fastidious pathogen that requires biosafety level 3 containment for isolation.
Companion animals	*Leptospira* spp.	More rapid and accurate than seroconversion. More readily available than culture or dark-field microscopy. Hampered by limited understanding of leptospiral shedding, potentially associated with false-negative results.
Wildlife	Avian influenza	Rapid screening for a pathogen that is of potentially profound public health significance. Shorter turnaround time and limited biosafety concerns compared to culture. Cost can limit widespread use.
Foreign and reportable diseases	*Brucella* spp.	Rapid screening to facilitate importation and reduce risk of entry into disease-free areas, and prompt identification of imported disease. High negative predictive value required.
Import screening	Limited use currently	Potential role of sensitive and rapid tests, potentially at point of import, to test for selected pathogens to decrease the risk of entry of pathogens and/or to have testing replace broad importation bans. High negative predictive value required.
Outbreak investigation	*Bacillus anthracis*	Rapid diagnosis for early intervention. Reduced laboratory safety concerns compared to culture. Lack of typing ability may be a limitation.
Differentiation of vaccine antigen from natural infection	Limited use currently	Few clearly demonstrated examples but a potential benefit to facilitate use of vaccination in outbreak response in situations where it is currently limited or prohibited.

zoonotic disease detection and control must be considered. Benefits include the ability to provide shorter turnaround times, something that could be critical from many standpoints, increased sensitivity, detection of fastidious organisms, and detection of pathogens without the need for culture and the associated biocontainment requirements. Advances in testing that facilitate field testing could also be of tremendous use, permitting decentralization of testing so that rapid testing could be performed on farms, in food production facilities, at veterinary facilities, in the field, and at other postcare or point-of-sampling locations.

Costs include the economic cost of testing, which is often greater than conventional (e.g., culture) methods, the need for specialized equipment, and greater technical demands. Yet advances in technology will probably reduce these limitations. Test development and validation costs can also be substantial, something that is often overlooked. An inability to provide an isolate for further testing and/or to characterize a detected pathogen may also limit some clinical and epidemiological applications of molecular methods.

Cost-benefit analyses are typically lacking and would vary greatly with the test, animal species or food product, disease, and region, and technological advances make this a rapidly moving target.

USE OF MOLECULAR DIAGNOSTIC TESTING IN DIFFERENT TYPES OF ANIMAL POPULATIONS AND SITUATIONS

Food

Food is an important vehicle for zoonotic pathogens, and the incidence of food-borne disease is high in both developed and developing regions. In the United States alone, an estimated 48 million food-borne illnesses occur yearly, with over 100,000 hospitalizations and 3,000 deaths (12). Testing of food may be performed at various levels in the production and distribution chain as a routine tool, with additional testing performed in response to outbreak concerns.

Traditionally, most food testing has involved culture, with molecular methods sometimes used for confirmatory testing or characterization of isolates. However, the potential for molecular diagnostic testing to increase sensitivity, decrease turnaround time, and increase throughput has been embraced, and there is increasing movement toward "point-of-care" molecular tests in food production (13). As with point-of-care testing in health care, movement of properly validated tests to food production sites (assuming proper test operation and quality control) can reduce turnaround times (facilitating test-and-hold procedures so that contaminated products are not released versus being recalled later, and facilitating more rapid investigation of causes of contamination to reduce further contamination), improve sensitivity of detection for some pathogens (e.g., *Campylobacter*), and potentially reduce costs (which might facilitate broader testing). Potential concerns include test sensitivity and specificity, the requirement for smaller facilities to have adequately trained personnel and quality control practices, limitations in the ability to detect broader population changes if there is no centralized reporting of results, and a lack of characterization (antibiogram, typing) for integration with clinical testing results. These can largely be overcome with proper test validation, a commitment to proper in-facility testing, communication of test results, and either performing secondary testing (i.e., culture) on selected positive samples, submitting subsets of

positive samples to another laboratory for secondary testing, or holding aliquots of positive samples for potential future testing. Testing for multiple pathogens through multiplex PCR, microarray, or panels of individual molecular tests can also be beneficial (14). Further, some molecular tests can provide additional and important information at the time of pathogen identification, such as detecting Shiga toxin-producing *Escherichia coli* rather than simply detecting *E. coli*. Yet while molecular methods have many potential advantages, the need for isolates to perform antimicrobial susceptibility testing and the need for some typing methods mean that a complete transition to culture-independent methods will not likely occur in the short term.

Integration of human diagnostic testing to identify food-borne disease, identify clonal outbreaks, and facilitate identification of the inciting food source is critical and can involve different types of conventional and molecular assays. The United States' PulseNet, an integrated network of public health and food surveillance laboratories, is an example of such a system. Integrated national surveillance programs like this can have a profound impact, and PulseNet is credited with prompting the recall of over 650 million kg of potentially contaminated food over the past 15 years (12) while identifying thousands of outbreaks and presumably preventing a large number of illnesses and deaths.

As opposed to tests used for many other situations described in this chapter, tests used on food have typically undergone more scrutiny and standardization. A wide range of validated methods has been published for a range of pathogens such as *Salmonella*, *Listeria*, and Shiga toxin-producing *E. coli* (15, 16). However, there are no universally accepted guidelines for performance standards. Obviously, a test that is 100% sensitive and specific is ideal, yet this is rare. Implications of false positives (e.g., resources required for follow-up investigation, condemnation of potentially large amounts of food, false disease burden estimates, consumer fear, economic impact on farm animal production) and false negatives (e.g., failure to identify a developing problem) must be considered alongside the expected population prevalence (which will impact the positive and negative predictive values).

Movement to molecular diagnostic tests, from the field or production facilities to national reference laboratories, is not without challenges, given the historical reliance on culture and the need for profound changes in equipment, skills, training and, indeed, mindsets. Beyond validation of tests, broader efforts to standardize both the tests and their applications (i.e., encouraging the use of the same standardized assays between laboratories) and to standardize data collection and reporting are required to facilitate prompt and accurate identification of outbreaks and food-borne disease threats. Harmonized molecular testing and data analysis, such as PulseNet's standardization of testing and classification protocols for various pathogens through methods such as pulsed-field gel electrophoresis (17, 18) or multilocus variable number tandem repeat analysis (19), are critical. International harmonization is less commonly performed but is similarly important because of the international distribution of the food supply chain and corresponding international outbreaks (20–23). Improvements in harmonization of protocols, along with coordination of typing nomenclature and data reporting, are required given the ever-expanding risk of international food-borne outbreaks.

An additional consideration for food (or animal) surveillance is the impact of changes in testing on disease trends. Since molecular diagnostic tests may have different (and often higher) sensitivities, changing test practices can be accompanied by spurious changes in disease rates. Therefore, it is critical that this be considered when investigating disease trends to avoid false conclusions.

The economics of food-borne pathogen testing play a major role in the selection of routine surveillance tests. In addition to accuracy, food testing requires a combination of rapidity (to minimize holding times and/or reduce the risk that food is sold before results are available), cost, and throughput (to allow for testing of large numbers of samples). Low limits of detection may be required for some pathogens that pose a particular risk and have very low infectious doses, such as *E. coli* O157:H7. Development of tests that have greater sensitivity but higher costs can create a quandary when resources are fixed, since movement to more expensive tests could result in smaller numbers of tested samples. Decreases in resources for laboratory detection and data analysis were commonly reported in a recent U.S. study (24), something that could hamper proper implementation of newer tests. The costs and benefits of any new test require careful study and may often be difficult to accurately quantify.

Food Animals

Food animals are important sources of zoonotic pathogens from direct contact, environmental contamination, and as the ultimate source of most food-borne pathogen contamination. The role of routine testing of food animals for the prevention of food-borne disease varies between animals and pathogens and is often limited. Testing to identify endemic pathogens (e.g., *Campylobacter* in poultry) is of little use in the absence of corresponding measures that would be taken in response to positive results, something that is typically the case. There is greater interest in testing for less common, high-impact pathogens (e.g., *E. coli* O157 in cattle), but as a routine measure, if there is no ability to intervene based on a positive result, screening is of limited impact, and control measures must be focused further along the production chain.

Accordingly, routine testing of food animals is rarely performed because of cost, logistics, the large number of food animals, and a lack of evidence for most pathogens that detection of a zoonotic agent in an animal on a farm represents a risk to the consumer. There are some situations, though, where testing could be justifiable. This would include outbreak investigation where a link to an individual farm is suspected (e.g., waterborne outbreak, petting zoo outbreak) or to investigate a developing zoonotic disease event such as hyperendemic Q fever in a region (25, 26). In these situations, farm-based surveillance would be justified for source attribution or to understand an apparent change in the epidemiology of disease. A situation where testing of healthy animals on farms is potentially useful and increasing is through national surveillance programs that monitor antimicrobial resistance in selected food-borne pathogens and indicator organisms (e.g., generic *E. coli*). Because of the need for antimicrobial susceptibility testing, these programs rely on conventional culture as primary tests, with molecular methods used on a more limited basis for isolate characterization and evaluation of virulence or antimicrobial resistance genes.

Molecular diagnostic tests are also of value for detection of pathogens that are fastidious and difficult or

impossible to isolate using conventional methods and those that require enhanced biosafety practices. A few examples of these are present in food animals, such as *C. burnetii*.

Beyond surveillance, testing of food animals is also performed diagnostically in response to disease. The approach to this varies greatly among species. For example, the low individual animal value of chickens means that testing of an individual chicken would be done not to determine an appropriate action to treat that individual, but rather in response to a concern about the entire flock. In contrast, individual animal testing for the purposes of both identifying a herd problem and determining an appropriate treatment course for the individual animal would be more economically justifiable in cattle. Therefore, test development for some species (e.g., poultry) focuses on high-impact pathogens that may have broad flock or herd effects, while there may be more justification for development of tests targeting sporadic diseases for more valuable animals (e.g., cattle).

Even with more valuable species, costs of testing can be a significant limitation because of the economics of food production and questions of who should pay for such testing (e.g., farm, manufacturer, government). Since diagnostic costs are almost exclusively borne by the producer, there must be a clear advantage to justify any changes in methods that might increase costs, and cost is a major reason that molecular testing has been used diagnostically on only a limited basis. Even in more economically valuable animals, testing for diagnosis of individual animal disease is rather limited at this time. Molecular tests are most commonly used for characterization of pathogens, mainly as part of surveillance activities (e.g., characterization of all *Salmonella* isolates from food animals) or outbreak investigations.

Harmonization of test methods, validation of tests, and integration of reporting systems are also important, as is discussed above for food. There are some strong examples of integrated surveillance systems, particularly for international avian influenza surveillance networks (27) and (mostly national) antimicrobial resistance surveillance programs (28–30).

Companion Animals

The potential role of companion animals in zoonotic pathogen transmission is often overlooked, despite the fact that pets are present in the majority of households in some countries (31). Further, the close nature of contact between people and domestic animals facilitates transmission of various zoonotic pathogens that are endemic or periodically encountered in companion animals. Companion animals are also able to act as bridging populations between wildlife and humans and act as intermediaries for transmission of wildlife-associated pathogens (e.g., avian influenza [32, 33], *Leptospira* [34]). There is also increasing evidence that companion animals can acquire pathogens typically associated with human infection from their human contacts (e.g., MRSA, *Clostridium difficile* [35–39]) and subsequently pose a potential risk to humans.

Most testing of companion animals is performed as part of clinical diagnostic testing by veterinarians in response to disease in an individual animal. Since a variety of diseases in companion animals are caused by potentially zoonotic pathogens (e.g., *Leptospira*, *Salmonella*, *Campylobacter*, dermatophytes, multidrug-resistant bacteria, *Giardia*), early and prompt diagnosis is important. Formal pathogen surveillance is limited, and it is rare that companion animal testing is performed in response to human disease events.

A wide range of molecular diagnostic tests are available through commercial diagnostic laboratories, and there is increasing use of molecular assays because of the perceived advantages of some tests, shorter turnaround times, and extensive marketing. Some molecular assays have proven advantages over conventional techniques, such as the potential to diagnose leptospirosis prior to seroconversion (40, 41). While many molecular assays may be more expensive than traditional methods, there are typically fewer economic constraints in companion animals because of the human-animal bond.

Broad national efforts are rarely directed at companion animals; however, zoonotic disease outbreaks associated with companion animals can be detected when they are caused by enteric pathogens that are tracked by established food-borne surveillance systems. For example, outbreaks of salmonellosis associated with feeder rodents, pet food, and pet frogs have been identified through PulseNet surveillance (42–44).

Wildlife

Comprehensive surveillance studies are performed on many wildlife species, including urban wildlife (45), wildlife that live in close proximity to livestock (46), wildlife that largely confine their range to local areas, and migratory species that can traverse countries or continents (47, 48). Many objectives are approached with wildlife surveillance, including zoonotic pathogen surveillance. Targeted surveillance activities may also be instituted in response to specific disease threats, such as wild bird screening for influenza A (49, 50), exploratory surveillance to identify natural hosts of pathogens such as Ebola virus (51) or the SARS coronavirus (52), investigation of animal sources in response to sporadic or epidemic disease in humans (e.g., leptospirosis), and evaluation of antimicrobial resistance in pathogens and commensal bacteria (53, 54).

Laboratory methods are not usually developed strictly for wildlife testing. Rather, wildlife testing is typically performed using methods established for other species or sample types. There is an increasing number of molecular tests for targeted pathogen detection (55, 56), broad-range methods to identify microbial populations (microbiotas) (57), or nonspecific methods to identify known and unknown organisms (e.g., next-generation sequence-based viral discovery approaches).

Various challenges can be encountered with wildlife surveillance, not the least of which is actually obtaining samples. Initial handling, processing, and shipping of wildlife samples may be more complicated than for other animal populations because sampling may be done in remote field locations, necessitating consideration of the impact of sample storage and shipping time and conditions (e.g., temperature) on test results. While some molecular methods may offer benefits by being more tolerant of roughly handled specimens, testing the impact of handling, storage, and shipping on specific samples used for specific tests is optimal, albeit uncommonly reported. The cost of testing is often a limiting factor in wildlife surveillance, particularly studies in which large numbers of animals are tested. Decreasing costs and increasing access to molecular methods, though, have facilitated the use of molecular assays, which can be used to both replace (e.g., pathogen detection assays) and supplement (e.g., molecular typing) traditional methods.

Emerging Diseases

Inherently, it is impossible to make specific plans for detecting emerging zoonotic diseases. Yet diagnostic testing plays a critical role in the identification, characterization, and containment of new disease threats. Newly identified pathogens can be identified through a variety of means, ranging from traditional culture to various molecular methods. Advances in next-generation sequencing and bioinformatics have revolutionized new pathogen discovery. In many ways, technology has surpassed knowledge, and finding new microorganisms is often easier than determining what those findings actually mean. Many new microorganisms of unknown or limited clinical relevance have been identified in animals (58–60), along with the realization that detection of a new organism and detection of a new pathogen are not necessarily synonymous. Therefore, once an emerging pathogen is identified, the ability to efficiently develop a practical and accurate test can play a major role in determining the relevance and risks and developing a response (when a response is indicated). Given the potential for rapid testing and increasingly accessible "broad-range" methods for pathogen discovery, molecular assays will likely be at the forefront of emerging zoonotic disease response.

Foreign and Reportable Diseases

Foreign animal diseases are diseases that are not established in a particular country or region. Reportable diseases are diseases that must be reported to regulatory bodies at the time of suspicion, time of diagnosis, or on a periodic basis. Many diseases in these categories are subject to scrutiny because of the potential for zoonotic transmission and/or establishment of an endemic presence in a region that is currently free of the pathogen. For both of these disease types, rapid diagnosis is important because prompt identification can help identify exposed individuals, reduce the risk of subsequent animal and human exposure, and facilitate a prompt containment response. High analytical sensitivity is important, but because of the potential implications of positive results (e.g., euthanasia of an animal or depopulation of a group of animals, importation restriction, prolonged quarantine, cost of investigation), specificity is also important, either through a primary assay that is both highly sensitive and highly specific or by using a two-step approach with a more specific assay to confirm positive screening results.

Import Screening

The amount of international movement of animals is poorly understood and typically underappreciated. Millions of animals cross borders, along with their microbial inhabitants. Some importation is performed with detailed regulations and scrutiny; however, this is largely restricted to food animals. Startlingly little scrutiny tends to be placed on companion animal species, and obviously, wildlife can cross borders at will.

It is unlikely that international movement of animals will decline. Severe restrictions will continue to be present for certain animal species in certain regions to prevent importation of known pathogens. Economic factors and free trade agreements often create pressures to decrease restrictions, yet border restrictions or prohibitions are frequently implemented in response to zoonotic disease concerns from exporting regions. The availability of highly sensitive, rapid testing methods can lead to altered regulatory restriction, facilitating a balance between adequate bioexclusion and limiting economic and political impacts. Conversely, a lack of adequate testing or poor-quality tests could result in import restrictions, something that can have a profound economic impact in some animal sectors.

Concerns are not restricted to food animals and food, although those remain the areas of greatest focus. The largely uncontrolled movement of companion animals has led to importation of pathogens such as rabies, *Leishmania*, and *Echinococcus multilocularis*. As awareness of these concerns is heightened, consideration of control measures is needed. Regulatory restriction is a less desirable approach and is impractical in regions where thousands of pets cross borders. As with food animals, the availability of highly sensitive testing that could be performed (potentially at the point of entry) could be a practical measure to reduce the risk of introduction of selected zoonotic pathogens. Test turnaround time is a critical factor for import testing. If testing is done prior to export, long turnaround times provide more of a chance for the individual to become infected between sampling and export or result in the need for a prolonged quarantine during the testing period. Similarly, if testing is done after importation, test results are ideally available before the animal is exposed to other animals in the country, so short turnaround times can limit quarantine times and identify infected animals as early as possible. Importation may be considered a somewhat marginal issue, but given the potential implications of introducing a foreign zoonotic pathogen into a naïve population, it is a subject that bears consideration.

Outbreak Investigation

The rapid nature of many molecular diagnostic tests, compared to traditional methods (e.g., culture), can facilitate a rapid and effective outbreak response. Further, culture-independent methods have advantages over isolation methods for pathogens that are not readily detected by culture and that require enhanced biocontainment for isolation (e.g., *Bacillus anthracis*, *Brucella* spp., *Yersinia pestis*, *Francisella tularensis*, *C. burnetii*, *Chlamydophila psittaci*, Hendra virus) or where testing is limited to specialized facilities (e.g., government reference laboratories). The use of proper molecular tests can potentially decrease turnaround time, increase the sensitivity of testing, and increase throughput, although these vary by test and pathogen. A major limitation of most molecular diagnostic tests is the inability to provide typing data, which are typically critical for outbreak investigation. However, these tests can still be incorporated into zoonotic disease investigations as rapid screening tests, in combination with culture, and as secondary tests to characterize recovered isolates. Newer methods that can both identify and characterize pathogens will likely become important outbreak investigation tools.

Differentiation of Vaccine Antigen from Natural Infection

Vaccination is used to control various infectious diseases in animal populations, yet recent vaccination can interfere with infection control responses with some vaccine and diagnostic test combinations. Confusion may arise when tests cannot differentiate natural infection from vaccine response. This is predominantly a concern with infectious (modified live) vaccines when testing is performed shortly after vaccination. Since these vaccines are designed to replicate *in vivo*, vaccine antigen may be present in the body in detectable levels for a variable amount of time

postvaccination. The potential for confusion has limited the use of vaccine for some animal diseases during outbreaks, because of interference with testing-based outbreak response (e.g., test and quarantine or test and slaughter measures). It is also a concern for endemic diseases when an animal develops clinical signs consistent with a zoonotic disease shortly after vaccination against that disease. In that situation, differentiating a positive result from vaccine antigen versus a positive result from natural infection is important to determine whether zoonotic risks may be present and whether further investigation or precautions are required. In some situations, molecular diagnostic assays can be used to determine the nature of the positive response, through detection of specific markers or with evidence-based thresholds that are consistent with natural infection versus the lower level of vaccine antigen.

USE OF MOLECULAR TYPING METHODS FOR ZOONOTIC DISEASE

While the focus is often on the use of molecular assays to identify a pathogen, characterization of the pathogen may be equally important in some situations, as has been discussed repeatedly above. This can involve detecting virulence factors (e.g., to differentiate clinically relevant from irrelevant types), detecting host-specific factors to infer the origin (e.g., evaluation of MRSA for the human-specific *scn*) (61, 62), providing molecular epidemiological data for outbreak investigation, and identifying important virulence factors (e.g., enterotoxin gene detection of *S. aureus* from food) (63). Genotypic characterization can also determine whether a zoonotic risk is present, such as with differentiation of nonzoonotic versus zoonotic *Giardia* assemblages (64–66).

Molecular typing is the most commonly used tool and can provide critically important information about some pathogens. This can involve various conventional molecular typing methods such as pulsed-field gel electrophoresis, multilocus variable number tandem repeat analysis, PCR ribotyping and sequence analysis of various genes, along with methods evaluating the presence or absence of specific (usually virulence) genes. It is likely that these will soon be supplanted by whole-genome sequence analysis, because sequence costs continue to plummet and bioinformatics throughput is increasing. Whole-genome sequence-based approaches to outbreak investigation have been reported for human pathogens (67) and have been used to differentiate human versus animal sources of potentially zoonotic pathogens (68).

While there has been limited use of whole-genome sequence analysis for zoonotic outbreak investigation, there is no reason that whole-genome sequence investigations will not become common, if not the norm, for a range of zoonotic pathogens in the near future.

CROSS-APPLICATION OF TESTS DEVELOPED FOR HUMANS

Tremendous effort, time, and expense go into the development and validation of assays that are used in humans. This amount of effort and cost are often unacceptable for development of tests to be used in animals because of an inability to recover costs in most situations. Yet the fact that the targeted organism is the same in humans and animals (otherwise it would not be a zoonotic pathogen) means that consideration can be given to cross-application of well-scrutinized human assays, when available. This has the potential to allow for more rapid development and implementation of tests for use in animals, but it does not mean that all development and validation steps can be bypassed, since identical performance of tests in different species cannot be guaranteed. While the target organism may be the same, there may be differences in the test samples that could impact the assay. This has received limited investigation; however, as an example, a commercial real-time PCR MRSA assay licensed for use in humans was identified as performing poorly in horses (69). Despite use of the same types of samples compared to humans (nasal swabs), the sensitivity of the assay was poor in horses. Reasons were not investigated but likely involved the presence of PCR inhibitors in equine nasals swabs. This assay was being evaluated as a potential tool for MRSA control in equine hospitals and on farms, and performance of this assay would have led to underdiagnosis and likely increased risk of MRSA transmission to other horses and humans. Thus, this assay would either be considered unacceptable for use in horses or would require further development to overcome the problems with test sensitivity. Accordingly, while cross-application of assays is a logical and potentially efficient approach, it is important that species- and sample-specific validation be performed to ensure that assays developed for humans can achieve their intended goals in animals.

ROLE OF VETERINARY ACADEMIC AND DIAGNOSTIC LABORATORIES IN ZOONOTIC DISEASE CONTROL

Commercial diagnostic laboratories and academic laboratories perform most diagnostic testing for animals. Those laboratories may also perform food testing, but food manufacturers do a substantial component of testing in-house. The disseminated nature of testing related to zoonotic diseases necessitates coordinated efforts involving reference (typically government) laboratories, human hospital laboratories, human commercial diagnostic laboratories, academic institutions, and veterinary diagnostic laboratories. This is often essential because of the need for parallel testing of clinical specimens from humans (human diagnostic laboratories), characterization of the pathogens (mainly reference and academic institutions), and testing of potential animal, food, or water sources (mainly government, academic, and veterinary laboratories). Proper investigations are dependent on an understanding of the testing methodologies, test performance, and laboratory performance across all partners, highlighting the need for proper and transparent testing practices in all sectors. While rigorous test development and quality control practices are in place for human diagnostic and reference laboratories, the same may not be true for academic and veterinary facilities, in part because of differences in the regulation of diagnostic testing.

REGULATION OF DIAGNOSTIC TESTING IN ANIMALS AND QUALITY CONTROL PRACTICES

The principles of laboratory quality assurance and quality control apply across species; however, application of those practices outside of accredited medical diagnostic laboratories is variable. There are no regulatory bodies for veterinary diagnostic laboratories (70) and no requirement for

laboratories to follow CLSI (or equivalent) guidelines, to evaluate and validate tests, to provide information about test performance parameters (e.g., limit of detection, target, sensitivity, specificity), or to have any form of quality control program. Voluntary accreditation such as through the American Association of Veterinary Laboratory Diagnosticians, development and implementation of quality control management systems such as the International Standards Organization 17025 standard (71), and/or use of guidelines developed by groups such as the American Society of Veterinary Clinical Pathology Quality Assurance and Laboratory Standards Committee (http://www.asvcp.org/about/committees/qas.cfm) can be achieved by veterinary diagnostic laboratories and can provide some degree of confidence in the quality of the laboratory. Guidelines are also available for point-of-care testing (72). Yet reliance on voluntary approaches such as these creates an environment where some laboratories use rigorous practices similar to those used in human diagnostic laboratories while others use highly questionable methods and assays.

While there are extensive validation and approval (e.g., U.S. Food and Drug Administration) requirements for assays used for clinical testing in humans, the same does not apply for most tests used on animals (70). Apart from tests used for specific regulatory purposes (e.g., international animal movement screening), there are typically no requirements for any degree of test development and validation. Many, if not most, veterinary molecular diagnostic tests have limited (or no) published supporting data about relevant aspects such as their sensitivity, specificity, repeatability and limit of detection. Any information, along with quality control practices, is often limited to internal use by companies and not made publicly available (ostensibly for proprietary purposes).

There has been limited independent investigation of the accuracy of testing performed in commercial veterinary diagnostic laboratories. One study compared results of feline immunodeficiency virus PCR from three different North American veterinary diagnostic laboratories and found that correct results were only reported in 58 to 90% of samples (73), with one laboratory's assay having a sensitivity of only 41%. A similar but small study of avian polyomavirus testing reported accuracy rates of 75 to 100% among five commercial laboratories, with two laboratories having testing sensitivities of 50 and 60% (74). Efforts directed toward development of transparent methods that can be applied across laboratories have mainly been limited to food-borne pathogens (15, 16). Overall, users often have limited guidance in selecting tests and interpreting results. This can be of particular importance for zoonotic pathogens because of the potential human health implications of the testing.

Related to the lack of regulation discussed above is the potential for tests to provide misleading results if the actual targets are not known or understood. This is particularly true for assays that are used for specimens outside of their normal sample types. An example is the use of real-time PCR assays on ticks found on dogs. Some commercial PCR assays target *Borrelia* spp. but are marketed as *Borrelia burgdorferi* (Lyme disease) tests because of the reasonable presumption than any *Borrelia* found in a dog (or human) would be *B. burgdorferi*. However, ticks can harbor other *Borrelia* spp., so positive results from a tick sample could have a poor positive predictive value and be clinically misleading. Another concerning example was reported identification of *Rickettsia rickettsii*, the cause of Rocky Mountain spotted fever, in a tick from a dog from a household with a child with an undiagnosed disease. While finding an infected tick on the dog could provide evidence of potential human exposure from the same source, the case occurred in an area far removed from where this pathogen and its host tick are known to occur. Subsequent investigation revealed that the PCR assay was nonspecific, being able to detect a range of *Rickettsia* species, including species that pose no risk to humans. Thus, any use of an assay outside of its intended sample type, without adequate scrutiny and without adequate understanding of the target (and the biological relevance of that target), could result in misleading information about human health risks.

Another concern related to commercial assays is tests without a biological basis for use of the test results to make reasonable decisions. Some veterinary commercial PCR laboratories offer panels of tests to identify zoonotic pathogens in healthy animals as part of "wellness screening." In addition to the issues discussed above, this can result in confusion or illogical actions because many potentially zoonotic pathogens are common commensals, and finding the pathogen in an animal often does not indicate the need for any response (e.g., treatment, altered management). This can lead to inappropriate actions such as unneeded antimicrobial therapy, fear of the pet, rehoming of the pet, or occasionally euthanasia. Screening of healthy animals for pathogen shedding is rarely recommended, even for animals that encounter high-risk individuals such as hospital visitation dogs (75) and animals owned by individuals with human immunodeficiency virus infection (76). Thus, the increased availability and decreasing cost of molecular screening assays can result in worsened care if inappropriately applied.

INTEGRATION OF TESTING AND REPORTING

The oft-discussed (but infrequently practiced) "one health" approach involves coordinated investigation, testing, and data reporting across species. Unfortunately, that is rarely present, particularly outside of food-borne pathogens. Apart from outbreak investigations that are coordinated and performed by regulatory agencies, disjointed information is typical. Even when regulatory bodies direct outbreak investigations, there may be inconsistencies, inefficiencies, and poor coordination when different groups are in charge of different facets (animal, human, food, water) of the investigation. Clearly, much improvement is needed.

CONCLUSIONS

As with most areas of infectious disease surveillance and diagnostic testing, there is increasing interest in molecular diagnostic assays because of a range of potential advantages. Molecular diagnostic tests are commonly and increasingly used in some animal sectors, both as primary pathogen detection tools and as secondary tests. These assays can provide important advances in the diagnosis, surveillance, and management of zoonotic diseases, but their use is complicated by variable (and often substandard) test development and validation and a lack of evidence regarding optimal use. Technological advances and the ever-present risk of emerging zoonoses will continue to drive interest in molecular assays. However, proper test development and validation, coordination of testing and reporting,

and a proper "one health" approach are required for optimal use of these potentially powerful diagnostic tools.

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section V

Syndromic Diagnostics

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Molecular Approaches to the Diagnosis of Meningitis and Encephalitis

KAREN C. BLOCH AND YI-WEI TANG

24

INTRODUCTION AND GENERAL DISCUSSION

Meningitis and encephalitis represent central nervous system (CNS) infections where rapid and accurate identification of an infectious agent is essential for management. CNS infections present a challenge for both the microbiologist and the clinician due to the vast number of pathogens associated with these syndromes (1, 2). Molecular diagnostic techniques such as PCR have replaced slower and less sensitive tests for a number of bacterial and viral causes of CNS infection (3). This chapter reviews various diagnostic tests available for CNS infections and provides a detailed discussion of specific molecular approaches to the most common organisms causing meningitis and encephalitis in the United States.

Pathophysiology of CNS Infections

The CNS has unique anatomic and immunologic characteristics that distinguish infection at this site from those involving other organ systems. The brain and spinal cord are contained within the bony confines of the calvarium and vertebral column. Cerebrospinal fluid (CSF) is produced in the choroid plexus of the ventricles and flows into the subarachnoid space at the cisterna magna and around the cerebral hemisphere, where it is reabsorbed by the arachnoid villi. CSF envelops the brain and spinal cord, providing cushioning and buoyancy for neural tissue. It carries essential metabolites into the neural tissue and cleanses the tissues of waste as it circulates around the brain, ventricles, and spinal cord. The blood-brain barrier (BBB) serves to limit the entry of microorganisms and inflammatory cells into the CNS. The absence of mediators of innate immunity in the CSF may contribute to the rapid progression and morbidity of CNS infections (4, 5). In addition, because of this anatomic barrier, antimicrobials used to treat CNS infections must have pharmacologic properties that allow them to penetrate the BBB, and maximal doses are usually required for bactericidal activity.

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Meningitis

Meningitis involves infection localized to the subarachnoid space, sparing the brain parenchyma. While neural tissue is not directly infected, secondary damage may occur as a result of the host inflammatory response. Leukocyte migration into the subarachnoid space can result in occlusion of cortical blood vessels and damage to nerve roots that traverse the subarachnoid space. Clinically, this manifests as cranial or spinal nerve dysfunction and hydrocephalus. Activation of white blood cells leads to an inflammatory cascade, with release of cytokines, oxidants, and proteolytic enzymes. At the cellular level, this chain of events results in disruption of the BBB and impaired cerebrovascular autoregulation (6). Increased intracranial pressure may lead to transtentorial herniation or tissue hypoxia due to decreased tissue perfusion.

Encephalitis

Encephalitis is defined by the presence of an inflammatory process in the brain, associated with clinical evidence of neurologic dysfunction (7). Encephalitis can present with purely parenchymal findings but more commonly is associated with meningeal inflammation, representing an overlap syndrome termed meningoencephalitis. Additionally, encephalitis can coexist with myelitis, inflammation of the spinal cord, to cause encephalomyelitis. These three syndromes are often grouped together due to the significant overlap in the epidemiology, microbiology, and approach to management.

Most infectious agents that cause encephalitis gain access to the CNS through hematogenous spread. The initial site of entry and replication varies depending on the organism and may include the respiratory tract (e.g., measles virus), gastrointestinal tract (e.g., enteroviruses), or skin (e.g., arboviruses). Viremia follows localized infection, ultimately leading to invasion and replication with the CNS.

Hematogenously spread CNS infections, such as arboviruses, generally cause diffuse brain involvement, with global impairment in neurologic function. Infections introduced through other routes typically involve specific areas of the brain. For instance, following the bite of an infected animal, rabies virus is inoculated subcutaneously, with retrograde progression along peripheral nerves into the brain stem (8). In contrast, the free-living amoeba *Naeglaria fowleri* enters the host transnasally, passing through the cribiform plate and invading the frontal lobes of the brain (9).

The pathogenesis of herpes simplex encephalitis (HSE) is believed to occur following reactivation of the virus in the trigeminal ganglion, with spread to the frontal and temporal lobes via the tentorial nerves (10).

Classification of CNS Infections

CNS infections are typically classified based on the anatomic site of infection. Infection of the meninges, brain, and spinal cord results in the syndromes of meningitis, encephalitis, and myelitis, respectively. Focal CNS infections include abscess, subdural empyema, or subarachnoid empyema. Infection may be limited to a single anatomic compartment or may involve multiple sites (e.g., meningoencephalitis, encephalomyelitis). Acute meningitis is characterized by the onset of the triad of fever, headache, and neck stiffness over a period of hours to days (11). The hallmark of encephalitis is alteration in mental status, ranging from lethargy to coma. Myelitis is characterized by inflammation of the spinal cord with symptoms including fever, headache, and paraparesis or paralysis.

Meningitis and encephalitis are the two most common CNS infections and are covered in detail in this chapter. Differentiation of these two syndromes is important, because both the pathophysiology and microbiology of meningitis and encephalitis are distinct. Encapsulated bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria meningitidis*, while common causes of acute meningitis, do not cause encephalitis. Viruses are important causes of both syndromes but often demonstrate species-specific localization to discrete sites in the CNS. Enteroviruses and mumps viruses usually infect the ependyma and tissues of the subarachnoid space, producing meningeal irritation. In contrast, rabies viruses almost always involve the parenchyma and cause encephalitis. Herpes simplex virus (HSV) 1 characteristically causes temporal lobe encephalitis, whereas HSV-2 more typically causes meningitis. Such affinities are not absolute. For example, enteroviruses, the leading cause of viral meningitis, are increasingly recognized as a common cause of encephalitis (12) and acute flaccid paralysis (13).

Encephalitis can be further subclassified into infectious and inflammatory causes (7). Infectious encephalitis results from direct CNS invasion by the agent, with infection of the brain parenchyma. In contrast, inflammatory encephalitis, which typically follows an antecedent viral infection or immunization, predominantly affects the white matter of the brain. The most common inflammatory condition is acute disseminated encephalomyelitis, a demylinating condition seen primarily in children. A newly described autoimmune syndrome termed anti-*N*-methyl, D-aspartate receptor encephalitis has been reported as a more frequent cause of encephalitis than all viral entities combined (14). Clinically, inflammatory encephalitis may resemble infectious encephalitis but is caused by immune-mediated demyelination, and diagnostic studies aimed at identifying a pathogen, such as nucleic acid amplification tests (NAATs), are negative. Distinguishing infectious and inflammatory causes of encephalitis is important, because the latter are typically treated with immunosuppressive agents.

Microbiology of CNS Infections

Just as meningitis and encephalitis localize to distinct compartments of the CNS, the pathogens causing these two syndromes differ, and the respective microbiology of meningitis and encephalitis will be discussed separately.

Meningitis

The acute meningitis syndrome can be subdivided into bacterial and nonbacterial (sometimes referred to as benign lymphocytic or aseptic meningitis) etiologies. These entities can usually be differentiated clinically by the acuity of symptom onset as well as specific laboratory findings. Peripheral blood leukocytosis (>10,000/mm^3), CSF pleocytosis (>1,000/mm^3) with a neutrophilic predominance, and CSF hypoglycorrhachia (<40 mg/dl) favor a bacterial cause (15). A CSF Gram stain showing a predominant organism is strong presumptive evidence of a bacterial process and can be confirmed by a positive culture.

"Aseptic meningitis" is a nonspecific term used to describe culture-negative acute meningitis. In addition to viruses, the differential diagnosis for culture-negative meningitis includes rickettsial infections, spirochetal infections (e.g., *Treponema pallidum* or *Borrelia burgdorferi*), mycobacterial or fungal infections, inflammation from a parameningeal infection, or partially treated bacterial infections. In addition, there are numerous noninfectious syndromes that cause CSF pleocytosis, which can mimic infectious meningitis.

While viruses are the most common cause of culture-negative meningitis, in clinical practice a specific pathogen is rarely isolated in viral cultures. Epidemiologic studies using a standard diagnostic panel including CSF PCR detect a specific viral pathogen in approximately two-thirds of cases (16, 17). Enteroviruses are the most common cause of viral meningitis, accounting for up to half of all cases, and are particularly prevalent in children and young adults, with peak incidence in summer and fall (18, 19). Other relatively common viral causes of meningitis include HSV-2, acute HIV infection, varicella-zoster virus (VZV), and lymphocytic choriomeningitis virus (CMV). Arboviruses are also a significant cause of viral meningitis; however, the specific virus varies based on the geographic distribution of the arthropod vector.

Encephalitis

The spectrum of agents causing encephalitis has shifted due to a decrease in vaccine-preventable conditions and the recognition of newly emerging pathogens such as West Nile virus (WNV). However, while the specific etiologies have changed, the overall incidence has remained stable (20). Other emerging causes of encephalitis include Nipah virus (21), Toscana virus (22), enterovirus 71 (23), *Balamuthia mandrillaris* (24), and Chandipura virus (25).

In light of the large number of potential pathogens, a practical approach is to categorize agents on the strength of causal association with encephalitis (2, 7). The most commonly identified etiologies in the United States are HSV-1, WNV, and the enteroviruses, followed by other herpesviruses (1) (Table 1). Other agents may be highly endemic regionally (e.g., La Crosse virus) or internationally (e.g., rabies virus, Japanese encephalitis virus). Bacterial agents, including *Ehrlichia* spp. and *Rickettsia rickettsii*, are potentially treatable causes of encephalitis, and prompt administration of antibiotics may be lifesaving (26, 27).

A second category includes agents that are less commonly identified but are neurotropic and are well-associated causes of sporadic encephalitis (Table 2). A number of these have well-defined geographic distributions (e.g., Chandipura virus in India, Nipah virus in Malaysia), and consideration of these agents is indicated if there is travel or residence in an area of endemicity. Agents in this category include uncommon pathogens with prominent CNS

TABLE 1 Common causes of meningoencephalitis in immunocompetent patients

Etiology	Epidemiology	Molecular test applicability[a,b]	Diagnosis[d]
Viruses			
Borrelia burgdorferi[c]	Tick-borne infection with meningitis and cranial neuropathy in early disseminated Lyme disease; encephalopathy in late disease	B	Serology (serial EIA and Western blot), CSF antibody index, CSF PCR
Enteroviruses[c]	Peak incidence in late summer and early fall Enterovirus 71 a cause of outbreaks of rhomboencephalitis, with children primarily affected	A	CSF PCR is the test of choice Stool or throat swab PCR or culture suggestive, but not diagnostic, of CNS involvement 5′-UTR PCR detects most members of enteroviruses but does not provide genotype-specific information
Epstein-Barr virus[c]	Either during acute infection or reactivation Cause of primary CNS lymphoma	A, B	Acute infection: serology Reactivation disease or primary CNS lymphoma: positive CSF PCR may indicate secondary viral reactivation
Herpes simplex virus (HSV) 1 and 2[c]	HSV-1 accounts for 5 to 10% of all cases of encephalitis, typically reactivation disease HSV-2 causes encephalitis in neonates, aseptic meningitis in older individuals	A	CSF PCR is "gold standard" for diagnosis
Japanese encephalitis virus	Mosquito-borne, most common worldwide cause of encephalitis Endemic throughout Asia Vaccine-preventable	C	Paired serology, CSF IgM, CSF antigen (serology cross-reacts with other flaviviruses)
La Crosse virus	Mosquito-borne Endemic in midwestern and eastern United States Peak incidence in school-aged children	C	Paired serology, serum IgM
Rabies virus[c]	Vaccine-preventable Most common vector is bats, and bites often unrecognized; dogs important in developing countries Worldwide distribution	B	Antibodies (serum, CSF); PCR of saliva, skin, or CSF; IFA of nuchal biopsy or CNS tissue Coordinate testing with local health department
St. Louis encephalitis virus	Mosquito-borne Endemic to western United States, with periodic outbreaks in central/eastern United States	C	Paired serology (cross-reacts with other flaviviruses), CSF IgM
Tick-borne encephalitis virus	Vaccine-preventable Transmitted via tick or ingestion of unpasteurized milk Endemic to Eastern and Central Europe, Far East	C	Serum IgM, paired serology
Varicella zoster virus[c]	Acute infection (chickenpox) or reactivation (shingles)	B	CSF PCR and CSF serology are indicated DFA, culture, or PCR of skin lesions suggestive, but not diagnostic, of CNS involvement Serum IgM (acute infection) Vasculopathy best diagnosed by intrathecal antibodies
West Nile virus[a]	Mosquito-borne cause of epidemic encephalitis throughout United States, Europe Peak incidence in adults >50 years	B	CSF IgM, paired serology (cross-reactivity with other flaviviruses)

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TABLE 1 Common causes of meningoencephalitis in immunocompetent patients (*Continued*)

Etiology	Epidemiology	Molecular test applicability[a,b]	Diagnosis[d]
			CSF PCR limited to diagnosis in immunocompromised patients who have impaired humoral response
Bacteria			
Bartonella henselae (and other *Bartonella* spp.)	Encephalopathy typically follows scratch or bite from cat or kitten Highest incidence in children	B	Serology (acute usually diagnostic), PCR of lymph node, CSF PCR not useful
Mycobacteria tuberculosis[c]	Most common in developing countries Disease in extremes of age or immunocompromised	B	CSF AFB smear, culture, PCR, positive respiratory cultures highly suggestive
Ehrlichia/Anaplasma[c]	Tick-borne bacteria causing human monocytic ehrlichiosis and human granulocytic anaplasmosis, respectively *Ehrlichia* endemic to southern and central United States, *Anaplasma* endemic to northeastern United States, and Midwest	A	Morulae in white blood cells, PCR of whole blood, paired serology CSF PCR may be positive but less sensitive than testing of whole blood
Rickettsia rickettsii[c]	Tick-borne infection found throughout North America, with peak incidence in southeastern and southcentral United States	B	Paired serology, PCR, or IHC on skin biopsy of rash PCR on CSF or blood not useful

[a]Of CSF or other biologic material (see diagnosis notes).
[b]A, molecular testing indicated in diagnostic evaluation; B, molecular testing may be indicated in selected cases or as an ancillary test along with the diagnostic methods; C, molecular testing has limited utility in diagnosis, and/or is only available in research laboratories.
[c]Detailed discussion of molecular diagnosis in text.
[d]Abbreviations: EIA, enzyme immunoassay; CSF, cerebrospinal fluid; CNS, central nervous system; UTR, untranslated region; IFA, indirect fluorescent antibody; DFA, direct fluorescent antibody; AFB, acid-fast bacilli; IHC, immunohistochemistry.

symptoms (e.g., eastern equine encephalitis virus, Venezuelan equine encephalitis virus) or relatively common infections in which meningoencephalitis occurs in a minority of cases (e.g., *B. burgdorferi*, *Coxiella burnetii*).

Most problematic is the third category of agents, those anecdotally associated with encephalitis but with minimal neurotropism and limited laboratory data confirming direct CNS invasion. For instance, *Mycoplasma pneumoniae* is the most commonly identified agent in several large case series of encephalitis (28, 29). However, the significance of serologic diagnosis of *M. pneumoniae* is unknown given the relatively high frequency of mycoplasma infection and limitations of commercial antibody testing (30). Rarely, mycoplasma may be identified in CSF by culture or PCR, which is diagnostic of direct CNS infection (29). Other organisms anecdotally associated with encephalitis but with similar difficulties in establishing causality include influenza virus (31), rotavirus (32), human herpes virus 6 (33), and parvovirus B-19 (34).

Perhaps the most challenging aspect for patients, families, and clinicians is that in approximately half of all encephalitis cases, no pathogen is identified, irrespective of geography or patient population (16, 20, 35–37). Illustrating the diagnostic challenges of encephalitis, a study enrolling >1,500 well-defined cases over a 7-year period using a standardized diagnostic algorithm including extensive molecular testing identified an etiology in only one-third of cases (1).

NONMOLECULAR LAB DIAGNOSTIC METHODS

The diagnosis of CNS infections can be challenging because the differential diagnosis is often extensive and includes infectious, postinfectious, and noninfectious causes (38). Diagnosis of CNS infection requires consideration of the most likely causative agents based on exposures, geography, and season, coupled with an understanding of the optimal diagnostic test and the highest-yield clinical specimen for testing. A thorough history and physical exam coupled with neuroimaging are critical for guiding testing (39). While this chapter will focus on the use of molecular assays for diagnosis of CNS infection, traditional microbiologic diagnostic approaches continue to play an important role in pathogen identification and are complementary to NAAT techniques (40–42).

Direct Microscopic Exam

A positive CSF Gram stain is highly suggestive of bacterial meningitis, although false-positive results have, on occasion, been reported (43, 44). The sensitivity depends on the bacterial pathogen: bacteria are observed by Gram stain in as many as 90% of infections with *S. pneumoniae* and in less than 50% for *Listeria monocytogenes*, and likely correlate with the relative burden of organisms in the CSF. The sensitivity of the Gram stain for the diagnosis of bacterial meningitis is 60 to 80% in patients who have not received antimicrobial therapy and 40 to 60% in patients who have received treatment (45). Rapid diagnosis may occasionally be made by visual detection of microorganisms by acid-fast bacilli stain, modified acid-fast bacilli stain, dark-field examination, wet mount, and India ink stain. Amoebae such as *Naeglaria* are best observed by examining thoroughly mixed sediment as a wet preparation under phase-contrast microscopy (43, 46).

Rarely, viral or rickettsial infections may be diagnosed by cytology or histopathology. For instance, visualization

TABLE 2 Less common causes of meningoencephalitis in immunocompetent patients[a]

Etiology	Epidemiology	Molecular test applicability[b]	Diagnosis
Viruses			
Eastern equine encephalitis virus	Coastal states (Atlantic and Gulf) Elderly disproportionately affected	C	Paired serology
Herpes B virus	Transmitted by bite of Old World macaque	A	Culture and PCR of vesicles, CSF
Human herpes virus 6[c]	Well-described cause of limbic encephalitis in bone marrow transplant patients Difficult to ascertain significance in immunocompetent individuals because latent infection is common	A	CSF PCR
Lymphocytic choriomeningitis virus	Peak incidence in fall and winter	C	Serology
Measles virus	Vaccine-preventable illness Measles inclusion body encephalitis onset 1–6 months after infection, subacute sclerosing panencephalitis a late manifestation (>5 years after infection)	C	CSF antibodies, CSF index, brain tissue PCR
Mumps virus	Vaccine-preventable illness	B	Serology, throat swab PCR, CSF culture or PCR
Nipah virus	Epidemics in Southeast Asia	C	Serology (Special Pathogens Branch, CDC)
Powassan virus	Tick-borne, endemic to New England, Canada	C	Paired serology
Rubella virus	Vaccine-preventable illness	C	Serology, CSF antibodies
Vaccinia	Infection or vaccination as precipitating event Thought to be autoimmune phenomenon	C	CSF antibodies, CSF index, serum IgM (natural infection)
Venezuelan equine encephalitis virus	Central, South America, rarely in border states (TX, AZ)	C	Paired serology, viral cultures (blood, oropharynx), CSF antibody
Western equine encephalitis virus	Summer and early fall onset, western United States and Canada, Central and South America	C	Paired serology
Bacteria			
Coxiella burnetti	Animal exposures (particularly placenta and amniotic fluid)	C	Paired serum
Treponema pallidum	Sexually transmitted disease, with meningoencephalitis seen in early disseminated disease and progressive dementia in late disease	C	CSF VDRL, serum RPR with confirmatory FTA-ABS
Tropheryma whipplei[d]	Chronic encephalitis often associated with osculomasticatory myorhythmia	A	CSF PCR, PAS positive cells in CSF, small bowel biopsy
Protozoa			
Balamuthia mandrillaris	Central America (natives and immigrants)	C	Serology (research labs), brain histopathology
Naeglaria fowleri	Summer months, children and adolescent boys at highest risk Swimming in fresh water, and particularly water sports, a risk factor	C	Mobile trophozoites on wet mount of warm CSF, brain histopathology

[a]Abbreviations: SSCE, subacute sclerosing panencephalitis; CSF, cerebrospinal fluid; RPR, rapid plasma reagin; FTA-ABS, fluorescent treponemal antibody-absorption; FTA-ABS; PAS, periodic acid-Schiff.

[b]Of CSF or other biologic material (see diagnosis notes). A, molecular testing indicated in diagnostic evaluation; B, molecular testing may be indicated in selected cases or as an ancillary test along with the diagnostic methods; C, molecular testing has limited utility in diagnosis, and/or is only available in research laboratories.

[c]Detailed discussion of molecular diagnosis in text.

of morulae, basophilic clusters found in the cytoplasm of monocytes, is highly suggestive of infection with *Ehrlichia chaffeensis* (47, 48). Pathologic examination of brain tissue may reveal Negri bodies with rabies virus infection or intranuclear eosinophilic amorphous bodies surrounded by a halo with HSV infection. Pathologic examination of the brain for trophozoites and cysts may be helpful in the diagnosis of *B. mandrillaris* and other free-living amebic infections.

Rapid Antigen

Bacterial antigen testing in CSF has limited utility due to poor sensitivity and specificity (49–51). One exception is the Binax NOW immunochromatographic test for *S. pneumoniae*, which has been demonstrated to be rapid and highly sensitive for the diagnosis of pneumococcal meningitis (52, 53). A study done in five countries in Africa and Asia indicated that the antigen assay enhanced diagnosis of pneumococcal meningitis, suggesting that previous studies have underestimated the proportion of pediatric bacterial meningitis cases caused by pneumococci (54). Similarly, viral antigen assays have largely been replaced by more sensitive molecular techniques. In contrast, detection of cryptococcal antigen in either CSF or serum of patients with HIV or other underlying immunocompromise is both highly sensitive and specific (55), and a positive antigen test would be an indication to begin directed antifungal therapy (56, 57). Detection of galactomannan antigen and 1,3-β-D-glucan in CSF may be helpful in establishing the diagnosis of CNS aspergillosis (58, 59). However, because the two antigens can also be expressed by fusarium species, their presence in CSF does not constitute a definitive diagnosis of CNS aspergillosis (60, 61). During a large national outbreak of fungal meningitis associated with spinal injection of contaminated methylprednisolone, detection of 1,3 beta-D-glucan in CSF was 100% sensitive and 98% specific for the presence of *Exserohilum rostratum* (62). Histoplasma antigen testing of CSF has a sensitivity ranging from 38 to 67%, with a CSF-to-urine antigen ratio >1, suggestive of intrathecal antigen production (63).

Culture

CSF culture allows definitive diagnosis of bacterial, mycobacterial, and fungal meningitis (43). The yield of CSF culture for bacterial meningitis ranges from 70 to 85% in untreated patients but decreases substantially after antibiotic treatment (64). The volume of CSF submitted correlates with the sensitivity of fungal and mycobacterial culture, with best results when ≥10 cc of sample is cultured (63).

A positive bacterial culture allows subsequent determination of the antimicrobial susceptibility of the causative agent. While the techniques used for antimicrobial susceptibility testing in CNS infections remain the same as those for isolates from other sites, criteria for determining antibiotic resistance may differ. Penicillin minimal inhibitory concentration breakpoints for pneumococcal CNS isolates, for example, are lower than those used for non-CNS isolates.

The application of monoclonal antibodies in shell vial culture has significantly increased the speed and specificity of viral culture; however, culture has largely been replaced by PCR (65–67). The sensitivity of CSF viral culture for enteroviruses is only 65 to 75% and requires up to 8 days for detection of cytopathic effect (68–70). Some serotypes of enteroviruses, especially coxsackievirus A strains, grow very poorly in tissue culture or are noncultivable (71). HSV culture sensitivity from CSF is very poor, especially in recurrent cases of HSV-2 meningitis or HSV-1 encephalitis (72).

Serology

Serology remains the test of choice for CNS infections caused by arboviruses, including WNV, and fastidious bacteria such as ehrlichia and rickettsia. Laboratory confirmation requires demonstration of a 4-fold increase in antibody levels (or seroconversion), requiring 2 to 3 weeks between the collection of acute- and convalescent-phase samples. The development of IgM capture methodology has allowed the presumptive identification of viral infections in the early stages of clinical diseases. For instance, West Nile neuroinvasive disease is diagnosed by detection of antibody in the CSF by IgM antibody-capture enzyme-linked immunosorbent assay (73). Neurosyphilis is suggested by a positive serum RPR and confirmed by a positive CSF VDRL test (74). While a positive serologic test for toxoplasma is not necessarily indicative of active infection, it is useful for guiding empiric therapy in an immunocompromised host with a compatible radiologic picture. Intrathecal production of antibody to VZV is increasingly recognized as the optimal diagnostic test for varicella vasculopathy (75).

For other organisms, serologic testing has a limited role in the diagnosis of CNS infection. For example, enteroviruses lack a common antigen among various serotypes, making IgM levels inconsistent in sensitivity (18, 41). Local production of HSV IgG antibodies in CSF can be used in diagnosis; however, the production of antibody is delayed until day 10 or 12 of infection and peaks at about day 20, limiting the usefulness of this test to retrospective diagnosis (76).

Demonstration of locally produced, pathogen-specific antibody strongly suggests the presence of intrathecal infection. To differentiate between peripheral and CNS infections, antibody titers in serum and CSF can be measured in parallel. With a peripheral infection, the antibody titer in the CSF should be less than 5% that of the peripheral blood, due to the protective effect of the BBB. However, antibody in CSF may be translocated from serum as a result of BBB breakdown, which can be indicated by elevated albumin in CSF. Antibody titers in CSF higher than 5% of serum levels in the presence of normal levels of CSF albumin are suggestive of intrathecal antibody synthesis. Peripheral blood contamination or BBB inflammation may skew this ratio and falsely suggest CNS infection. For instance, intrathecal antibody index for *B. burgdorferi* may be useful for the diagnosis of neuroborreliosis (77).

MOLECULAR TECHNIQUES FOR DIAGNOSIS

Nucleic acid amplification-based molecular diagnostic techniques have dramatically improved the ability to diagnose CNS infections. Landmark studies published in the early 1990s confirmed the excellent performance characteristics of CSF PCR for diagnosis of HSV encephalitis (78, 79), and subsequently PCR has replaced brain biopsy as the diagnostic method of choice (67). Since that time, NAAT techniques have been proven to be the test of choice for diagnosis of CNS infection with other herpes group viruses (80–82) and enteroviruses (18, 80, 83, 84).

CSF is the specimen of choice for detecting pathogenic organisms causing CNS infections. Usually three or four

tubes of CSF are collected by lumbar puncture for diagnostic studies. The first tube has the highest potential for contamination with skin flora and should not be sent to the microbiology laboratory for direct smears, culture, or molecular studies (85). Nucleic acids are extracted from CSF for *in vitro* amplification and identification. Several commercial methods for the extraction of viral nucleic acid for amplification procedures are available (86). Inhibitors may be inadvertently extracted along with the nucleic acids and can interfere with the amplification process, reducing efficiency and lowering the sensitivity of the PCR assay (87). Several "Boom" technique-based (88), automatic extraction devices are commercially available for nucleic acid extraction from various clinical specimens including CSF (89–91).

PCR-led NAAT assays have been widely used in the detection and identification of microbial pathogens in CSF (67, 76, 78, 79). Microorganism loads in CNS are usually low; any techniques that maximize test sensitivity while retaining specificity are desirable. Multiple commercial NAAT devices for the detection of viral pathogens in CSF have been developed, but FDA-cleared tests are extremely limited. For HSV detection, after almost a quarter of a century, and probably due to the small commercial market size, there is only one FDA-cleared commercial HSV assay designated for CSF specimens (92). In contrast, quite a few commercial NAATs are now available for detection and identification of HSV in cutaneous and mucocutaneous lesions (93). Among them, MultiCode-RTx HSV 1 & 2 (Luminex Corp.), PROBETEC HSV (Becton Dickinson) (94), IsoAmp HSV (BioHelix Corp.) (95), and AmpliVue HSV 1+2 Assay (Quidel Corp.) are FDA-cleared *in vivo* diagnostics (96). The GeneXpert Dx system (Cepheid, Sunnyvale, CA) is a fully integrated and automated nucleic acid sample preparation, amplification, and real-time detection system. The system-based enterovirus assay, which is used to provide rapid and on-demand diagnosis of aseptic meningitis, has received approval by the FDA (83).

Given the breadth of pathogens implicated in CNS disease, more comprehensive molecular panels for the agents of meningoencephalitis would bring added value to commercially available tests. Multiplex design enables simultaneous detection and identification of multiple targets in a single reaction. Relative simplicity, powerful multiplexing capabilities, and high-throughput detection make multiplex assays most attractive for screening and detection of a panel of candidate pathogens in CSF. This is particularly important in immunocompromised hosts. In addition, multiplex assays provide potent tools to detect mixed infections. Numerous multiplex NAAT assays have been developed for simultaneous detection and identification of microbial pathogens in CSF (97–111). In May 2014, the FDA cleared a multiplex assay (Lyra Direct HSV 1 + 2/VZV Assay, Quidel Corp.) for detection and differentiation of HSV-1, HSV-2, and VZV for cutaneous or mucocutaneous lesion samples in symptomatic patients (112). When it was used in CSF specimens, however, the sensitivities were significantly lower than those of other monoplex assays such as Simplexa HSV 1 & 2 Direct (113).

ADDITIONAL CONSIDERATIONS IN MOLECULAR TESTING

Identifying an etiologic agent in patients with encephalitis requires consideration of the most likely causative organisms, the optimal diagnostic tests for these agents, and the highest-yield clinical specimens for testing (2, 7). Knowledge of the epidemiology and clinical presentation of specific agents is critical in selecting which diagnostic tests are appropriate for a given patient. In particular, animal or vector exposures, geographic location, recent travel history, season of the year, exposure to ill contacts, and occupation need to be considered. Further diagnostic testing may be appropriate based on individualized exposures or risk factors.

Recently, a retrospective 11-year experience of clinical application of viral CSF PCR testing for diagnosis of CNS disorders in Europe was reported. The analysis covered CSF PCR data of 481 pediatric and 2,604 adult patients, including HSV, VZV, Epstein-Barr virus (EBV), cytomegalovirus (CMV), human herpesvirus 6 (HHV-6), and enteroviruses. Nucleic acid of EBV was detected in 1.6%, of VZV in 1.3%, of HSV in 1.24%, of enteroviruses in 0.4%, of HHV-6 in 0.17%, and of CMV in 0.2% of the patients. Newborns and elderly people showed the highest infection rates. HSV, VZV, and enterovirus prevailed in typical infectious CNS diseases; EBV, in further inflammatory neurological diseases; HSV and EBV, in immunocompromised patients; and EBV, HSV, and HHV-6, in other noninflammatory neurological diseases. Rapid viral clearance was typical for HSV, VZV, CMV, and enterovirus infections, suggesting that the detection of HSV, VZV, CMV, and enteroviruses strongly indicates symptomatic viral CNS disease. Secondary viral reactivation mostly underlies positive EBV and HHV-6 findings. Their detection does not rule out clinical impact but points to the need to search for additional underlying conditions (81).

Issues regarding molecular diagnosis of specific pathogens are outlined in the following sections, but several general concepts should be considered. CSF is the optimal specimen for PCR testing in patients with encephalitis, with nucleic amplification testing of other specimens providing indirect evidence of infection (e.g., PCR of lymph node tissue in patients suspected to have *Bartonella* encephalopathy). The timing of specimen acquisition is an important variable in interpreting results. For instance, HSV PCR may be falsely negative if performed very early (114) or late (3) in the course of HSE. Host features also contribute to testing characteristics. Immunocompromised patients are at risk for a much wider array of opportunistic pathogens (e.g., HHV-6 encephalitis in bone marrow transplant patients, CMV encephalitis in HIV patients) as well as more severe clinical manifestations with more common pathogens (e.g., WNV in transplant patients).

Finally, interpretation of a positive NAAT result is complicated by the fact that some viruses survive latently in macrophages (e.g., EBV) or neurologic tissues (e.g., HHV-6) and may be incidentally detected using sensitive molecular techniques. For this reason, while a positive qualitative PCR result indicates that pathogen-specific nucleic acid is detected in CSF, it is not necessarily indicative of infection. For instance, EBV can be integrated in host genes, resulting in positive PCR results (40), and not infrequently, EBV may be amplified from the CSF of patients with an alternative CNS infection (115).

APPLICATION OF MOLECULAR METHODS TO DETECT COMMON CNS PATHOGENS

Viruses

HSVs

HSE is the most common cause of sporadic encephalitis in the United States, accounting for 10 to 20% of all viral

encephalitides (116, 117). HSV-2 is a well-established cause of neonatal brain infection, but beyond infancy more than 95% of HSE cases are due to HSV-1 (118). Approximately two-thirds of HSE cases result from reactivation of latent HSV-1, while the other third are a result of primary infection (119). HSE at the time of primary infection is thought to infect olfactory neurons followed by retrograde spread to the brain, while reactivation of latent virus in the trigeminal ganglia with spread via tentorial nerves to the frontal and temporal lobes accounts for the remainder. Alteration of consciousness, fever, and headache are common clinical presentations. Personality changes and aphasia are also frequently observed at presentation and may be helpful clues in considering the diagnosis. Neuroimaging typically shows unilateral or bilateral temporal lobe involvement, with magnetic resonance imaging being more sensitive than computed tomography (120). HSV-2 is a rare cause of encephalitis but a frequent cause of a self-limited lymphocytic meningitis in adolescents and adults, with 20% of patients developing recurrent episodes of aseptic meningitis (previously termed Mollaret's meningitis) (121).

Early recognition of HSE is important since treatment with acyclovir significantly decreases morbidity and mortality (122). Historically, brain biopsy was the diagnostic test of choice, but molecular techniques have revolutionized the diagnostic approach for patients with suspected HSE. Molecular testing of CSF offers the advantage of a minimally invasive approach coupled with a turnaround time of less than 1 day if colorimetric enzyme immunoassay or real-time PCR is used (123). Several multiplex PCR-based assays were described to cover a panel of herpesviruses including HSV-1/2 (100–104, 107–109). In experienced laboratories, the sensitivity of HSV CSF PCR in adults with HSE approaches 100%, with a specificity of 94%, and results remain positive for up to 1 week, even with antiviral treatment (3). The sensitivity of CSF HSV PCR for neonates and infants is lower, ranging from 75 to 100% (124). The Simplexa HSV 1 & 2 Direct assay (Quest/Focus Diagnostics) has received FDA clearance for detection of HSV-1/2 in CSF specimens (92).

Due to the differing performance characteristics of various HSV PCR assays, results need to be interpreted in the context of the clinical illness. False negatives are well documented, particularly early in the disease course (114, 125). For patients with a clinical presentation suggestive of HSE (e.g., temporal lobe enhancement on magnetic resonance imaging), repeat testing on a second specimen obtained 3 to 7 days after onset of symptoms is recommended prior to discontinuation of empiric acyclovir (2).

An additional cause of a false-negative HSV PCR result is the presence of large numbers of red blood cells in the CSF, because porphyrin can inhibit the PCR reaction even with as many as 20,000 copies of HSV DNA (3). False-negative results happened on CSF specimens with low concentrations of HSV-2 (102).

Many uncertainties remain regarding the optimal use of PCR for prognosis and monitoring therapy. Quantification of HSV in CSF has been reported to be a predictor of prognosis in HSE, but this test is not widely available, and its utility remains unknown (126, 127). A follow-up CSF analysis as well as quantification of HSV-1 was recommended to individualize the duration of acyclovir treatment (128). Some authorities recommend repeating HSV PCR on a second CSF specimen obtained 10 to 14 days after initiation of acyclovir, with prolongation of therapy for persistently positive results (129). In neonates, persistent detection of HSV by PCR at the end of treatment has been shown to be a poor prognostic sign (130).

VZV

Initial infection with VZV results in primary varicella (chickenpox). After infection, the virus remains latent in cranial-nerve and dorsal-root ganglia, with reactivation causing dermatomal, or in the immunocompromised host, disseminated zoster (shingles) (131). Before 1995, when the varicella vaccine became widely available, approximately 90% of primary VZV infections occurred before adolescence (132).

VZV meningoencephalitis prior to the advent of molecular testing was diagnosed solely on a temporal relationship between a typical vesicular rash and neurologic symptoms. With the availability of molecular testing, a broader clinical spectrum of VZV-related neurologic disease has been identified, often in patients without cutaneous findings (132, 133). In a multicenter Finnish study of 174 patients with confirmed or probable VZV CNS infection, 27% and 65% of patients with encephalitis or meningitis, respectively, did not have skin lesions, a disease termed *herpes sine herpete* (134). In one study of immunocompetent adults with VZV meningitis, rash was a late finding, following the onset of symptoms by a median of 6 days (135).

Several discrete neurologic syndromes have been attributed to VZV infection. Acute cerebellar ataxia is a complication of primary varicella infection and does not occur with viral reactivation (133). Patients with acute cerebellar ataxia develop acute gait ataxia, nystagmus, vomiting, tremor, and headache, although they usually have intact cognition. Symptoms typically begin in the 10 days following cutaneous eruption, but rarely there may be up to a 3-week latency between rash and onset of cerebellar symptoms (136). A syndrome of diffuse encephalitis due to either acute VZV infection or reactivation has also been recognized (137).

VZV is increasingly reported as a cause of CNS vasculitis involving either large or small blood vessels, termed VZV vasculopathy. Large vessel unifocal granulomatous arteritis is mainly a disease of the elderly and is characterized by acute focal deficit (motor or sensory) that develops weeks or months after zoster (or varicella) in a contralateral distribution (131). Recurrent ischemic episodes have been documented with this syndrome, and the mortality rate is approximately 25% (138). Small vessel multifocal vasculopathy is a subacute condition, seen more frequently in immunocompromised patients (139, 140). With both of these conditions, PCR testing has identified VZV in CNS vascular endothelium.

The test characteristics of VZV PCR vary based on the neurologic syndrome, duration of symptoms, and host immune status. In one study of patients with a clinical suspicion for CNS VZV infection, the sensitivity of CSF VZV PCR was 44% for patients with a dermatomal rash and meningoradiculitis compared to 66% for patients with a generalized rash and encephalitis (141). Timing of sample collection appeared to be an important variable in this study, because VZV CSF PCR was positive for 62% of CSF samples collected <7 days after the onset of rash, compared to 25% of those collected >7 days after skin eruption. Other investigators have demonstrated that only 25% of CSF specimens are positive for VZV DNA in patients with neurologic manifestations associated with either primary

VZV infection or reactivation, and CSF PCR was positive only among cases sampled <9 days after dermatologic onset (134). In another small study of children with acute cerebellar ataxia, 3 of 5 cases (60%) had a positive CSF VZV PCR result (142). The diagnostic yield of CSF PCR among patients with VZV vasculopathy has been reported to be 28%, with the decreased sensitivity attributed to the significant delay (average 4.2 months) between the onset of neurologic symptoms and molecular testing in these cases (140). In patients suspected to have VZV vasculopathy, detection of intrathecal antibodies may be a superior diagnostic test. Quantitative VZV PCR is not widely available, but in one study of patients with meningitis or encephalitis due to VZV reactivation, higher levels of virus in the CSF were found with encephalitis compared to meningitis and were predictive of a more severe illness (143). In another study, a significant correlation between VZV viral load in CSF and the severity and duration of neurological disease was observed (144).

VZV DNA can be amplified from the CSF in 2.5 to 7% of HIV-infected patients with new neurologic signs or symptoms (145, 146). The significance of this finding is not always clear, because VZV may accompany other CNS pathogens, suggesting subclinical reactivation in some cases (146). Cutaneous findings are variably present in HIV-infected patients with VZV CNS infection (147, 148). Among patients with VZV vasculopathy, PCR was positive in more than half of immunocompromised patients with vasculopathy, as compared to only 16% of immunocompetent patients (140).

EBV

By adulthood, over 90% of the general population has serologic evidence of prior EBV infection (149). The most common clinical presentation of acute EBV infection is infectious mononucleosis, characterized by pharyngitis, cervical lymphadnenopathy, and fever; however, asymptomatic seroconversion occurs in the majority of acute infections (150). Commonly associated symptoms of acute mononucleosis, such as headache and neck stiffness, do not necessarily imply invasive neurologic infection, because these are frequent accompaniments to viremia. Following acute infection, EBV is immortalized in B-lymphocytes, with symptomatic reactivation occurring primarily in individuals with significant impairment in T-cell immunity (151).

Neurologic manifestations can be seen with either acute EBV infection or reactivation, with specific syndromes including meningitis, encephalitis, myelitis, and acute cerebellar ataxia (152).

Detection of EBV DNA in CSF may reflect either CNS infection or viral reactivation in the setting of an inflammatory response induced by another agent or condition (115, 149). Secondary viral reactivation mostly underlies positive EBV findings (81). Quantitative EBV viral loads may prove useful in discriminating infection from reactivation due to an alternative pathogen, but a precise cutoff value has not been determined (153).

The significance of detection of EBV in the CSF of patients with HIV deserves special mention. Primary CNS lymphoma (PCNSL), a relatively common complication of HIV infection prior to the availability of highly active antiretroviral therapy, is caused by localized reactivation of EBV in the CNS, with clonal proliferation of lymphocytes (154). In HIV-infected patients, EBV amplification from the CSF has been proposed as a marker for PCNSL among patients with CNS mass lesions (155) and as a predictor of the later development of PCNSL among patients without radiologic findings (156, 157). This approach has been called into question by more recent studies documenting a positive predictive value of only 10 to 29% for EBV PCR from the CSF of HIV-infected patients with neurologic disease (158, 159). While the test characteristics may be improved slightly by using a quantitative cut-point of 10,000 copies/ml (158), EBV viral load does not reliably discriminate between PCNSL and EBV encephalitis (153), and pathologic review of brain tissue is still required for definitive diagnosis of PCNSL.

Other Herpesviruses

The role of other herpesviruses in causing encephalitis is controversial. CMV is an important cause of encephalitis in the immunocompromised host but does not appear to be a significant cause in the general population (160). Similarly, HHV-6 has been established as a cause of encephalitis in immunocompromised patients, particularly stem cell transplant recipients, but its role in the normal host is controversial (161, 162).

WNV

WNV is a mosquito-borne virus that was first detected in the Northern Hemisphere in 1999 and has since emerged as the most common form of endemic encephalitis in the United States (163). The clinical spectrum of WNV infection ranges from asymptomatic seroconversion to encephalomyelitis. Symptoms are present in ~20% of cases, with neuroinvasive disease reported in <1% of cases. Neurologic involvement is more common among the elderly and the immunocompromised. Although there is no pathognomonic presentation of WNV encephalitis, weakness, including acute flaccid paralysis, is a characteristic finding (164).

WNV viremia begins within 1 to 3 days of mosquito inoculation but is typically short-lived (165). Constitutional symptoms precede neurologic involvement, which develops 7 to 9 days after infection, at which time the virus has been cleared from the bloodstream (166, 167). For this reason, NAAT has an important role in the screening of asymptomatic blood donors for the presence of WNV, where reverse transcriptase PCR or nucleic acid sequence-based amplification can detect as few as 50 viral copies/ml (168). Molecular amplification techniques have been less successful in diagnosing symptomatic patients. During a large community outbreak of WNV in Canada, only 45% of plasma samples from patients with serologically confirmed WNV infection had detectable virus by PCR (169). Predictors of a positive plasma PCR result included testing within 8 days of symptom onset and West Nile fever (36% positive) rather than West Nile neuroinvasive disease (9.5% positive). Plasma WNV viral load did not correlate with either the duration of symptoms or the presence of CNS involvement.

Similarly, amplification of WNV from CSF is of limited utility, with only 57% of patients with serologically confirmed West Nile neuroinvasive disease having positive CSF PCR by real-time PCR, and 0% of specimens positive by conventional PCR (170). Immunocompromised hosts, a population at high risk for severe neurologic disease and adverse outcomes from West Nile neuroinvasive disease, represent the exception. Case reports have documented positive CSF WNV NAAT in transplant patients and those with hematologic malignancies (171, 172). In these cases, virus in the blood and CSF may persist for a

prolonged time, congruent with the delay in production of detectable neutralizing antibody (173).

Enteroviruses

The *Enterovirus* genus comprises >90 subtypes differentiated by a numeric designation (e.g., enterovirus 71) (174, 175). Two former members of this genus, echoviruses 22 and 23, have been reassigned as human parechovirus 1 and 2, which have been demonstrated to cause meningitis in young children (176). Humans are the only natural hosts for the enteroviruses, and the principal mode of human-to-human transmission is fecal-oral (133). In 2014, enterovirus D68 was recognized as the cause of an outbreak of severe respiratory infection in children in the Midwest (177).

Enteroviruses are the most common cause of aseptic meningitis in the United States, accounting for 80 to 92% of cases in which an etiologic agent is identified (18), with a seasonal predilection extending from June to October (178). Less commonly, these viruses can cause severe, life-threatening manifestations, such as encephalitis, paralysis, myopericarditis, and neonatal sepsis (178). In a large study evaluating causes of encephalitis, enteroviruses were the leading infectious agents identified, with 75% of cases occurring among children (1). Large outbreaks of enterovirus 71 in Southeast Asia have been associated with brain stem encephalitis in pediatric patients, causing a high mortality and considerable cognitive morbidity among survivors (179, 180).

Enterovirus-specific reverse transcriptase PCR has a sensitivity and specificity that approach 100%. Numerous monoplex and multiplex PCR-based assays have been developed (18, 81, 83, 97), and most of them target the highly conserved 5′-nontranslated region, allowing amplification of all human enteroviruses. The disadvantages of this approach are that these assays (i) cross-react with rhinoviruses and (ii) do not differentiate enterovirus genotypes. Several molecular devices have been cleared by the FDA for detection of enteroviruses in CSF including the Cepheid Xpert EV assay, which is a fully integrated and automated system with a test turnaround time of approximately 70 minutes (83). Other nucleic acid detection methodologies directed to the enteroviral capsid protein VP1 (181) or other capsid regions of the genome are less cross-reactive but allow the product to be sequenced for serotype identification.

Rabies Virus

Rabies virus is an almost universally fatal cause of encephalitis. While this infection is relatively uncommon in the United States, more than 50,000 cases are estimated to occur worldwide, mainly from wild-animal bites (182).

Human rabies can present in two general forms: encephalitic or furious rabies (80 to 85% of cases) or the less common paralytic or catatonic form. Patients frequently complain of paresthesias at the site of inoculation. This prodrome may last only a couple of days before an acute neurologic syndrome consisting of excessive salivation, agitation, hydrophobia, and nuchal rigidity begins, often accompanied by autonomic nervous system involvement (183). Antirabies vaccine or rabies immune globulin is not effective once symptoms are present, and death typically occurs within 1 to 2 weeks of symptom onset.

Antemortem diagnosis of rabies is important for establishing prognosis and coordinating postexposure treatment of contacts. For this reason, testing for suspected cases of rabies should be coordinated with the local or state health department. Several studies have explored the application of NAAT tests in CSF for the diagnosis of rabies. A small study evaluating rabies virus real-time PCR on both CSF and saliva among patients with confirmed infection identified a sensitivity of 9% and 30%, respectively (184). Another study comparing conventional PCR to real-time PCR of saliva found a sensitivity of 37% for the former, compared to 75% for the latter (185). A recent study reported that real-time PCR alone could achieve antemortem rabies diagnosis in 11/13 (84.6%) cases; combined with rabies viral neutralizing antibody detection in CSF, antemortem rabies diagnosis could be achieved in all 13 (100%) cases (186).

Acute Bacterial Meningitis

The syndrome of acute bacterial meningitis is a medical emergency, requiring rapid diagnostic evaluation and institution of empiric therapy (2). Even with this approach, the mortality among adults with community-acquired acute bacterial meningitis exceeds 25% (187). Predictors of an adverse outcome include impaired level of consciousness, seizures, and hypotension (188, 189).

Definitive diagnosis of bacterial meningitis requires laboratory confirmation, because involvement of the CNS by other pathogens (e.g., viruses, fungi, mycobacteria) and noninfectious processes (e.g., subarachnoid hemorrhage) may produce identical syndromes. CSF findings suggestive of bacterial meningitis include neutrophilic pleocytosis, hypoglycorrhachia, and elevated protein levels, but these are nonspecific. A positive CSF Gram stain is indicative of acute bacterial meningitis, but the sensitivity of this test ranges from 60 to 90% and falls to <20% following administration of antibiotics (2, 190, 191). A positive CSF culture is definitive evidence for acute bacterial meningitis, but the time to a positive test is >24 hours, and the yield of CSF culture decreases sharply as early as 15 minutes following administration of antibiotics (192, 193).

There has been significant interest in evaluating molecular techniques for the diagnosis of acute bacterial meningitis. Several studies have evaluated a multiplex PCR assay directed at DNA sequences of the most common pathogens causing acute bacterial meningitis (98, 110). A study comparing broad-range bacterial PCR with culture of CSF among 74 patients with acute bacterial meningitis reported a sensitivity of PCR of 59% compared with 43% for culture (194). Notably, 15 of 19 patients (79%) with positive PCR results but negative cultures had received antibiotics prior to lumbar puncture. Other studies have confirmed the utility of broad-range PCR for culture-negative purulent meningitis following antibiotic administration (195–198). A recent study from China compared bacteriological culture, monoplex, and multiplex PCR in the diagnosis of bacterial neonatal meningitis. The study indicated that both monoplex and multiplex PCR assays had better sensitivities than bacterial culture with the capacity of detecting the pathogens in CSF samples with negative culture results (199).

Tick-Borne Rickettsial Diseases

Tick-borne rickettsial diseases (TBRDs), including Rocky Mountain spotted fever (caused by *R. rickettsii*), human monocytic ehrlichiosis (caused by *E. chaffeensis*), and human granulocytic anaplasmosis (caused by *Anaplasma phagocytophilum*), are important and treatable causes of meningoencephalitis in the United States. The distribution of these bacteria differs with respect to vector and geography; however, all

three cause a similar febrile syndrome, typically associated with headache. Altered mental status is estimated to occur in as many as 20% of cases (26, 200).

Confirmation of TBRD presents a diagnostic challenge. Detection of a 4-fold rise in antibody titer is definitive evidence of a TBRD but is only useful for retrospective diagnosis. The sensitivity of PCR for the different pathogens that cause TBRD is variable. *R. rickettsii* infects vascular endothelial cells, and PCR of fresh tissue obtained through skin biopsy or autopsy may be diagnostic (26, 200). Molecular assays for *R. rickettsii* DNA can be performed on whole blood but are not widely available, and the low sensitivity limits the clinical use of this test (201, 202).

In contrast, several studies have validated PCR for the rapid diagnosis of ehrlichiosis. Whole-blood PCR has been reported to have a sensitivity of 56 to 100% for human monocytic ehrlichiosis (203, 204) and 60 to 70% for human granulocytic anaplasmosis (205). The diagnostic yield is highest early in the course of infection, prior to initiation of antibiotic treatment. *Ehrlichia* species can rarely be amplified from CSF, but the yield of this specimen compared to whole blood has not been evaluated (48, 206). A molecular device coupling broad-range PCR and mass spectrometry demonstrated rapid and accurate detection of *Ehrlichia* species in both peripheral blood and CSF (207).

Whipple's Disease

Whipple's disease is an uncommon syndrome caused by the fastidious bacterium *Tropheryma whipplei*. Clinical manifestations are protean but include weight loss, uveitis, diarrhea, and arthropathy, with CNS manifestations in 20 to 40% of cases (208–210). Occult CNS involvement is common, because 70 to 90% of patients without neurologic symptoms have detectable bacteria on postmortem brain examination or CSF analysis (211–213). CNS symptoms include altered mentation, seizures, or oculomasticatory myorhythmia, and cranial nerve abnormalities (208, 214).

Classically, diagnosis of Whipple's disease is through histological identification of periodic acid-Schiff staining bacilli in enterocytes on duodenal biopsy. Recently, a noninvasive approach has been advocated for screening suspect cases through tandem PCR of saliva and stool samples for *T. whipplei*. While bacteria may be amplified from these specimens in asymptomatic carriers (215, 216), a positive test on both specimens is associated with a positive predictive value for Whipple's disease of 95%, increasing to 100% if the bacterial load in stool exceeds 10^4 copies (217). False-positive results have been reported using broad-range 16S rRNA primers, with studies proving the superiority of probes specific for repeat gene sequences unique to *T. whipplei* (218, 219).

Isolated or localized Whipple's disease represents a particular diagnostic challenge. In this subgroup, histologic examination of small bowel biopsy is negative, and the sensitivity of tandem PCR of saliva and stool decreases to 58% (217). For isolated CNS infections, CSF PCR is extremely specific, but the sensitivity is not known (217). In cases where a high suspicion for CNS Whipple's disease persists despite a negative CSF PCR, brain tissue may be diagnostic (220–222). A retrospective analysis of molecular assays was performed for the diagnosis of CNS Whipple's disease over a 12-year period in a reference center in France. *T. whipplei* was recovered from CSF of 6% of patients. The number of patients with a positive PCR result for *T. whipplei* has increased significantly over the last 12 years (223).

Tuberculous Meningitis

Tuberculous meningitis (TBM) is a relatively infrequent complication following pulmonary infection but is associated with significant morbidity and mortality (224, 225). The outcome is dependent on time to starting effective antimycobacterial therapy, but both the clinical presentation and CSF findings of TBM are nonspecific, which may lead to delays in initiating treatment (226, 227). For this reason, there is significant interest in identifying diagnostic techniques that would allow rapid confirmation of TBM.

Nucleic acid amplification has limited utility for the diagnosis of pulmonary tuberculosis (228, 229), but CSF lacks the inhibitors present in sputum. A meta-analysis of commercial nucleic acid amplification assays on CSF reported a sensitivity of 56% for the diagnosis of TBM, but the specificity approached 100% (230). Most assays target insertion sequence IS*6110* through a single-step amplification reaction (231, 232). In proficiency panels, false-negative PCR results may be seen at low bacterial concentrations (<2 CFU/ml) (233). The Cepheid Xpert MTB/RIF assay has been cleared by the FDA for the detection of pulmonary tuberculosis and the determination of multidrug resistance. A recent systematic review and meta-analysis indicated that the assay had pooled sensitivity of 80.5% against culture and 62.8% against a composite reference standard in CSF (234). Given the suboptimal sensitivity, the current role of CSF PCR is to confirm a diagnosis of TBM, but a negative result is not useful in excluding disease or in discontinuing treatment.

Lyme Disease

Neurological manifestations may be seen in early disseminated Lyme disease as cranial neuropathy or meningitis, while late neurologic findings, which are less common, include encephalomyelitis or peripheral neuropathy. Molecular testing is not useful in late neuroborreliosis but has a limited role in laboratory confirmation of early neurologic disease (235). Most assays target the outer surface protein A gene (236). The sensitivity of CSF PCR among patients with neuroborreliosis ranges from 15 to 79% (237–240). One study reported a CSF PCR sensitivity of 50% among patients with intrathecal antibody production, but PCR failed to detect organisms in any patient lacking CSF antibodies, despite the presence of positive serum antibody and CSF pleocytosis (241). A positive CSF PCR result in a seronegative patient is suggestive of a false-positive result. Among children with clinical and serologic evidence of Lyme meningitis or facial palsy, CSF PCR was positive in only 5% and 12% of cases, respectively (242, 243).

CNS Aspergillosis

The recent outbreak of *E. rostratum* meningitis linked to epidural injections of methylprednisolone acetate has brought renewed attention to mold infections of the CNS (60). CNS invasive aspergillosis (IA) is an almost uniformly fatal complication in immunocompromised patients. Confirming the diagnosis is challenging because invasive procedures are impaired by neutropenia and low platelet count. Several PCR assays have been developed for detection of *Aspergillus*-specific nucleic acids in CSF (244–246). Reinwald et al. recently reported that the use of a nested PCR assay enhanced diagnosis of CNS aspergillosis in immunocompromised patients (245). The retrospective analysis of CSF samples from patients with suspected CNS IA yielded a high sensitivity of the nested

PCR assay. One study suggested PCR testing of CSF samples for patients for whom CNS IA is suspected, especially for those whose clinical condition does not allow invasive procedures because a positive PCR result makes the presence of CNS IA in that patient population highly likely (245). *Aspergillus* PCR may not be widely available, and *Aspergillus* galactomannan testing of CSF would be an alternative option to allow earlier and more sensitive diagnosis of CNS IA compared to fungal culture (247).

CONCLUSION

Nucleic acid amplification techniques have markedly improved the identification of CNS infections caused by viral and fastidious bacterial pathogens. Molecular techniques such as PCR allow rapid diagnosis with consequent improvement in outcomes and cost savings. However, the proper testing and interpretation of results requires insight into the strengths and limitations of these tests. Results of molecular diagnostic testing for CNS infections must be interpreted in the context of the individual patient presentation and clinical illness, and close cooperation between the laboratory and the clinician is required for optimal use of these technologies.

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Using Nucleic Acid Amplification Techniques in a Syndrome-Oriented Approach: Detection of Respiratory Agents

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25

Lower respiratory tract infections (LRTIs) are an important problem. They occur frequently, are associated with significant morbidity and mortality, are present in a variety of health care settings, and impose a considerable cost to European health care services.

In developed countries, while mortality declined spectacularly during the 20th century, LRTIs remain a leading cause of death. WHO statistics estimate that mortality from respiratory infections is 48/100,000 worldwide and ranges from 40 to 50/100,000 in Europe (WHO's 2002 annual report at http://www.who.org/).

Such figures stress the importance of early recognition of those patients who are severely ill or at risk of becoming severely ill. Of all RTIs about one third are thought to involve the lower respiratory tract (LRT), with ±10% community-acquired pneumonia (CAP), the remaining two-thirds affecting the upper respiratory tract.

At present there is still a great deficit in the etiologic diagnosis of community-acquired LRTIs: in most studies more than 50% of cases remain without an etiologic diagnosis, resulting in unnecessary or inappropriate antibiotic prescribing.

A wide variety of diagnostic procedures and techniques are applied to the detection of the etiologic pathogens of community-acquired LRTI. Traditional diagnostic culture methods above all lack sensitivity, are not feasible in many contexts, and focus only on a few of the large number of etiologic agents. For example, for years, viruses were rarely thought of as the etiologic agent of LRTIs except for cases involving children and immunocompromised patients. However, it has been demonstrated that viruses, in particular RNA viruses, may cause LRTIs and even pneumonia in otherwise healthy adults. Multiple pathogen infections are also increasingly detected (1).

For the so-called atypical bacterial causes—*Mycoplasma pneumoniae*, *Chlamydophila pneumoniae*, and *Legionella pneumophila*—traditional diagnostic methods are too insensitive and too slow, producing a result only after several days.

Therefore, alternative diagnostic procedures were developed: antigen detection by latex agglutination or immunofluorescence, enzyme-linked immunosorbent assay, immunochromatography, and nucleic acid amplification techniques (NAATs), particularly PCR and NASBA (nucleic acid sequence-based amplification).

Over the past 2 decades, NAATs have been revolutionizing the diagnostic procedures for the management of patients with LRTI, resulting from a combination of improved sensitivity and specificity, a potential for automatization, and the production of very rapid results. NAATs have already become the "gold standard" in some diagnostic fields, but not many assays have been approved by the FDA for the detection of respiratory pathogens, and fewer still have entered the daily routine diagnosis and management of patients. This can be ascribed to the rapid evolution of the technology, the cost of this technology, and the large number of etiological agents, bacterial as well as viral, responsible for community-acquired LRTI.

This overview will therefore provide a look at the general principles, advantages, diagnostic value, and limitations of the most currently used new amplification techniques for the etiological diagnosis of RTIs as they evolve from research to daily practice.

OPTIMAL SAMPLING FOR THE DETECTION OF RESPIRATORY AGENTS

Specimens that are useful for the detection of etiologic agents in LRTIs may be collected from different body sites, especially in the case of suspected pneumococcal pneumonia, or may be different kinds of specimens from the respiratory tract. It should be noted here that for a number of respiratory etiologies there is currently no consensus on the optimal sampling compartment. A review of possible samples to be used for PCR-based tests or PCR combined with other detection methods was provided by Loens et al. (2).

Streptococcus pyogenes

Swab specimens obtained from the posterior pharynx and tonsils are most widely used as a sampling method for group A streptococci (GAS) pharyngitis testing. This convention is based on recommendations published by the Infectious Diseases Society of America, which defines specimens obtained from these sites to be the only adequate samples to test for the presence of *S. pyogenes* (3). However, these recommendations are based on two small

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studies conducted more than 20 years ago and evaluating only 32 patients (4, 5). A study conducted by Fox et al. (6) investigated the use of newer techniques of rapid direct carbohydrate antigen and nucleic acid probe detection to identify GAS in samples obtained prospectively from the oral cavities and posterior pharynges of pediatric patients with sore throats. Their study validated the recommendations by the Infectious Diseases Society of America to obtain a sample from the posterior pharynges and tonsils of patients suspected of having GAS pharyngitis. On throat specimens from symptomatic children, both traditional as well as newer tests used to detect the presence of GAS performed significantly better.

Streptococcus pneumoniae

Although blood cultures are still considered culture-based diagnosis of pneumococcal pneumonia because the organisms are recovered from a normally sterile source, PCR-based diagnosis on blood specimens is so far not being used routinely. Recent studies, however, show that the detection of bacterial DNA load in whole blood supports the diagnosis of *S. pneumoniae* infection in patients with CAP: high genomic bacterial load (7) for *S. pneumoniae* may be a useful tool for severity assessment. Systematic comprehensive studies need to be performed to confirm the clinical usefulness of PCR on plasma or whole blood.

The specificity of respiratory specimens for the diagnosis of bacterial pathogens, either by culture or by amplification-based techniques, in the LRTI is not high because of contamination with the upper airway biota. Several techniques have been proposed to achieve accurate discrimination between colonization and infection. Diagnostic accuracy is improved by the use of bronchoalveolar lavage (BAL) specimens in severely ill hospitalized patients.

It is generally accepted that the best nonsterile respiratory specimen for the recovery of *S. pneumoniae* is sputum. Sputum specimens must be representative of lower respiratory secretions. The most widely used method to assess the acceptability in this regard is based on cytologic criteria (8).

M. pneumoniae, *C. pneumoniae*, and *L. pneumophila*

Because of its fastidious nature, *M. pneumoniae* is not routinely cultured from respiratory specimens. Most studies are PCR-based on both LRT and upper respiratory tract (URT) specimens. Different specimens have been used such as sputum, nasopharyngeal swabs (NPSs), oropharyngeal swabs or washes, BAL, or pleural fluid as described in the review by Loens et al. (2).

The superiority of sputum compared to other respiratory specimens for the molecular detection of *M. pneumoniae* has been demonstrated in several studies. The high diagnostic sensitivity of sputum PCR could be explained by the higher number of *M. pneumoniae* organisms in the pulmonary alveoli than on the epithelium of the URT, which has been demonstrated in experimentally infected hamsters. Consequently, if a sputum sample is available, it might be the best specimen for *M. pneumoniae* detection. An oropharyngeal swab or nasopharyngeal aspirate (NPA) might be the second best option for analysis by NAATs (2). The choice of the respiratory specimen may also have a major impact on the sensitivity of *C. pneumoniae* isolation and PCR. Based on a literature search, it is clear that sputum or an NPS may be the preferred specimen for detection of *C. pneumoniae* by NAATs (2).

In patients with community-acquired LRTI caused by *L. pneumophila*, bronchoscopically taken specimens such as BAL are the preferred specimens in severely ill patients admitted to the hospital. Sputum specimens are generally considered to be the best alternative specimens for the isolation of *L. pneumophila* in non-severely ill patients. Combining test results from more than one site appears to improve the diagnostic accuracy. Based on the available data, Loens et al. concluded that no anatomic site or method is clearly superior for optimal detection of *Legionella* in respiratory specimens. Combining test results from more than one site appears to improve the diagnostic accuracy (2).

Bordetella pertussis

NPAs are the optimal sample for infants; they offer superior sensitivity compared to swabs (9). Nasopharyngeal swabs provide valid specimens from older children, adolescents, and adults. Sputum samples or throat washes may represent an alternative for adolescents and adults. The sensitivity of detecting *Bordetella* DNA in these materials has to be validated because, for culture, throat washes were found less suitable (10).

Respiratory Viruses

Specimens for viral tests are not fundamentally different from specimens for bacterial tests, but they are governed by three mandatory rules. They must be taken early, taken from respiratory epithelium (nose, trachea, bronchi), and packed in viral transport medium. At present, sputum is not acceptable for direct virus testing, and BAL is useful for respiratory infections in immunocompromised patients. Serology is not very useful for diagnosis because antibodies appear too late, and the tests themselves perform poorly.

Although not optimal for the detection of typical bacterial pathogens, it is generally assumed that for respiratory infections due to viruses, the optimal specimen is the NPA; this might not be true for all respiratory viruses and for all detection techniques applied. Branche et al. applied real-time quantitative reverse transcriptase PCR (RT-PCR) on dunked sputum (dunking a cotton swab in sputum and swirling it in sterile water) and nose and throat swabs from 965 patients for the detection of respiratory viruses (11). A viral infection was identified in 295/965 patients by in-house uniplex real-time RT-PCR assays. By nose and throat swab alone, 73 (24%) were positive, 105 (35%) were positive by sputum alone, and 124 (41%) were positive in both the nose and throat swab and sputum. Although rhinovirus is considered a cause of URT infections (URTIs), 36/44 (82%) rhinovirus infections that were detected had detectable virus in the sputum sample, 16 (36%) of which were only detected in sputum. The same authors also applied real-time quantitative RT-PCR and found that for the majority of samples and for all four viruses tested (influenza A, respiratory syncytial virus [RSV], human metapneumovirus, and human coronavirus [HCoV] OC43), the quantitative load of the sputum as such was slightly greater than that of the dunked sputum, and both types of sputum contained higher loads than the nose and throat swab.

It is difficult to compare recovery rates of respiratory pathogens from swabs because different types of swabs and transport media have been used. Since 2003, studies have been performed to evaluate the recovery of different organisms in new transport and storage media. For virus transport systems, no real standard has been defined. Different respiratory specimens and types of swabs and transport media were

used. The Copan system, combining flocked swabs and universal transport medium (Copan, Brescia, Italy) for collection and transport, is a universal system compatible with antigen detection kits, direct fluorescent antibody, culture, and PCR. Nasal swabbing with the new flocked swabs is equivalent to traditional rayon NPS with less patient discomfort. Significantly more epithelial cells are collected by these flocked swabs, providing better specimens for diagnosis. Furthermore, NPSs collected with flocked swabs detect a higher number of positives than NPSs collected with Dacron swabs (12).

DIAGNOSIS BY NAAT FOR INDIVIDUAL BACTERIAL RESPIRATORY PATHOGENS

S. pyogenes

S. pyogenes (GAS) is the cause of a wide range of invasive and noninvasive diseases: It is the most common bacterial cause of pharyngitis in school-age children and adolescents, affecting approximately 1 in 10 children per year (13). In children, pulmonary infections caused by GAS vary between 4 and 60% (14). On the other hand, GAS infections were found in 11% of pleuropulmonary infections in a mixed population in France (15). Detection of the organism is based on culture on 5% blood agar, a rapid antigen detection test, and/or PCR.

Based on several studies summarized by Kellogg et al. (16), a standard throat culture reliably detects 90 to 95% of GAS in symptomatic patients. Roe et al. (17) described a lower sensitivity (85%) of throat swab cultures. Nerbrand et al. (18) used both enrichment broth and PCR in addition to standard plating cultures. This resulted in a plate culture sensitivity of only 82%. A much higher sensitivity (99.4%) of a throat culture was obtained by Chapin et al. (19) based on comparison to a parallel rapid antigen detection test.

Compared to other bacterial pathogens, very few PCR assays have been described for the detection of GAS in throat swabs or other respiratory specimens (20–25). Gene targets that are being used include the DNase B gene, SpeB, *parE* gene, spy1258, spy1857, *ptsI* gene, MF gene, 16S rDNA, and *rpoB* gene (Table 1).

Dunne et al. (22) compared six qualitative PCR assays and also investigated the sensitivity and specificity of two quantitative PCRs. In this study, the SpeB quantitative PCR was found to be highly sensitive and specific for the detection of GAS in throat swabs. In addition, the authors concluded that differentiation between an acute GAS infection and carriership remains a challenge.

TABLE 1 Summary of single-plex PCR assays for detection of *S. pyogenes* and previously validated assays used as comparators[a]

Assay year (reference)	Assay type	Detection format	Gene target (product size)	PCR assay used as comparator for new assay	Non-PCR comparator test	Specimens tested for validation of sensitivity and/or specificity
1997 (23)	PCR	Agarose gel electrophoresis	MF gene (419)		Optical immunoassay, agar culture, broth-enhanced culture, latex agglutination	Various bacterial strains Throat swabs from patients being evaluated for pharyngitis
1991 (240)	PCR	Agarose gel electrophoresis	SpeB (257)			34 *S. pyogenes* strains
2003 (25)	PCR	Real-time	ptsI gene (198)		Culture, rapid antigen immunoassay	Various bacterial strains 384 throat swabs
2003 (241)	PCR	ELISA	NS (824)			Various bacterial strains, serial dilutions
2004 (242)	PCR	Sequencing	rpoB (NS)			Various bacterial strains
2004 (243)	PCR	Array	parE (139)		Culture	Various bacterial strains
2005 (244)	PCR		Spy1258 (450)			Various bacterial strains
2008 (245)	PCR	Sequencing				
2009 (21)	PCR	Real-time	NS (80)		Culture and latex agglutination	8 specimens known to be GAS positive, throat swabs
2011 (24)	PCR	Real-time using Tm	DNase B gene (NS)		Culture	Various bacterial strains, archived throat swabs, 344 swabs from a swab comparison study
2013 (29)	PCR	Turbidity		Roche GAS assay	Throat swab cultures, rapid antigen test	437 throat swabs
2013 (22)	PCR	Real-time	SpeB (346)	Comparison of 6 PCRs	Culture	Various bacterial strains
2013 (22)	qPCR	Real-time	SpeB (77)	Comparison of 6 PCRs	Culture	Various bacterial strains Throat swabs
2013 (22)	qPCR	Real-time	Spy1258 (141)	Comparison of 6 PCRs	Culture	Various bacterial strains Throat swabs
2013 (22)	PCR	Real-time	Spy1857 (155)	Comparison of 6 PCRs	Culture	Various bacterial strains

[a]Abbreviations: ELISA, enzyme-linked immunosorbent assay; qPCR, quantitative PCR.

As for the other bacteria previously described, the real-time multiplex PCR (MX-PCR) developed by Morozumi et al. (26) for the simultaneous detection of the atypical bacteria and *S. pneumoniae* and *S. pyogenes* has been applied to clinical specimens from patients with CAP. The assay was found to be 100% sensitive and specific compared to conventional culture results.

Uhl et al. (25) compared the performance of the LightCycler StrepA assay to that of the Directigen 1-2-3 Group A Strep Test Kit and standard culture for the detection of GAS in 384 throat swabs. The LightCycler PCR produced more positive results than either culture or the Directigen immunoassay. The sensitivities and specificities for this comparison were 55% and 99% for the Directigen method and 93% and 98% for the LightCycler assay, respectively. In a study conducted by Atlas et al. (27), comparing culture and a point-of-care test on specimens from patients with acute pharyngitis, a sensitivity of 92.1% and a specificity of 100% were obtained with the point-of-care test. The performance of two rapid antigen detection assays (QuickVue+ streptococcus A test and the Directigen EZ group A streptococcus test) for *S. pyogenes* detection in pleural fluid samples from pediatric patients with empyema was compared with culture, Gram stain, and PCR by Zheng et al. (28). Each rapid antigen test was able to detect 90% of the positive PCR samples with a 100% specificity.

Finally, the Illumigene Group A Streptococcus assay is a new commercially available molecular method which is cleared by the FDA. The whole process time from preparation to result reading for up to 10 specimens is finalized within approximately 1 hour. In a prospective study, the Illumigene assay was compared to (i) standard bacterial culture, (ii) a rapid antigen test using 437 consecutive throat swabs, and (iii) PCR targeting the *ptsI* gene in 161 samples to evaluate its clinical performance (29). Compared to culture, a sensitivity and a specificity, respectively, of 100% and 95.9% were obtained. A sensitivity and specificity of 100% and 99.2% were obtained when compared to PCR. In contrast, the GAS rapid antigen assay resulted in 73.3% sensitivity and 89.1% specificity in this study. Despite these results, culture back-up is still recommended.

More and more, the importance of group C and G *Streptococcus dysgalactiae* subsp. *equisimilis* as a significant pathogen is being recognized (30). No differential diagnosis can be made between an *S. pyogenes* infection and an *S. dysgalactiae* infection on the basis of clinical signs and symptoms. Therefore, the Lyra Direct Strep Assay (Quidel Corp., San Diego, CA), a real-time PCR test for the rapid and qualitative detection and differentiation of group A β-hemolytic streptococci (*S. pyogenes*) and pyogenic group C and G β-hemolytic streptococci (*S. dysgalactiae*, *Streptococcus equi*, *Streptococcus canis*), has been developed. The assay has been applied to nucleic acids isolated from throat swab specimens obtained from symptomatic patients.

S. pneumoniae

S. pneumoniae is the most frequent microbial agent to be found in bacteriologically documented pneumonia in both elderly and middle-aged adults. Of all etiologic agents detected in CAP, *S. pneumoniae* is responsible for more than 20 to 30%, both in the community and in hospitalized patients. The risk of pneumonia due to *S. pneumoniae* is higher in old people than in the general population. The occurrence of pneumonia due to *S. pneumoniae* is more frequent in institutionalized patients than in noninstitutionalized subjects of the same age, and the relative risk of pneumococcal infection increases linearly with age. Early and accurate diagnosis of pneumococcal pneumonia remains difficult due to the limitations of conventional diagnostic methods. Detection by conventional culture is cheap and facilitates antibiotic resistance determination. Blood and sputum cultures are, however, time-consuming and lack sensitivity, particularly for patients with antecedent antibiotic exposure. Sputum culture is also difficult to interpret due to oropharyngeal contamination. Several PCRs have been employed with varying degrees of success, using primers specific to repetitive regions and genes encoding rRNA (31–33), pneumococcal surface adhesion A molecule (*psaA*) (34, 35), pneumolysin (*ply*) (36), penicillin-binding protein (37), 16S rDNA (38), putative sugar-specific permease and intergenic spacer (39), a DNA-dependent ATPase (*recA*) (40), virulence factors such as CpsA (41), and autolysin (*lytA*) (42–45). In recent years, real-time PCR has improved diagnostics. The loop-mediated isothermal amplification (LAMP) technique has also been applied for the successful detection of *S. pneumoniae* in clinical specimens (46). Nevertheless, the identification of *S. pneumoniae* is more complicated than previously assumed (47–49). Several reports have shown that strains that are genotypically closely related to oral streptococci harbor the *S. pneumoniae* virulence factor genes *lytA* and *ply* (49–51). PCR methods based on the *ply* gene are not specific and show high detection rates in saliva from culture-negative healthy individuals (52). Recently, a new *S. pneumoniae*-specific target, the gene fragment Spn9802, has been reported (52, 53).

In addition to some specificity problems, studies using qualitative PCRs have reported difficulties in determining whether positive PCR findings represent colonization or infection, limiting clinical applicability (54, 55).

When testing sputum samples, reported PCR positivity rates have ranged from 68 to 100% for samples from patients with pneumonia (56), although it is unclear how often this reflects colonization of the URT rather than infection (55).

Also, the data obtained by using MX-PCR to detect, among others, the *S. pneumoniae ply* gene, show that asymptomatic carriage makes the use of molecular assays problematic to detect *S. pneumoniae* in URT specimens (57).

According to the European Respiratory Society (8) LRTI guidelines, qualitative nucleic acid amplification tests for *S. pneumoniae* on pleural fluid, peripheral blood, and sputum add little to the existing diagnostic tests in sputum and are unable to distinguish colonization from infection.

One approach that may help to differentiate between carriage and infection with a higher bacterial burden in invasive pneumococcal disease than in a carrier state is the use of quantitative PCRs (RQ-PCR). In recent years, more and more real-time RQ-PCRs have been evaluated (51, 53, 58–60). Although this has not been systematically evaluated, initial data suggest that this might be worth exploring further (59, 61). Yang et al. (51) evaluated, in a first prospective study, the clinical utility of an RQ-PCR assay targeting the *ply* gene to detect *S. pneumoniae* in sputum from adult pneumonia patients. RQ-PCR findings were compared with those of a composite reference standard comprising Gram stain of sputum and sputum/blood cultures. Based on receiver operating characteristic curve analysis, the log-transformed threshold cycle (C_T) giving maximal sensitivity and specificity for the PCR assay was

28.96, corresponding to approximately 3.7×10^4 genomic equivalents of *S. pneumoniae* per ml of sputum. At this threshold, the assay sensitivity and specificity were 90.0% and 80.0%, respectively. Targeting the same gene in another study, Kais et al. (59) examined respiratory tract specimens from 203 patients with and without infection by RQ-PCR and quantitative culture. Significant findings in RQ-PCR were defined as amounts of DNA corresponding to 10^5 CFU/ml. In patients with infection, significant pathogens were found in 32/135 samples with culture and in 51/135 with RQ-PCR, resulting in an increase from 23.7% to 37.7%. The same assay was used for the quantitative detection of *S. pneumoniae* in sputum from 184 CAP patients (58). *S. pneumoniae* was, with all methods used, found to be responsible for 38% of the cases; RQ-PCR, with a cut-off level corresponding to 10^5 CFU/ml, together with urinary antigen detection, was the method that most efficiently contributed to determination of pneumococcal etiology and was shown to be particularly valuable in patients previously treated with antibiotics. On the other hand, the data obtained by using MX-PCR to detect, among others, the *S. pneumoniae ply* gene show that asymptomatic carriage makes the use of molecular assays problematic in detecting *S. pneumoniae* in URT specimens (57). These results were confirmed by Abdeldaim et al. (62).

Albrich et al. investigated the applicability of quantitative *lytA* real-time PCR results from induced sputum and NPSs to distinguish pneumococcal community-acquired pneumonia (CAP) from asymptomatic colonization in HIV-infected South African adults (63). *S. pneumoniae* was considered the cause of pneumococcal CAP if blood culture, induced-sputum culture or Gram stain, urine antigen test, or whole-blood *lytA* real-time PCR revealed pneumococcus or if *lytA* real-time PCR from NPSs gave a result of >8,000 copies/ml. The *lytA* real-time PCR was also performed on induced sputum. The *lytA* real-time PCR from sputum detected *S. pneumoniae* in 149 (67.1%) of 222 patients with available induced sputum, whereas the results of either Gram stain or culture of sputum were positive in 105 of 229 patients (45.9%; $P < 0.001$). Against the composite diagnostic standard, a cutoff value of 10,000 copies/ml for good-quality sputum *lytA* real-time PCR had a sensitivity of 78.1% and a specificity of 80.0%. This cutoff value performed similarly to the previously identified cutoff value of 8,000 copies/ml for NPS *lytA* real-time PCR (64). Overall, *lytA* real-time PCR performs similarly well on induced sputum as on NPSs for most patients but performs slightly better if good-quality sputum can be obtained (64).

Abdeldaim (53) et al. compared the performance of RQ-PCR targeting the Spn9802 sequence to *lytA* PCR and culture on NPAs from 166 CAP patients and from 84 controls. Against an expanded gold standard, sensitivities and specificities for Spn9802 PCR were 94% and 98%, respectively, and those for *lytA* PCR were 82% and 97%, respectively.

Sputum and NPAs, on the one hand, and PCR targets *lytA* and Spn9802, on the other hand, were compared in a quantitative PCR for the rapid detection of pneumococcal pneumonia by Stralin et al. (65). The following RQ-PCR positivity rates were found in CAP patients with and without pneumococcal etiology according to conventional diagnosis: 96% and 15% (sputum *lytA*), 96% and 17% (sputum Spn9802), 81% and 11% (NPA *lytA*), and 81% and 20% (NPA Spn9802). The best-performing test, the sputum *lytA* RQ-PCR, showed the highest sensitivity (94%) and specificity (96%), with a cutoff value of 10^5 DNA copies/ml.

The detection of *S. pneumoniae*-specific targets by real-time PCR assays, such as Spn9802 or *lytA* in plasma, is also useful for the rapid detection of bacteremic pneumococcal pneumonia (7). Detection of bacterial DNA load in whole blood supports the diagnosis of *S. pneumoniae* infection in patients with CAP (66). Bacterial load is associated with the likelihood of death, the risk of septic shock, and the need for mechanical ventilation (67, 68). High genomic bacterial load for *S. pneumoniae* may be a useful tool for severity assessment.

A new promising trend is the application of MX-PCR for the identification and detection of markers that identify specific serotypes of *S. pneumoniae* in clinical specimens. This approach was found to be successful when applied to sterile specimens such as blood (69, 70) or pleural fluid (71–73). The applied assays were sensitive; e.g., pneumococcal DNA was detected in 87.5% of culture-negative pleural fluid (73) and in 97% of culture-positive pleural fluid (72). A similar strategy was applied to nasopharyngeal samples (74–77) from patients and healthy individuals. The assay detected all culture-positive samples and 22/23 serotypes matched with the serotypes identified by the conventional method (74) from pneumonia patients. An MX-PCR was also evaluated against culture on 793 nasopharyngeal samples from children with LRTI by Ahn et al. (78). In this study, 19.3% of the samples were found to be *S. pneumoniae* positive by MX-PCR compared to 10.2% positives by conventional techniques. A recent review presents additional data on the laboratory diagnosis of *S. pneumoniae* pneumonia (79).

In conclusion, *S. pneumoniae* may be rapidly diagnosed by analyzing sputum by RQ-PCR and may be particularly valuable in patients in whom antibiotic therapy was initiated before sampling. Furthermore, it would be able to distinguish between colonization and infection. RQ-PCR warrants further evaluation in clinical settings.

M. pneumoniae

Among atypical infections, *M. pneumoniae* is the most common, followed in frequency by *Chlamydophila* spp. and *Legionella*. *M. pneumoniae* belongs to the class of the *Mollicutes* and has been associated with a wide variety of acute and chronic diseases. RTIs with *M. pneumoniae* occur worldwide and in all age groups.

The proportion of LRTIs, including CAP, in children and adults associated with *M. pneumoniae* infection during the past 10 years has ranged from 0% to more than 50%, varying with age and the geographic location of the population examined and the diagnostic methods used. Determining the true role of *M. pneumoniae* in RTIs remains a challenge, given the wide variations of data from studies with equally wide variation of and lack of standardized diagnostic methods.

Serological methods in particular, such as the complement fixation test and enzyme immunoassays, are the most widely used to diagnose an *M. pneumoniae* infection. The application of PCR is increasingly accepted as a rapid diagnostic test since culture is too slow and too insensitive to be therapeutically relevant. None of the currently available NAATs have been extensively validated against culture, which remains the reference standard despite its low sensitivity and variable yield, depending on the specimens tested and the isolation protocols used. The sensitivity of NAATs is almost always superior to that of the traditional

procedures, and they are increasingly considered as the new gold standard. However, different studies have used not only different diagnostic tools or combinations thereof, but also different diagnostic criteria for diagnosing an infection, thereby making comparison between studies difficult. Most importantly, the lack of standardization has resulted in a wide variation of interlaboratory test performance, even when using the same test and criteria (80).

A growing body of literature describing the use of in-house NAATs for detecting *M. pneumoniae* DNA or RNA in various diseases is now available with a great variation of methods used from study to study, including variability of target (P1 gene, 16S rRNA, ATPase gene, *parE* gene, *tuf* gene; monoplex versus multiplex targets) and of NAAT (conventional, nested, and real-time; RNA versus DNA targets; and PCR and NASBA technologies) and detection formats (agarose gel electrophoresis, SYBR green, TaqMan probe, hybridization probes, molecular beacons, and microchip electrophoresis).

An overview of the literature on the use of NAATs to detect *M. pneumoniae* since 1989 is given in two reviews by Loens et al. (81, 82) with a description of the currently available molecular amplification methods. Topics discussed include specimen collection and transport, preparation of nucleic acid from clinical specimens, choice of the target sequence, and detection of the amplicons. Methods to recognize and prevent false-positive and false-negative results, the results of NAATs compared with results obtained by conventional diagnostic tests, and clinical applications are also reviewed. The availability of the very sensitive NAATs has in recent years also put the often-used serological tests in perspective and allows a better interpretation of the serological test results and their limitations, such as the low sensitivity of IgM antibodies in acute-phase specimens and the importance of the delay between two serum samples. The clinical significance of a serologic test, for both IgM and IgG, should be defined by studies of patients with a documented infection and for whom detailed information concerning the time lapse between onset of disease and the collection of the serum specimens is known. Studies in which NAATs are also used on respiratory specimens allow a better interpretation of the serological test results.

Data analysis of different studies indicates that no single available test was sensitive enough for the identification of *M. pneumoniae* in CAP. A combination of serology and PCR proved to be the most reliable approach for identifying *M. pneumoniae* in CAP.

Data from recent studies using PCR-based methods and serology published during the last 5 years in different patient populations from around the world are summarized in a recent review by Ieven and Loens (83), and the role of both techniques is discussed.

Since the previous review (285), new tests have become commercially available, such as the Illumigene kit (84) (Table 2). A new trend is the simultaneous detection of the organism and mutations associated with macrolide resistance directly in clinical specimens (85, 86).

C. pneumoniae

C. pneumoniae, an obligate intracellular bacterium, has been associated with a variety of diseases but most importantly with respiratory infections that occur worldwide and in all age groups. Studies of *C. pneumoniae* published since the 1990s declare the organism to be associated with less than 5% to up to more than 40% of LRTI in both children and adults, varying with age, geographical location, the population studied, and especially the diagnostic methods used.

For the detection of this organism as well, PCR holds promise as a diagnostic tool. The major outer membrane protein gene and 16S rRNA gene are often used targets for amplification.

Most importantly, among the early studies published, none of the available methods have been standardized, which has resulted in a wide variation of interlaboratory test performance. In an effort to standardize diagnostic assays for *C. pneumoniae*, Dowell et al. (87) published recommendations for diagnostic testing in 2001. In 2007, Kimar et al. (88) published an excellent overview of the current status of diagnostic methods for *C. pneumoniae* since the Dowell publication. They conclude that significant limitations remain in the accurate and reliable diagnosis of *C. pneumoniae*, and the availability of commercial assays may enable standardization. However, they await further validation: the sensitivity of all these assays is dependent on DNA copy numbers, and further evaluations on clinical specimens that may have low bacterial loads are needed. In addition, for acute LRTI with *C. pneumoniae* a combination of PCR detection and specific single-serum IgM measurement seems to be called for.

A multicenter prospective study investigating the incidence of *C. pneumoniae* infections in adult ambulatory (n = 182) and hospitalized (n = 364) patients with CAP in the German Competence Network for Community Acquired Pneumonia (CAPNETZ) Project used microimmunofluorescence (MIF) testing to determine single IgM, IgG, or IgA titers, as well as three previously published PCR protocols (89). Significant variation in PCR results was reported among the three laboratories, each independently experienced in the use of PCR assays. Via the use of PCR positivity in at least two of the three laboratories to define acute infections, *C. pneumoniae* infections were identified in 5 (0.9%) of 546 case patients. MIF tests were performed for three of these five case patients, and all results were negative for anti-*C. pneumoniae* IgG, IgM, or IgA antibodies. These data confirm the lack of correlation of serological methods with culture and/or PCR tests reported earlier (90).

Hvidsten et al. (91) studied the importance of methodology in relation to timing of sampling for detecting *C. pneumoniae*. Nasopharyngeal swabs for PCR-based detection and sera for single IgM and IgG seroconversion by MIF were obtained from 127 military conscripts during a *C. pneumoniae* outbreak. Serum drawn many months before the outbreak provided the baseline antibody status. The sensitivities were 79%, 85%, 88%, and 68%, respectively, and the specificities were 86%, 84%, 78%, and 93%, respectively, for MIF IgM, enzyme immunoassay IgM, recombinant-enzyme-linked immunosorbent assay IgM, and PCR. In two subjects, acute infection was diagnosed on the basis of IgG seroconversion alone. The lower sensitivity of PCR detection was explained by the late sampling or clearance of the organisms following antibiotic therapy and the timing of sampling for the different test principles used. On the basis of their findings, the authors recommend a combination of nasopharyngeal swabbing for PCR and specific single-serum IgM measurement. Benitez et al. assessed the performance of real-time PCR and MIF for the detection of *C. pneumoniae* during an outbreak (92). They concluded that (i) application of serology alone would likely have been misleading since the rate of seropositivity

TABLE 2 Summary of recent single-plex PCR assays for detection of *M. pneumoniae*, *C. pneumoniae*, and *B. pertussis* published since the previous review and previously validated assays used as comparators[a]

Assay year (reference)	Assay type	Detection format	Gene target (product size)	PCR assay used as comparator for new assay	Non-PCR comparator test	Specimens tested for validation of sensitivity and/or specificity
M. pneumoniae						
2009 (246)	PCR	Real-time	P1 gene (342)	Affigene Cp/Mp Tracer	IgM and IgG	50 BAL specimens
2011 (247)	PCR	Oligochromatographic test	P1 gene (NS)	Cepheid	IgG seroconversion/ significant rise	Samples from 145 patients
2012 (248)	PCR	Real-time	P1 gene (72)	repMp1 and Mp181		Various bacterial species, bacterial dilution series, well-defined clinical specimens
2012 (249)	LAMP	Turbidity	P1 operon		IgG seroconversion/ significant rise	Samples from 368 pneumonia patients
2013 (250)	PCR	Real-time	P1 gene (534)	Conventional PCR (NS)	IgM, IgG, and IgA serology	Dilution series, respiratory samples from CAP patients
2013 (251)	PCR	Real-time	ptsI (160)	LightMix kit *M. pneumoniae* (TIB MOLBIO), *M. pneumoniae* analyte-specific reagent (Focus Diagnostics) (252)		Bacterial dilution series, spiked clinical specimens, well-defined clinical specimens
2013 (253)	PCR	Quantitative fluorescence	16S rDNA (Daan Gene kit) (NS)		IgG and IgM serology, culture	Specimens from CAP patients
2014 (86)	PCR	Cycleave	23S rDNA	(254)		Various bacterial species, bacterial dilution series, clinical specimens
2014 (84)	LAMP	Turbidity	Illumigene assay (NS)	2nd real-time PCR and sequencing	Culture	Various bacterial species, bacterial dilution series, 214 culture positive/ negative specimens
C. pneumoniae						
2009 (246)	PCR	Real-time	Polymorphic membrane protein G (140)	Affigene Cp/Mp Tracer	MIF	50 BAL specimens
2011 (255)	PCR	Real-time	16S rDNA (207–215)	(206)		Various bacterial species, plasmid dilution series, well-defined archived specimens, 442 prospectively collected nasopharyngeal swabs if positive confirmed by sequencing
B. pertussis						
2010 (256)	PCR	Dipstick	GenoQuick *Bordetella* assay			Clinical specimens from suspected cases
2012 (257)	PCR	Real-time	NS	(258)	Culture	Clinical specimens from suspected cases

[a]Abbreviations: A-test, passive agglutination test; LAMP, loop-mediated isothermal amplification.

among noncases was extremely high and (ii) real-time PCR is a more useful diagnostic tool for confirmation of a *C. pneumoniae* infection.

Padalko et al. (93) analyzed the yield of PCRs for the detection of *C. pneumoniae* in respiratory specimens. Their data were based on routine analysis of respiratory samples submitted for *C. pneumoniae* detection collected in four large Belgian hospitals during 2 consecutive years. Only 0.2% of the 3,560 samples were found to be *C. pneumoniae* positive. The authors concluded that a critical evaluation of *C. pneumoniae* as a cause of respiratory infections is needed.

Since our review in the 2nd edition of this volume (285), only a few new assays have been added (Table 2).

L. pneumophila

Legionella spp. are aerobic, nutritionally fastidious Gram-negative rods. *Legionella* is commonly found in cooling towers, humidifiers, and potable water distribution systems. Since the organism was first identified in 1976 during an outbreak at an American Legion Convention in Philadelphia, *Legionella* has been recognized as a relatively common cause of both community-acquired and hospital-acquired pneumonia, with *L. pneumophila* causing more than 90% of the cases of Legionnaires' disease. Legionnaires' disease is a form of bacterial pneumonia that is characterized by fever, chills, and a dry cough associated with muscle aches and occasional diarrhea. Severity ranges from mild to fatal, with an average mortality rate of 15%, especially observed in immunocompromised patients.

Serologic tests, although often used, can never offer an early diagnosis of legionellosis. *Legionella* antibody tests have a sensitivity of 61 to 64% depending on the assay applied and do not substantially improve the diagnosis of legionellosis (94). They therefore are an epidemiological rather than a diagnostic tool.

NAATs enable specific amplification of minute amounts of *Legionella* DNA. NAATs also have the potential to detect infections caused by any *Legionella* species or serogroup, as presented in Table 3. Diagnostic PCR assays have principally targeted specific regions within the 16S rRNA gene, the 23S-5S spacer region, 5S rDNA, or the *mip* gene (Table 3). Thus far, encouraging results obtained mostly from *in vivo* evaluations and small patient series have been reported. A new commercial test (BD ProbeTec ET *L. pneumophila*; Becton Dickinson) that detects *L. pneumophila* serotypes 1 to 14 in sputum is now cleared by the FDA, but published data on performance characteristics are lacking.

The added value of real-time PCR for diagnosis of Legionnaires' disease in routine clinical practice was studied by Diederen et al. (95). Patients were evaluated if, in addition to PCR, the results of at least one of the following diagnostic tests were available: (i) culture for *Legionella* spp. on specific media and (ii) detection of *L. pneumophila* antigen in urine specimens. Of the 151 evaluated patients, 37 (25%) fulfilled the European Working Group on *Legionella* Infections criteria. An estimated sensitivity and specificity of 86% and 95% were found for 16S rRNA-based PCR, and corresponding values of 92% and 98%, respectively, were found for the *mip* gene-based PCR. A total of 35 patients were diagnosed by using the urinary antigen test, and 34 were diagnosed by the 16S rRNA-based PCR. With the combined urinary antigen test and *mip* gene PCR, Legionnaires' disease was diagnosed in four more patients than with the use of the urinary antigen test alone.

The CAPNETZ project also studied the incidence, clinical characteristics, and outcome of *Legionella* pneumonia in 2,503 adult patients with CAP (96). The following tests were used: culture on selective media, genus-specific PCR targeting the 16S rRNA gene, and urinary antigen tests. Unfortunately, not all specimens were available from all patients. Respiratory specimens were available for 35% of patients. *Legionella* pneumonia was diagnosed in 94 patients (3.8%) and was found to be equally common among ambulatory and hospitalized patients. The predominant species was *L. pneumophila*. Diagnosis of a *Legionella* infection was based on a positive culture result for *L. pneumophila* in 3 patients, a positive PCR result from respiratory samples in 52 patients, and/or a positive urinary antigen test result in 48 patients. Of these, culture and PCR were positive in two patients, culture and urinary antigen test in one patient, PCR and urinary antigen test in six patients, and culture, PCR, and urinary antigen test in one patient.

The influence of antimicrobial therapy on the sensitivity of *Legionella* DNA detection by PCR in LRT samples was studied in three cases (97). The data presented by Korosec et al. suggest that within a few days, specific antimicrobial therapy induces a significant decrease of the bacterial

TABLE 3 Summary of recent single-plex PCR assays for detection of *Legionella*, published since the previous review, and previously validated assays used as comparators[a]

Assay year (reference)	Assay type	Detection format	Gene target (product size)	PCR assay used as comparator for new assay	Non-PCR comparator test	Specimens tested for validation of sensitivity and/or specificity
2009 (259)	PCR	Real-time	23S-5S rDNA spacer (NS)		Culture	Various bacterial species, DNA dilution series, clinical specimens
2011 (107)	PCR	Real-time	Sg1 (294)	iQcheck Quanti Legionella (Bio-Rad)		Various bacterial strains, samples from confirmed legionellosis cases
	qPCR	Real-time	Sg1 (75)			
2011 (260)	PCR	Agarose gel electrophoresis	ompS (849)		Ag ELISA	DNA dilution series
2011 (261)	PCR	Agarose gel electrophoresis	gyrB (224)			Various bacterial species, DNA dilution series
2012 (106)	qPCR	Real-time	mip (NS)		Culture, uAg	Various bacterial species, DNA dilution series, clinical samples

Abbreviations: ELISA, enzyme-linked immunosorbent assay; ICT-strip, immunochromatography; qPCR, quantitative PCR; uAg, urinary antigen test.

concentration in respiratory specimens, reaching the detection limit of the assay. Similarly, Diederen et al. (98) quantified *Legionella* DNA in serum from two patients with legionellosis by real-time PCR during the course of illness. The results obtained mirrored both the clinical condition and C-reactive protein values during the course of illness.

Legionella DNA can be detected in urine, serum, and leukocyte samples obtained from patients with Legionnaires' disease with sensitivities varying between 30 and 86% (99–104). Although the sensitivity of the detection of *Legionella* DNA in serum is rather low (50 to 60%), sensitivity might be higher in more severe cases (99, 100). This issue needs further study.

Recently quantitative assays have been developed and validated for the detection of *L. pneumophila* and/or *Legionella* spp. in clinical specimens (105–107). By applying RQ-PCR to respiratory specimens from patients admitted to the hospital, Maurin et al. demonstrated that a high bacterial load was significantly associated with higher Fine classes (based on the presence and severity of five symptoms [cough, fatigue, dyspnea, sputum, and chest pain] (108), a need for hospitalization, and admission to an intensive care unit and prolonged hospitalization (105). Mentasti et al. applied RQ-PCR to serum, urine, and respiratory specimens from confirmed cases (106). All *L. pneumophila* culture-positive respiratory specimens were also positive with RQ-PCR. In addition, of 80 culture-negative samples tested, 47 were positive by quantitative PCR. Furthermore, the authors showed that RQ-PCR was far more sensitive than culture for samples taken at ≤2 days of hospitalization (94.7% versus 79.6%), with the difference being even more marked for samples taken between 3 and 14 days. The bacterial load was high but varied significantly in positive culture samples. The quantity of DNA in negative culture specimens was significantly lower. When applied to serum, the quantity of bacterial DNA was generally low. None of the urine samples was found to be *L. pneumophila* positive. An overview of newly described assays is presented in Table 3.

B. pertussis

B. pertussis, a small Gram-negative bacterium, is responsible for the vast majority of pertussis or "whooping cough" cases; *Bordetella parapertussis* causes a mild pertussis-like illness. Other species, including *Bordetella bronchiseptica*, may infrequently cause respiratory infections in humans, mostly in patients who are immunocompromised. In the present vaccine era, the overall incidence of pertussis has been reduced dramatically.

While pertussis has generally been considered an infection of children associated with considerable morbidity and mortality, it has been increasingly recognized over the past 2 decades in adults. The increase in reported pertussis over the last 2 decades is mainly due to a greater awareness of pertussis and perhaps to the use of several less efficacious vaccines.

The use of a *B. pertussis*-specific PCR in combination with single-serum serology has been shown to increase the sensitivity for pertussis diagnosis. Recommendations for the use of PCR in the diagnosis of pertussis have been published previously (109). Since then, real-time PCR formats have emerged and are now widely used. Block-based PCR (110) and real-time PCR (Table 4) are generally more sensitive than culture for the detection of *B. pertussis* and *B. parapertussis*, especially in the late stage of the disease and after antibiotic treatment has started (111, 112). Similar to culture, the sensitivity of PCR decreases with the duration of cough; however, due to its higher sensitivity, it may be a useful tool for diagnosis not only for the first 3 to 4 weeks of coughing, but even longer (113).

A frequently used target for PCR detection of *B. pertussis* is the repetitive element IS*481* (Table 4), which is absent in *B. parapertussis* but found in approximately 50 to a few hundred copies in *B. pertussis* (114, 115). Although IS*481* is generally regarded as specific for *B. pertussis*, a few studies detected it also in *B. bronchiseptica*. Although the latter organism is primarily associated with disease in mammals other than humans, human illness and carriage are on the rise, particularly in infants or immunocompromised hosts with exposure to carrier animals (116–121). While rare, disease in immunocompetent adults may also occur. A review of the literature covering PCR methods used to identify *B. pertussis* reveals that "diagnostic" amplicons were obtained from *B. bronchiseptica* isolates in at least five investigations using primers for the 5′ end of the repetitive element (114, 122–125) but by none using primers targeting the 3′ end (126–129). Register et al. (130) reported that the prevalence of IS*481* in *B. bronchiseptica* is approximately 5% and suggested that PCR targeting IS*481* may not be sufficiently specific for reliable identification of *B. pertussis*. There has also been concern about the specificity of detection of *B. pertussis* due to sequence identity with *Bordetella holmesii* (131). Register et al. (132) also reported that the *B. pertussis* pertactin gene sequence for the region that encompasses the RT-PCR primers and probe described by Vincart (133) is nearly identical to that of many *B. bronchiseptica* strains of human and avian origin. By using the Vincart assay, 4/6 *B. bronchiseptica* strains were falsely identified as *B. pertussis*.

Although the pertussis toxin operon is present in *B. pertussis*, *B. parapertussis*, and *B. bronchiseptica*, the pertussis toxin promoter is a target for *B. pertussis*-specific assays using real-time PCR. It was, however, consistently less sensitive than IS*481* (134). Some reference laboratories use both targets.

During a pertussis outbreak, a multitarget real-time PCR (135) for the detection of *B. pertussis* was evaluated targeting IS*481*, IS*1001*, and an IS*1001*-like element as well as pertussis toxin subunit S1 (136). All patients with an IS*481* Ct of <30 were also positive by the ptxS1 assay and clinical pertussis cases. No patients with IS*481* Ct values of ≥40 tested positive by culture.

The EUVAC.NET network evaluated the performance of PCR tests used across Europe in 2011 for the detection of *B. pertussis* (137). The participants were 24 national reference centers from 19 countries. A wide variation in methods used for PCR-based diagnosis was found. Several laboratories were not able to discriminate between DNA samples from different *Bordetella* spp.

Four commercial real-time PCR assays for the detection of *Bordetella* spp. in NPAs (Simplexa *B. pertussis*/*B. parapertussis* [Focus Diagnostics], the SmartCycler *B. pertussis*/*parapertussis* assay [Cepheid], the *Bordetella* R-gene [Argene], and the *B. pertussis* real-time kit [Shangai ZJ Bio-Tech]) were compared with real-time in-house PCR by Lanotte et al. (138). Sensitivities over 90% were obtained for the former three kits, whereas the kit from Shangai ZJ Bio-Tech proved to be unsuitable for routine clinical use unless significant improvements are made.

An overview of pertussis diagnostics was provided in 2014 by Wirsing von König (139). An overview of assays published since the previous review is given in Table 2.

TABLE 4 Summary of recent in-house multiplex PCR assays for detection of respiratory pathogens, published since the previous review (285)

Assay year (reference)	Assay type	Detection format	Specimens tested for validation of sensitivity and/or specificity
2009 (262)	MX-PCR	Liquid array	Various bacterial species, DNA dilution series, clinical specimens Pathogens targeted: *Streptococcus pneumoniae*, *Neisseria meningitidis*, *Escherichia coli*, *Staphylococcus aureus*, *Listeria monocytogenes*, *Streptococcus agalactiae*, herpes simplex virus type 1 and 2, varicella zoster virus
2009 (263)	MX-PCR	Real-time	Clinical specimens from patients with uAg test available Pathogens targeted: *S. pneumoniae* pneumolysin and autolysin gene
2010 (264)	MX-qPCR	Real-time	Various bacterial species, DNA dilution series, well-defined clinical samples Pathogens targeted: *S. pneumoniae*, *Haemophilus influenzae*, *N. meningitidis*
2010 (265)	MX-PCR	Microarray	Viral/bacterial dilution series, clinical samples analyzed by a second PCR Pathogens targeted: HAdV, enterovirus, HBoV, hMPV; HCoV 229E, OC43, HKU1; influenza A, influenza B, influenza C, PIV1-4, RSV A/B, rhinovirus, *Chlamydophila pneumoniae*, *Mycoplasma pneumoniae*
2010 (266)	MX-PCR	Microarray	Various bacterial species, plasmid DNA dilution series, 14 sputa analyzed by a second PCR Pathogens targeted: *S. pneumoniae*, *Streptococcus pyogenes*, *Moraxella catarrhalis*, *Haemophilus* spp., *C. pneumoniae*, *M. pneumoniae*, *Legionella pneumophila*, *Coxiella burnetii*, *Pseudomonas aeruginosa*
2010 (267)	MX-PCR	Real-time	Various bacterial species, bacterial dilution series, clinical specimens Pathogens targeted: *Bordetella pertussis* and *Bordetella parapertussis*
2011 (268)	MX-PCR	Real-time	*Bordetella* species, bacterial dilution series, clinical specimens Pathogens targeted: *B. pertussis*, *B. parapertussis*, *Bordetella holmesii*, *Bordetella bronchiseptica*
2011 (135)	MX-PCR	Real-time	Various bacterial strains, bacterial dilution series, spiked specimens, clinical specimens also analyzed by culture and ptxS1 PCR
2011 (269)	MX-PCR	Real-time	Various bacterial species, bacterial dilution series, clinical specimens Pathogens targeted: *S. pneumoniae*, *Burkholderia pseudomallei*
2011 (261)	MX-PCR	Agarose gel electrophoresis	Various bacterial species, DNA dilution series Pathogens targeted: *Legionella* spp.
2011 (270)	MX-PCR	Real-time	Various bacterial species, clinical specimens Pathogens targeted: influenza A, influenza A H1, influenza A H3, influenza B, RSV, PIV 1-3, hMPV, rhinovirus, enterovirus, parechovirus, HAdV, *Legionella* spp., *L. pneumophila*, *H. influenzae*, *S. pneumoniae*, *S. pyogenes*, *M. pneumoniae*, *C. pneumoniae*, *B. pertussis*
2011 (269)	MX-PCR	Real-time	Various bacterial species, bacterial dilution series, clinical specimens Pathogens targeted: *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*
2012 (271)	MX-LAMP	Turbidity and digestion	Various bacterial species, bacterial dilution series Pathogens targeted: *S. pneumoniae*, *Streptococcus suis*, *S. agalactiae*, *S. aureus*
2012 (272)	MX-PCR	Real-time	Various respiratory pathogens, positive control dilution series, clinical specimens Pathogens targeted: influenza and RSV
2012 (273)	MX-PCR	Mass spectrometry	Pathogens targeted: RSV, influenza A and B; PIV 1-4; hMPV; HAdV; HCoV NL63, HKU1, 229E, and OC43; HBoV
2012 (274)	MX-PCR	Capillary electrophoresis	Various respiratory pathogens, DNA dilution series, clinical specimens Pathogens targeted: influenza A and B; influenza A H1N1; PIV 1-3; rhinovirus; hMPV; HAdV; RSV; HCoV OC43, 229E, NL63, and HKU1; HBoV
2012 (275)	MX-PCR	Agarose gel electrophoresis	Various bacterial species, DNA dilution series, well-defined sputa Pathogens targeted: *S. pneumoniae*, *H. influenzae* type b, *Mycobacterium tuberculosis*
2013 (276)	MX-PCR	ELISA	Culture supernatants of the organisms, clinical specimens from frozen stocks, prospectively included nasopharyngeal aspirates

(*Continued on next page*)

TABLE 4 Summary of recent in-house multiplex PCR assays for detection of respiratory pathogens, published since the previous review (285) (*Continued*)

Assay year (reference)	Assay type	Detection format	Specimens tested for validation of sensitivity and/or specificity
			Pathogens targeted: enterovirus, influenza A, influenza B, RSV, PIV 1-4, HAdV, rhinovirus, hMPV, HCoV, reovirus, *M. pneumoniae*, *C. pneumoniae*, *B. pertussis*, *B. parapertussis*, *L. pneumophila*
2013 (277)	MX-PCR	Affimetrix Chip-image file	Clinical specimens simultaneously investigated by culture and 2 commercially available assays: the Eragen assay and the Luminex RVP
			Pathogens targeted: 72 pathogens
2013 (170)	MX-PCR	Microarray	Well-defined clinical specimens analyzed by individual real-time PCRs
			Pathogens targeted: HAdV; hMPV; PIV1-4; influenza A; influenza B; influenza C; RSV; rhinovirus; HCoV OC43, 229E, NL63, and HKU1; enterovirus; *B. pertussis*; *C. pneumoniae*; *H. influenza*; *L. pneumophila*; *M. pneumoniae*; *S. pneumoniae*; *S. pyogenes*
2013 (278)	MX-PCR	Luminex technology	Reference material, dilution series of positive specimens, 1,065 respiratory specimens analyzed by a second PCR
			Pathogens targeted: influenza A, influenza B, influenza A H3N2, influenza H1N1, and pandemic influenza A H1N1
2013 (279)	MX-PCR	Real-time	Various respiratory viruses, reference materials, *in vitro* RNA dilution series, well-defined clinical specimens, clinical specimens analyzed by second PCR
			Pathogens targeted: influenza A; influenza B; influenza A H1 seasonal; influenza A H1N1; influenza A H3; RSV A/B; hMPV; PIV1-4; rhino/enterovirus; HCoV OC43, HKU1, NL63, and 229E
2013 (280)	MX-PCR	Electrophoresis	Various respiratory viruses, dilution series of RNA transcripts, dilution series of plasmids, analysis by two commercially available assays (Luminex RVP Fast and Seeplex RV15 ACE)
			Pathogens targeted: influenza A; influenza B; influenza A H1N1; PIV1-3; rhinovirus; hMPV; HAdV; RSV A/B; HBoV; HCoV NL63, OC43, 229E, and HKU1
2013 (233)	MX-PCR	Dipstick	Various respiratory viruses, virus dilution series, well-defined clinical specimens
			Pathogens targeted: influenza A and B
2013 (281)	MX-PCR	Agarose gel electrophoresis	Various bacterial species, DNA dilution series, well-defined clinical specimens
			Pathogens targeted: *S. pneumoniae*, *Streptococcus mitis*, *Streptococcus oralis*
2013 (282)	MX-PCR	Real-time	Various bacterial species, bacterial dilution series, well-defined clinical specimens
			Pathogens targeted: *S. pneumoniae*, *N. meningitidis*, *H. influenzae*, *S. agalactiae*, *L. monocytogenes*, *Cryptococcus neoformans*
2014 (283)	MX-PCR	Real-time	Various bacterial pathogens, bacterial dilution series, spiked specimens, well-defined clinical specimens
			Pathogens targeted: *B. pertussis*, *B. parapertussis*
2014 (284)	MX-PCR	Real-time	DNA dilution series, well-defined clinical specimens from CAP patients
			Pathogens targeted: *S. pneumoniae*, *H. influenza*, *M. catarrhalis*, *P. aeruginosa*, *Klebsiella pneumoniae*, *E. coli*, *S. aureus*, *M. pneumoniae*, *C. pneumoniae*, *Chlamydophila psittaci*, *C. burnetii*, *Legionella* spp., *L. pneumophila*, *B. pertussis*, *M. tuberculosis*, *Mycobacterium intracellulare*, *Mycobacterium avium*, *Mycobacterium kansasii*, *Pneumocystis jeroveci*, *Nocardia* spp., metallo-β-lactamase, MRSA
2014 (85)	MX-PCR	Agarose gel electrophoresis	Various bacterial pathogens, bacterial dilution series, confirmation by sequencing, confirmation by second PCR, well-defined clinical specimens
			Pathogens targeted: *M. pneumoniae* and associated macrolide resistance

[a]Abbreviations: A-test, passive agglutination test; ELISA, enzyme-linked immunosorbent assay; HAdV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus, hMPV, human metapneumovirus; MRSA, methicillin-resistant *S. aureus*; PIV, parainfluenza virus; MX-PCR, multiplex PCR; NASBA, nucleic acid sequence-based amplification; RSV, respiratory syncytial virus; uAg, urinary antigen test.

Bidet et al. (140) measured the persistence of *B. pertussis* DNA in NPAs of 21 infants treated for pertussis. After 5 days of treatment, PCR was positive for all 21 patients. After 14 and 21 days, PCR was still positive for 83% and 66%, respectively, of assessable patients. One patient was tested 1 month after treatment initiation, and *B. pertussis* DNA was still detectable. Quantitative analysis showed that the DNA concentration diminished during treatment in all except one case. The authors concluded that real-time PCR can be used to diagnose pertussis even 3 weeks after treatment initiation in infants. However, whether or not this applies to older children and adults as well remains to be investigated. Furthermore, serial real-time PCR might be useful for the prediction of treatment failure and comparison of efficacies of different antibiotics, although further studies are needed.

Palmer et al. analyzed PCR positivity and duration of illness in children less than 5 years of age (141) and found that PCR positivity mirrored the understood length of infectivity with regard to both catarrhal symptoms and paroxysmal cough, namely, that positive PCR results were obtained at least 21 days following onset of catarrhal symptoms and at least 14 days following onset of paroxysmal cough.

NAAT FOR INDIVIDUAL VIRAL RESPIRATORY PATHOGENS

The importance of viruses as causal agents has been confirmed in LRTI. In CAP the most common etiological agents after *S. pneumoniae* are respiratory viruses, which are involved in 5 to >30% of infections (8). A multitude of reports have appeared on the epidemiology of viruses in acute respiratory infection (ARI), but most are restricted to a few viruses (influenza, sometimes together with RSV, and rhino-, metapneumo-, or coronaviruses) and/or to some population groups, e.g., children, adults, or the elderly (142–149). Great variations occur in function of time, place, and the age groups studied.

Influenza viruses consist of three different types: A, B (150), and C (151). Within influenza A, distinct subtypes determined by the hemagglutinin and neuraminidase genes can be distinguished. Influenza A causes annual epidemics, the epidemic period varying usually between 3 and 8 weeks. Pandemics can occur after the emergence of a novel virus subtype as a result of genetic reassortment of hemagglutinin and neuraminidase genes occurring when two different subtypes coinfect the same host (152).

Influenza is a febrile illness characterized by a sudden increase in temperature, headache, malaise and myalgia, and respiratory symptoms such as sore throat and cough. During the seasonal epidemics, influenza infections are responsible for a significant number of hospitalizations each year; people over the age of 65 are most at risk, followed by children under the age of 5.

RSV is the most common cause of viral LTRI in very young children and is the major cause of bronchiolitis and pneumonia in infants below 2 years of age (153). Two subtypes can be distinguished: groups A and B, of which RSV A seems to be responsible for the most severe infections. Disease often begins with upper respiratory symptoms but rapidly progresses to bronchiolitis or pneumonia with wheezing and respiratory distress. Not only in very young children but also in the elderly, RSV is now commonly associated with LRTI and pneumonia (146, 154–156). Peak prevalences of RSV infections are usually seen in the winter between December and March.

Parainfluenza viruses are divided into four serotypes (HPIV-1 to HPIV-4) that are all capable of causing respiratory infections in humans (157–159). HPIV-1 is the most important etiologic agent in croup in very young children but can also be responsible for milder RTIs. HPIV-2 has also been associated with croup, but less frequently than HPIV-1. HPIV-3 is more associated with severe LRTI. HPIV-4 is the least common virus of this group. Parainfluenza viruses occur any time of the year, but small outbreaks can be observed in autumn, mostly due to HPIV-1, and spring, mostly due to HPIV-3.

Rhinoviruses, with more than 200 serotypes, have until recently only been associated with the common cold. However, they are now detected with an increasing importance and have been associated with asthma exacerbations in both children and adults, wheezing, acute respiratory distress, serious LRTI, and even CAP in children, the elderly, and immunocompromised patients (160–164).

Adenoviruses, of which at least 51 serotypes have been described, can cause a variety of infections ranging from respiratory infections to conjunctivitis, keratoconjunctivitis, and gastroenteritis. The most common serotypes involved in human respiratory infections are serotypes 1 to 5, which cause mostly mild symptoms, but more severe disease such as bronchiolitis or even pneumonia may also occur occasionally, especially in pediatric transplant patients (165–168).

Coronaviruses are also more prevalent than previously thought (169, 170). In 2012, an emerging coronavirus, Middle East respiratory syndrome coronavirus (MERS-CoV), was reported to cause respiratory infections in humans. Cunha et al. published a recent review on this new virus (171). As time progresses, the importance of the more recently discovered human bocavirus, human metapneumovirus infections, and new coronaviruses is becoming more evident. Based on signs and symptoms, human metapneumovirus is very similar to RSV, causing both URTI and LRTI, especially bronchiolitis but also pneumonia, most frequently in very young children (172, 173). The peak incidence of human metapneumovirus infections usually follows RSV, but both peaks overlap frequently. Although the role of some of these new viruses becomes more clear in specific patient populations, more studies are needed to identify the clinical relevance of some others such as bocavirus (174, 175).

Antigens of the most common respiratory viruses such as influenza, RSV, adenovirus, and parainfluenza viruses can be detected by direct immunofluorescence or by commercially available enzyme immunoassays. The sensitivities of these tests vary from 50 to >90%, and the sensitivity of the direct immunofluorescence test is lower in adults than in children. Culture procedures for other viruses and fastidious bacteria are too insensitive and are superseded by NAATs, preferably in real-time formats and in combination with automated nucleic acid extraction. This approach allows laboratories to obtain a result within 4 to 5 hours.

Multiple protocols are more frequent for virus groups that contain multiple virus types. Several amplification protocols were developed to cover particular types or groups among the adenoviruses. More than 200 rhinovirus types and their close relationship with enteroviruses constitute a special challenge. The judicious choice of primers and particularly of the hybridization probes should ensure a

satisfactory coverage of the rhinovirus types. Side-by-side comparison of two amplification protocols comparing two different conventional molecular amplification techniques applied to a considerable number of clinical specimens is rare. Combined with conventional methods, most PCR protocols have a better sensitivity; e.g., all RSV procedures have a sensitivity of 94.5 to 97% compared with cell culture and immunofluorescence, but PCR is definitely more sensitive for adults and the elderly as a result of the lower virus production in these patients.

Since excellent reviews on the detection of respiratory viruses (176–180) are available, and more and more multiplex assays are applied for the detection of respiratory viruses, this section will not describe in detail the different mono-assays available for the detection of individual viruses but will focus on recent trends and emerging new viruses such as emerging influenza A viruses and MERS-CoV.

With the emergence of human infections due to an influenza A H3N2 variant in 2009 in North America, influenza A H1N1 worldwide in 2009, and more recently avian influenza A H7N9 in China, it is important to compare the commercially available point-of-care tests as well as in-house and commercially available molecular amplification tests. Chan et al. compared the analytical sensitivity for the detection of influenza A H7N9 and influenza A H3N2v of seven point-of-care influenza tests and the Resplex II plus v2.0 and the xTAG RVP Fast (181), whereas Hatchette et al. compared the performance of in-house PCR using the CDC primers against the performance of the RealStar Influenza S&T RT-PCR v3.0, the Quidel Molecular Influenza A+B assay, the Simplexa Flu A/B& RSV assay, the Seegene RV15 One Step ACE detection kit with agarose gel detection, the xTAG RVP Fast v1, the Seegene RV15 One Step ACE detection kit with capillary electrophoresis, and the xTAG RVP Classic (182) for the detection of influenza A H7N9. The main conclusion of both papers is that all (molecular) tests are not equal. According to Hatchette et al. (182), the analytical sensitivity of the commercially available assays is highly variable compared to that of the CDC-based in-house NAATs for the detection of influenza A H7N9. RealStar Influenza S&T v3 and the Quidel Molecular influenza A+B assay were only slightly less sensitive. A poor sensitivity was obtained with the xTAG RVP Classic, which is a concern since this test is FDA approved. Chan et al. (181) published similar findings. In addition, they reported that the analytical sensitivity of the point-of-care tests for H7N9 and H3N2v was comparable to that for the influenza A H1N1 pandemic virus and similar to the sensitivity of both PCRs.

Corman et al. described real-time RT-PCRs for the detection of MERS-CoV, of which one, targeting the *upE* gene, has been used as a first-line diagnostic assay (183). In view of growing knowledge, the WHO has continuously updated the guidelines for laboratory testing. This resulted in two additional RT-PCRs targeting the *RdRp* gene and the *N*-gene (184). Since then, other PCRs have been developed and validated (185, 186), including a commercial assay (187).

Molecular theranostics for infectious disease is an emerging concept in which molecular microbiology tools are needed to provide accurate and informative data, thus enabling better therapeutic interventions (188). In the field of viral respiratory infections such as an influenza virus infection, this would include—in addition to detection, typing, and subtyping of the virus—quantification of the virus present in the respiratory specimen (189) and the detection of antiviral markers (190). The availability of these combined data will allow a rapid decision on whether or not to start or continue antiviral treatment.

The quantitative detection of influenza showed that when respiratory secretion collection was done within 5 days after the onset of symptoms, the viral load was greater than 1×10^6 RNA copies/ml (191). Schibler et al. (192) validated a real-time PCR assay for the detection and quantification of rhinovirus and enterovirus RNA. They concluded that several difficulties limit the use of this assay for the quantitative detection of the absolute viral load in respiratory specimens: (i) the lack of an accurately quantified international reference RNA, (ii) the variability related to sampling procedures, and (iii) the rhinovirus genotype being amplified. However, despite these limitations, with an estimated variability of below 10% the assay proved to be applicable for the comparison of rhinovirus RNA amounts in clinical specimens. Also, assays have been developed for the molecular quantitative detection of RSV (193), RSV subtype A, influenza A, human metapneumovirus, and HCoV OC43 (11).

During 2007–2008, increased resistance to oseltamivir, which became prevalent worldwide, was reported among seasonal influenza A (H1N1) virus strains in many countries. This was found to be associated with a specific mutation causing a histidine-to-tyrosine substitution (H275Y) in neuraminidase. Nearly all sporadic cases of oseltamivir-resistant 2009 H1N1 virus infections identified to date have also been associated with this H275Y mutation (194–199).

Another trend is the direct amplification of nucleic acid without prior extraction such as the Simplexa Flu A/B and RSV Direct assay (Focus Diagnostics, USA) (200, 201). Compared with traditional methods, the overall positive/negative percent agreement was determined to be 96.6%/98.1% for influenza A, 98.4%/99.9% for influenza B, and 99.3%/98.8% for RSV. Compared to a nucleic acid extraction-dependent assay, the positive percent agreement was 90% for influenza A, 92% for influenza B and 98% for RSV (200). Woodberry et al. (201) applied the same assay to 498 NPSs and compared the results to those obtained with an in-house RT-PCR. Concordance rates were 96.6%, 97.6%, and 99.2% for influenza A, influenza B, and RSV, respectively.

MULTIPLEX NAATS

As diagnostic techniques improve, it becomes clear that multiple organisms may be found in adults, as has been described in youngsters. Pediatric studies have found polymicrobial infections in CAP: dual viral infection is present in 0 to 14%, dual bacterial infection in 0 to 14%, and mixed viral-bacterial infection in 3 to 30%. Polymicrobial CAP occurred in up to 6% of hospitalized adult, non-immunocompromised patients, at all ages, as well as in inpatients and outpatients; the most frequent combinations were those of bacteria with an atypical organism such as *M. pneumoniae* or *C. pneumoniae* and of two bacteria such as *S. pneumoniae* and a Gram-negative bacterial species. Patients with mixed pneumonia likely have more comorbidities and a more altered outcome due to treatment failure of monotherapy. In the approximately 10% of patients with mixed CAP, *S. pneumoniae* is the most prevalent microorganism.

Respiratory viruses and the so-called atypical bacteria are all responsible for RTIs that may produce clinically

similar manifestations. To reduce costs and hands-on time, multiplex NAATs for the simultaneous detection of two, three, or more different respiratory pathogens in one tube with a mixture of primers have been developed by some groups. For example, in a recent study that applied five separate multiplex PCRs for the detection of 20 respiratory pathogens, including *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*, in respiratory specimens from 475 hospitalized children with CAP, the overall positivity rate was found to be twice as high as when conventional tests were used (202). Currently, some assays detect up to 22 targets (203). However, comparison between mono- and multiplex assays has rarely been performed. Findings and conclusions result frequently in contradictory and conflicting data concerning the sensitivity and specificity of the multiplex NAATs compared to the mono-NAAT. There are indications that increasing the number of targets in one reaction results in loss of sensitivity (204, 205). This is not unexpected since the presence of several pairs of primers may increase the probability of mispairing, resulting in nonspecific amplification products and the formation of primer-dimers. Furthermore, enzymes, primers, and salt concentrations as well as temperature cyclings required for each target may be slightly different. The results of the proficiency panels described previously in this chapter seem to confirm that multiplex assays are somewhat less sensitive than monoplex assays, but until the number of organisms present in clinical specimens of diseased individuals is known, it is impossible to state whether the degree of sensitivity attained is clinically acceptable.

These techniques are therefore in competition with, and in many cases gradually replaced by, real-time multiplex reactions because of their greater user friendliness. The number of agents that can be detected simultaneously in one in-house real-time multiplex reaction tube is restricted by the number of available wavelengths in existing equipment (at least three at present); e.g., Lightcycler 2.0 uses three dyes and the LightCycler 480 uses four. But several reaction tubes can be run in parallel. The major drawback of this approach is the reduction of the amount of neuraminidase that can be introduced in each amplification and the longer hands-on time required to manipulate all the tubes. Once more, the thermocycling may be suboptimal for some agents involved in the assay. In all simultaneous assays a compromise will have to be made between the optimal temperature cycling requirements and the sensitivity of each component.

Real-time multiplex NAATs have been applied to two or three agents simultaneously. Welti (206) developed one of the first real-time multiplex PCRs. The PCR was done in two separate reactions: in the first reaction *M. pneumoniae* and *C. pneumoniae* were detected, and in the second reaction *L. pneumophila* was detected together with a commercial internal control (IPL Applied Biosystems). Loens et al. (207) developed a real-time multiplex NASBA for the diagnosis of *M. pneumoniae*, *C. pneumoniae*, and *L. pneumophila*. The multiplex NASBA was performed in one tube. Both groups compared the multiplex assays with the corresponding mono-assays. The sensitivity of the multiplex PCR was identical to that of the conventional PCR, but the multiplex NASBA assay was less sensitive than the corresponding real-time mono-NASBA procedure. A loss of sensitivity in a conventional multiplex PCR for these agents, as mentioned above, was also mentioned by Tong et al. (204). Templeton et al. (208) covered 15 agents by six multiplex real-time reactions. More research is needed to identify those reactions that can be combined with a minimal loss in sensitivity.

It has been proposed that industry-produced assays in kit form may enable standardization. A comparative analysis of the limits of detection of the ResPlex I assay (Table 5) and real-time single PCR assays demonstrated that the MX-PCR assay is 10-fold less sensitive in detecting *M. pneumoniae* (209). Furthermore, the ResPlex I assay was performed on 49 NPS specimens known to be positive by real-time PCR for three pathogens (*C. pneumoniae*, *M. pneumoniae*, and *S. pneumoniae*) and detected 50%, 59%, and 81% of the *C. pneumoniae*-, *M. pneumoniae*-, and *S. pneumoniae*-positive samples, respectively.

Another evolution combines conventional PCR with microarray detection. Davignon et al. (210) developed a resequencing oligonucleotide microarray for the identification of *S. pyogenes* and associated antibiotic resistance determinants. The identification was based on SpeB and provided an accurate detection of *S. pyogenes* and the *erm (B)*, *erm(TR)*, and *mef* genes involved in erythromycin resistance detection.

Li et al. (211) evaluated two commercial multiplex panels, NGEN and ResPlex II, detecting 6 and 12 respiratory viruses or virus groups, respectively, by microarray and Luminex liquid chip hybridization and identification, respectively. Sensitivities of these two assays were lower than those of the monoplex real-time RT-PCR assays, most noticeably for RSV and PIV-3. Although these might be improved by further primer/probe optimization, changes in primer/probe sequences could negatively influence other assays targeted in the multiplexed reaction. Although hands-on time of these tests is only approximately 60 minutes, turnaround times are still 6 hours for the ResPlex II and 9 hours for the NGEN.

However, since the calculation of the sensitivities of the industry-produced multiplex assays was mainly dependent on DNA copy number, further evaluation and standardization using an extended number of clinical specimens that may have a low load of the organism are needed.

The Argene Respiratory MWSr-gene concept allows the detection of numerous pathogens (influenza A/B, RSV/hMPV, rhino&EV, AdV/hBoV, Chla/Myco pneumo, HCoV/PIV, *Bordetella*, *B. parapertussis*) in the same run. In addition, the diagnostic strategy can be adapted to the season: searching for the most likely pathogens can be considered in the first approach, the remaining pathogens being searched for systematically in the second stage.

Other commercial assays aimed at the detection of a large panel of respiratory viruses include, for example, the Seeplex RV assay (Seegene Inc., Seoul, South Korea); the MultiCode-PLx RVP assay (EraGen Biosciences), which detects 17 different respiratory viruses; and the xTag RVP assay (Luminex Molecular Diagnostics, Toronto, Canada) for the detection of 19 respiratory viruses including the newly described coronaviruses. Both these assays use a fluidic microbead array and the Luminex xMap detection system. The xTAG RVP assay was the first assay that was cleared by the FDA for the detection of a number of respiratory viruses.

Since the calculation of the sensitivities of the commercial multiplex assays was mainly dependent on DNA copy number, further evaluation and standardization using an extended number of clinical specimens that may have a low load of the organism are needed.

Balada-Llasat et al. (212) evaluated the ResPlex II v2.0, multicode-Plx, and xTAG respiratory panels using 202

TABLE 5 Summary of commercially available PCR assays for detection of respiratory pathogens[a]

Kit	Manufacturer	Assay type	Detection procedure	Pathogens targeted
Alere I influenza system	Alere	Nicking endonuclease amplification		Influenza A and B
Mycoplasma pneumoniae BDProbeTec ET	BD	SDA	Fluorescence	*M. pneumoniae*
Bordetella pertussis BDProbeTec ET	BD	SDA	Fluorescence	*B. pertussis*
Legionella pneumophila BDProbeTec ET	BD	SDA	Fluorescence	*L. pneumophila*
Chlamydiaceae BDProbeTec ET	BD	SDA	Fluorescence	*Chlamydiaceae*
Microproof *Streptococcus pneumoniae* detection kit	Biotecon	PCR	Real-time	*S. pneumoniae*
iQcheck *Legionella*	BioRad	qPCR	Real-time	*Legionella* spp.
ExiStation SP real-time PCR kit	CBIOTECH	PCR	Real-time	*S. pneumoniae*
ASR RSV	Cepheid	PCR	Real-time	RSV
ASR MPN	Cepheid	PCR	Real-time	*M. pneumoniae*
Xpert Flu A	Cepheid	PCR	Real-time	Influenza A H1N1
Xpert Flu B	Cepheid	PCR	Real-time	Influenza B
R-DiaBor	Diagenode	PCR	Real-time	*B. pertussis*, *B. parapertussis*
R-DiaFlu	Diagenode	PCR	Real-time	Influenza A and B
R-DiaLeg	Diagenode	PCR	Real-time	*Legionella* spp., *L. pneumophila*
R-DiaMPV	Diagenode	PCR	Real-time	hMPV
R-DiaPara13	Diagenode	PCR	Real-time	PIV 1 and 3
R-DiaPara24	Diagenode	PCR	Real-time	PIV 2 and 4
R-DiaRhino	Diagenode	PCR	Real-time	Rhinovirus
R-DiaRSV	Diagenode	PCR	Real-time	RSV
O-DiaADV	Diagenode	PCR	Real-time	HAdV
Simplex *Mycoplasma pneumoniae*	Focus Diagnostics	PCR	Real-time	*M. pneumoniae*
Focus H1N1	Focus Diagnostics	PCR	Real-time	Influenza A H1N1
ProhMPV+	Hologic	PCR	Real-time	hMPV
Chlamydia pneumoniae RG quantitative real-time PCR kit	Genome Diagnostics	PCR	Real-time	*C. pneumoniae*
S. pneumoniae RG quantitative real-time PCR kit	Genome Diagnostics	PCR	Real-time	*S. pneumoniae*
AccuPower SP real-time PCR kit	Goffin Molecular Technologies	PCR	Real-time	*S. pneumoniae*
GenoQuick *Bordetella* assay	Hain Life Sciences	PCR	Dipstick	*B. pertussis* and *B. parapertussis*
R.A.P.I.D influenza	bioMérieux	PCR	Real-time	Influenza A
MutaPLATE *C. pneumoniae*	Immunodiagnostik	PCR	Real-time	*C. pneumoniae*
S. pneumoniae real-time PCR kit	Liferiver	PCR	Real-time	*S. pneumoniae*
Group A *Streptococcus* real-time PCR kit	Liferiver	PCR	Real-time	Group A streptococci
Illumigene group A *Streptococcus*	Meridian BioBioScience	LAMP	Turbidity	Group A streptococci
Illumigene *Mycoplasma*	Meridian BioBioScience	LAMP	Turbidity	*M. pneumoniae*
Venor MP	Minerva BioLabs	PCR	Agarose gel electrophoresis and real-time	*M. pneumoniae*
Onar LP	Minerva BioLabs	PCR	Real-time	*L. pneumophila*
L. pneumophila LightMix kit	TIB MolBIOL	PCR	Real-time	*L. pneumophila*
Influenza A/H1N1	Roche	PCR	Real-time	Influenza A H1N1
LightCycler Strep-A assay	Roche	PCR	Real-time	Group A streptococci
Influenza A LightMix kit	TIB MolBIOL	PCR	Real-time	Influenza A
Influenza B LightMix kit	TIB MolBIOL	PCR	Real-time	Influenza B

(*Continued*)

TABLE 5 (*Continued*)

Kit	Manufacturer	Assay type	Detection procedure	Pathogens targeted
Tamiflu LightMix kit	TIB MolBIOL	PCR	Real-time	Tamiflu resistance detection
RSV LightMix kit	TIB MolBIOL	PCR	Real-time	RSV
hMPV LightMix kit	TIB MolBIOL	PCR	Real-time	hMPV
Adeno LightMix kit	TIB MolBIOL	PCR	Real-time	HAdV
PIV 1-3 LightMix kit	TIB MolBIOL	PCR	Real-time	PIV 1-3
M. pneumoniae LightMix kit	TIB MolBIOL	PCR	Real-time	*M. pneumoniae*
C. pneumoniae LightMix kit	TIB MolBIOL	PCR	Real-time	*C. pneumoniae*
B. pertussis/parapertussis LightMix kit	TIB MolBIOL	PCR	Real-time	*B. pertussis* and *B. parapertussis*
L. pneumophila LightMix kit	TIB MolBIOL	PCR	Real-time	*L. pneumophila*
MERS coronavirus	TIB MolBIOL	PCR	Real-time	MERS HCoV
Microbial DNA qPCR Multi-Assay kit *S. pneumoniae* pathogenicity	Qiagen	PCR	Real-time	*S. pneumoniae* and virulence factors
Influenza LC RT-PCR	Artus (Qiagen)	PCR	Real-time	Influenza A and B
Plex-ID flu assay	Abbott Molecular Inc.	MX-PCR	Electrospray ionization-mass spectrometry	Influenza A (H1N1-p, H1N1-s, H3N2) and influenza B
Cp/Mp tracer	Affigene	MX-PCR	Real-time	*M. pneumoniae*, *C. pneumoniae*
AID CAP viral assay	AID GmbH	MX-PCR	ICT	Influenza A and B, PIV 1-3, RSV A/B, HAdV
AID CAP juvenile assay	AID GmbH	MX-PCR	ICT	*S. pneumoniae*, *Haemophilus influenzae*, *B. pertussis*, *B. parapertussis*
AID CAP bacterial assay	AID GmbH	MX-PCR	ICT	*S. pneumoniae*, *H. influenzae*, *Moraxella catarrhalis*, *C. pneumoniae*, *M. pneumoniae*, *L. pneumophila*
RealStar Influenza S&T RT-PCR	Altona Diagnostics	MX-PCR	Real-time	Influenza A and B
Chlamylege	Argene	MX-PCR	Hybridization	*C. pneumoniae*, *M. pneumoniae*, *Legionella* spp.
Bordetella LC PCR kit	Artus (Qiagen)	MX-PCR	Real-time	*B. pertussis*, *B. parapertussis*, *B. bronchiseptica*
EasyPlex respiratory pathogens B and C	Ausdiagnostics	MX-PCR	Real-time	Influenza A, influenza A H1, influenza A H3, influenza A H5, influenza B, RSV, rhinovirus, enterovirus, PIV 1-3, HAdV, hMPV, HCoV 229E and OC43, *B. pertussis*, *M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *Legionella longbeachae*, *Pneumocystis*, *H. influenzae*, *S. pneumoniae*
Respiratory Multi Well System	bioMérieux	5 duplex PCRs	Real-time	Influenza A, influenza B, RSV, hBoV, HAdV, hMPV, rhino/enterovirus, *C. pneumoniae*, *M. pneumoniae*
ASR Flu A/B	Cepheid	MX-PCR	Real-time	Influenza A and B
ASRBP2	Cepheid	MX-PCR	Real-time	*B. pertussis*, *B. parapertussis*
ASRFLU	Cepheid	MX-PCR	Real-time	Influenza A and B
RESRNA	Diagenode	MX-PCR	Real-time	Influenza A and B, RSV, hMPV, rhinovirus, PIV1-4
MultiCode-PLx RVP	EraGen Biosciences	MX-PCR	Luminex technology	Influenza A and B, RSV A/B, HAdV, PIV 1-4, rhinovirus, 3 HCoV, hMPV A and B

(*Continued on next page*)

TABLE 5 Summary of commercially available PCR assays for detection of respiratory pathogens[a] (*Continued*)

Kit	Manufacturer	Assay type	Detection procedure	Pathogens targeted
Fast Track Respiratory Pathogen assay	Fast-Track Diagnostics	MX-PCR	Real-time	*S. pneumoniae*, *S. aureus*, *H. influenzae*, *M. catarrhalis*, *Legionella* spp., *M. pneumoniae*, *C. pneumoniae*
Simplexa flu A/B&RSV direct assay	Focus Diagnostics	3 amplification disks	Real-time	Influenza A and B, RSV
Genmark e-Sensor RVP	GenMark Dx	MX-PCR on microfluidic platform	Electrochemical detection	Influenza A, influenza A H1, influenza A H3, influenza A 2009 HN, influenza B, RSV A/B, PIV 1-3, hMPV, human rhinovirus, HAdV B/E and C
Clart PneumoVir	Genomica	MX-PCR	Microarray	Influenza A/B/C, RSV A/B, HAdV, PIV 1-4, rhinovirus, HCoV, hMPV A and B, HBoV, echovirus
Clart Flu A Vir	Genomica	MX-PCR	Microarray	Seasonal influenza A H1N1, outbreak influenza A H1N1, influenza A H3N2
ProPneumo-1	Hologic	MX-PCR	Real-time	*M. pneumoniae*, *C. pneumoniae*
ProParaflu+	Hologic	MX-PCR	Real-time	PIV 1-3
ProFlu+	Hologic	MX-PCR	Real-time	Influenza A and B, RSV
Pro hMPV+	Hologic	MX-PCR	Real-time	hMPV
Pro Adeno+	Hologic	MX-PCR	Real-time	HAdV 1-51
ProFast+	Hologic	MX-PCR	Real-time	Influenza A: seasonal A/H1, seasonal A/H3, and 2009 H1N1
ProPneumo1+	Hologic	MX-PCR	Real-time	*M. pneumoniae*, *C. pneumoniae*
BioFire	bioMérieux	MX-PCR	Microarray	Influenza A H1N1; influenza A H1; influenza A H3; influenza B; RSV; hMPV; HCoV NL63, OC43, 229E, HKU1; HAdV PIV 1-4; HBoV; rhino/enterovirus; *B. pertussis*; *M. pneumoniae*; *C. pneumoniae*
AdvanSure respiratory virus kit	LG Life Science	MX-PCR	Real-time	Influenza A and B; PIV 1-3; RSV; HAdV; HBoV; hMPV; HCoV OC43, 229E, and NL63; rhinovirus
xTAG RVP	Luminex Corp.	MX-PCR	Luminex technology	Influenza A (H1, H3, H5, nonspecific) and B; RSV A/B; PIV 1-4; HAdV; rhinovirus/enterovirus; HCoV OC43, NL63, 229E, HKU1, and SARS; hMPV
Luminex RVP Fast	Luminex Corp.	MX-PCR	Luminex technology	RSV, influenza A and B viruses; PIV 1-4; hMPV; HAdV; enterovirus-rhinovirus; HCoV NL63, HKU1, 229E, and OC43; HBoV
NGEN RVA ASR	Nanogen Inc.	MX-PCR	Microarray	Influenza A and B, RSV A/B, PIV 1-3
MGB Eclipse Flu A/B RUO	Nanogen Inc.	MX-PCR	Real-time	Influenza A and B
Verigene RV+	Nanosphere Inc.	MX-PCR	Nanoparticle probe technology	Influenza A and B, RSV A/B

(*Continued*)

TABLE 5 (*Continued*)

Kit	Manufacturer	Assay type	Detection procedure	Pathogens targeted
RespiFinder	Patho Finder	MX-PCR	Capillary electrophoresis or microfluidics	Influenza A (H5, nonspecific) and B, RSV A/B, PIV 1-4, rhinovirus, 3 HCoV, hMPV, HAdV
RespiFinder plus	Patho Finder	MX-PCR	Capillary electrophoresis	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, influenza A (H5, nonspecific) and B, RSV A/B, PIV 1-4, rhinovirus, 3 HCoV, hMPV, HAdV
RespiFinder focus	Patho Finder	MX-PCR	Capillary electrophoresis or microfluidics	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, influenza A and B, RSV A/B, hMPV, HAdV
SmartFinder	Patho Finder	MX-PCR	Real-time	*M. pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*, influenza A and B, RSV A/B, PIV 1-4, HAdV, rhinovirus, 3 HCoV, hMPV, HBoV
ResPlex II	Qiagen	MX-PCR	Luminex technology	Influenza A and B, RSV A/B, PIV 1-4, hMPV, rhinovirus, coxsackie and echovirus
Influenza A+B assay	Quidel	MX-PCR	Real-time	Influenza A and B
Lyra influenza A and B	Quidel	MX-PCR	Real-time	Influenza A and B
Lyra RSV and hMPV assay	Quidel	MX-PCR	Real-time	RSV and hMPV
Lyra Direct Strep assay	Quidel	MX-PCR	Real-time	Streptococcus from group A, C, and G
Seeplex RV15 ACE	Seegene Inc.	MX-PCR	Capillary electrophoresis	Influenza A and B, RSV A/B, PIV 1-4, rhinovirus, 3 HCoV, HAdV, HBoV, enterovirus
Seeplex PneumoBacter ACE	Seegene Inc.	MX-PCR	Capillary electrophoresis	*S. pneumoniae*, *H. influenzae*, M. *pneumoniae*, *C. pneumoniae*, *L. pneumophila*, *B. pertussis*
Seeplex RV/PB18 ASE	Seegene Inc.	MX-PCR	Capillary electrophoresis	*S. pneumoniae*, *H. influenzae*, M. *pneumoniae*, *C. pneumoniae*, *L. pneumophila*, influenza A and B, RSV A/B, PIV 1-3, rhinovirus, 3 HCoV, HAdV, HBoV, enterovirus

[a]Abbreviations: HAdV, human adenovirus; HBoV, human bocavirus; HCoV, human coronavirus; hMPV, human metapneumovirus; ICT, immunochromatographic test; LAMP, loop-mediated isothermal amplification method; MERS, Middle East respiratory syndrome; MX-PCR, multiplex PCR; PIV, parainfluenzavirus; qPCR, quantitative PCR; RSV, respiratory syncytial virus; SARS, severe acute respiratory syndrome; SDA, strand displacement analysis. For further details on the evaluation and/or validation of the most important commercial tests, see text above. Boldface type indicates FDA-approved tests.

specimens from adult patients with an RTI. Viral culture and xTag were used as standards to calculate the sensitivities and specificities. Overall, the multiplex assays detected more viruses than culture detected. As compared to culture, the xTAG RVP had a sensitivity and specificity of 100% and 91%, compared to MultiCode-PLx with 89% and 87% and Resplex II with 89% and 94%, respectively. Each assay showed differences in sensitivities for individual viruses.

In a comparison of mono real-time PCRs with the Seeplex RV detection kit and the RespiFinder DC TwoStep kit (PathoFinder, Maastricht, The Netherlands), using a panel of 168 positive and negative culture samples (213), 77% of the samples were positive as detected by at least one method. Forty-one percent of the samples were positive by cell culture, 69% by mono-PCR, 76% by the RespiFinder kit, and 60% by the Seeplex RV kit. Furthermore, the RespFinder test yielded results in one attempt for all samples, while 7.2% of samples had to be repeated by the Seeplex RV kit due to inconclusive results.

Another important study compared the Resplex II Panel v2.0, the Seeplex RV15, the xTAG RVP, and xTAG RVP Fast with direct fluorescent antibody staining and viral isolation on 750 NPSs from children. Of 750 specimens, 288 were positive by direct fluorescent antibody or viral isolation, while an additional 214 were positive by multiplex PCR, for a total positivity rate of 66.9%. Compared with a composite reference standard, the interassay accuracy of the multiplex PCRs varied, but all were superior to conventional diagnostic methods. The authors

(169) found a sensitivity of ≥96.9% for all targets except entero/rhinovirus (71.7%). Resplex II v2.0 had good sensitivity for influenza A and B (≥96.9%) but lower sensitivity for the other viruses (82.0 to 84.0%). Resplex II v2.0 sensitivity fell further for adenovirus and bocavirus. The xTAG RVP showed a high sensitivity for influenza A and B and metapneumovirus (≥98.4%) but lower sensitivities for parainfluenza virus, adenovirus, and RSV (85.4 to 88.2%) and the lowest sensitivity for coronaviruses (48.1%). The RVP Fast had good sensitivities for most viruses but demonstrated a significantly reduced sensitivity in the detection of adenovirus, coronaviruses, influenza B, and parainfluenza viruses (52.4 to 5.8%). The specificity was excellent for all assays.

Another test approved by the FDA for the detection of a number of respiratory viruses is the BioFire Respiratory panel. The BioFire is a small desktop closed single-piece flow real-time PCR system. It includes automation of nucleic acid extraction, an initial RT- and multiplex PCR, followed by single-plex second-stage PCR reactions for the detection of 15 viral agents: adenovirus; coronavirus HKU1; coronavirus NL63; human metapneumovirus; rhinovirus/enterovirus; influenza A; influenza A H1; influenza AH1 2009; influenza A H3; influenza B; parainfluenza 1, 2, 3, 4; and RSV (214). The test requires 5 minutes of hands-on time and 65 minutes of instrumentation time. The implementation of this first version in a core laboratory was described by Xu et al. (215). As in other studies (216), the implementation of the assay significantly decreased the time required to report results. Drawbacks reported were the relatively low sensitivity for adenovirus (147, 216–219) and the inability to separate rhinovirus and enterovirus. In May 2012, the FDA expanded the uses for the BioFire respiratory panel with the addition of *B. pertussis*, *M. pneumoniae*, and *C. pneumoniae*. The expanded panel now detects a total of 17 viruses and 3 bacteria. In 2013, a new version of the BioFire (version 1.7) was released with improved sensitivity for adenoviruses. Doern et al. (220) evaluated this new version and confirmed the improved sensitivity for adenovirus: from 39 clinically relevant serotypes, 8 were not detected by the previous version, and only 1 was not detected by version 1.7.

Popowitch et al. (221) compared the four FDA-cleared assays: Biofire RP, Genmark eSensor RVP, Luminex xTAG RVPv1, and Luminex RVP Fast Multiplex. The overall sensitivities and specificities for each panel were as follows: BioFire RP, 84.5% and 100%; eSensor RVP, 98.3% and 99.2%; xTAG RVPv1, 92.7% and 99.8%; and xTAG RVP Fast, 84.4% and 99.9%. Fluctuations in sensitivity were virus dependent. The greatest discrepancies were noted for adenovirus and influenza B virus detection. Ruggiero et al. (222) compared virus culture with the BioFire RP and the Genmark eSensor RVP on LRT specimens. The overall agreement between both MX-assays was 89.5%. The overall sensitivities of viral culture, the BioFire, and the eSensor RVP were 89.5%, 100%, and 100%, respectively, with specificities varying between 82.6 and 90.5%. A recent study applied the BioFire and uniplex quantitative PCR to archived sputa, dunked sputa, and nose and throat swabs (11). A respiratory virus was detected in 31% of 965 illnesses using uniplex quantitative RT-PCR. For 105 subjects, sputum was the only positive sample, including 35% of influenza cases. A total diagnostic yield of 197/965 was obtained when uniplex RT-PCR was applied to nose and throat swabs alone, versus 302/965 when sputum results were included as well. Biofire also found 99/108 sputa to be positive. The results of RQ-PCR revealed higher mean viral loads in dunked sputum compared to nose and throat samples for influenza A, RSV, and human metapneumovirus.

Considerable investment and research efforts have been made toward the development of rapid new diagnostics for influenza A and B viruses. The Xpert Flu assay (Cepheid, California) has emerged as one of the most promising and is FDA cleared. In a review, Salez et al. summarize the current knowledge of the Xpert Flu test, discuss its potential value as a point-of-care test, and outline the potential leads for future development (223).

All these studies have shown that multiplex increases the sensitivity of detection of respiratory viruses while maintaining excellent specificity. However, it will be important to develop more effective clinical and laboratory algorithms for their timely and optimal use and to study their impact on patient care in different populations in different settings.

LATEST TECHNOLOGICAL DEVELOPMENTS

Newer technologies such as microfluidics and nanotechnology offer the potential for even more rapid detection of important pathogens, allowing even near-patient testing. Since these technologies do not require viable organisms, and thus avoid any adverse effect of longer specimen transport, they can be successfully applied to both the in- and outpatient settings. Several companies currently possess the technical expertise and research infrastructure to bring a useful diagnostic testing approach to the clinical trial stage soon. More and more microfluidic devices are being developed for the rapid identification of pathogenic organisms. For example, de la Rosa et al. (224) describe a miniaturized microfluidic device that facilitates electromanipulation of *B. pertussis*. The device provides sample preparatory capability by exploiting positive dielectrophoresis in conjunction with pulsed voltage for manipulation and disruption of *B. pertussis* cells without damaging the nucleic acid. This technique could be an important step in sample preparation on-chip involving respiratory pathogens. Another example, described by Easley et al. (225), is an integrated microfluidic device capable of accepting whole-blood or nasal aspirate as starting materials. The device carries out on-chip DNA purification and PCR amplification, followed by separation and detection in a manner that allows for microliter samples to be screened for infectious pathogens with sample-in–answer-out results in <30 minutes. It was successfully used for the detection of *B. pertussis* in 1 μl of nasal aspirate from a patient suspected of having whooping cough.

Cao et al. (226) developed a single-use microfluidic chip that integrates solid-phase extraction and RT-PCR to amplify influenza A RNA in less than 3 hours. Based on the analysis of 146 specimens, the microfluidic test was found to be more sensitive than two rapid immunoassays (Xpect Flu and Binax Now) and comparable to direct fluorescent antibody.

The group of Campuzano et al. (227–229) presented a successful proof-of-concept for the detection of *S. pneumoniae* by disposable amperometric magneto-sensor. The assay was validated both on strains and on clinical samples with sensitivity and specificity around 90%.

Molsa et al. (230) evaluated a field-compatible, portable PCR instrument (the RAZOR EX-thermocycler) for the rapid near-patient detection of influenza A viruses in clinical specimens. Results were achieved within 90 minutes, including 45 minutes for sample preparation, and they were in agreement with results obtained by standard real-time PCR under laboratory conditions.

The Alere I Influenza system (Alere Scarborough, Scarborough, ME) assay incorporates a nicking endonuclease amplification reaction technique for the detection and differentiation of FluA and FluB in NPS specimens. The system requires only 2 minutes hands-on time to process and set up one sample, with results becoming available within 15 minutes. Nie et al. (231) evaluated the performance of this test against the Filmarray. Sensitivities of 87.2%, 92.5%, 25%, and 97.4% for influenza A H1N1, influenza A H3N2, untypeable influenza, and FluB, respectively, were obtained. The specificities were 100% for both influenza A and B. According to the authors, due to the integrated, rapid (less than 15 minutes), and simple characteristics of the Alere I Influenza assay, there is a potential for point-of-care testing.

The SAMBA Flu duplex test was developed to provide a fast (2 hours, 15 minutes), easy-to-use solution for the detection of influenza A and B (232, 233). This duplex test couples isothermal amplification with visual detection on a dipstick in a closed semiautomated system. Compared to quantitative in-house PCR, the sensitivity and specificity of the SAMBA flu test were 100% and 97.9% for influenza A and 100% and 100% for influenza B.

Slinger et al. (234) successfully performed a proof-of-concept study to determine if *B. pertussis*, *B. parapertussis*, and RSV could be detected in clinical specimens using nanoliter real-time PCR. Over 3,000 nanoliter PCR reactions can be performed on a single plate on the commercially available nanoliter-volume real-time PCR platform from the OpenArray Real-time PCR System (Life Technologies, USA), making this platform potentially useful for the detection of multiple targets simultaneously.

Dunne et al. (235) postulated that by the year 2025 sophisticated samplers will be available that painlessly obtain the necessary material, followed by automated analyzers to simultaneously process DNA, RNA, proteins, glycopeptides, and exopolysacharides to detect any of a possible 168 pathogenic microbes as well as toxins and resistance genotypes—all completed and yielding a diagnosis within 15 minutes.

CONCLUDING REMARKS

With the use of new tools such as NAATs, a greater understanding of the etiology and epidemiology of RTIs and other bacterial infections is possible. The combined use of single-target assays or of multiplex assays has increased the diagnostic yield in respiratory infections by 30 to 50%. When this is combined with traditional bacteriologic techniques to diagnose *S. pneumoniae* infections, more than 50%, and in some studies of CAP up to 70%, of etiologic agents can be detected.

The wider application of multiplex reactions in recent years also resulted in the detection of numerous simultaneous viral infections with widely varying incidences: from 3 to 23% and even >50% in some studies. The differences in incidence may result from the variety of diagnostic panels applied. There were no preferential combinations of the viruses. Only a few studies found combined infections to be associated with a more severe clinical status. The clinical significance of combined infections remains to be further clarified.

As more epidemiological information on the role of a panel of respiratory viral pathogens becomes available, it is clear that screening for these viruses in specific patient populations such as transplant patients, immunocompromised hosts, very young children, and the elderly is desirable, and preventive and therapeutic recommendations may take this information into account.

However, a number of subjects remain to be investigated. At present, the impact of NAAT detection to understand the etiology of RTIs is limited to a few pathogens, but with new tools, a greater understanding of the etiology and epidemiology of RTIs and other bacterial infections is possible. Since there is little scientific information on how to diagnose the etiology of CAP in a cost-effective manner in daily practice, further studies are needed. Despite the probability that improved patient outcome and reduced cost of antimicrobial agents, reduced use of less sensitive and specific tests, and reduced length of hospital stay will outweigh the increased laboratory costs incurred through the use of molecular testing, such savings are difficult to document. However, the delivery of rapid, sensitive, and specific results makes the implementation of complete respiratory screens beneficial even without a full cost-benefit analysis.

NAATs are at present more expensive than conventional approaches, with the most expensive being the fluorogenic-based real-time detection systems. However, improvements in standardization and automation for sample preparation and technical advances in thermocyclers that allow the performance of multiple runs of PCR simultaneously or in a very short time will lead to increased use of amplification methods and cost reductions to rates competitive with conventional methods.

Whether a comprehensive approach is patient friendly, allows streamlining of management, is cost effective, and reduces the antibiotic pressure in the hospital and the community remains to be further studied.

Several studies tended to show cost efficiency of rapid diagnosis of ARI resulting from reduced antibiotic use and complementary laboratory investigations but most significantly from shorter hospitalization and reduced isolation periods of patients.

During epidemics it may be important to rule out a particular infection. A considerable saving in diagnostic procedures in ARI is made possible by the abolishment of tissue cultures and serologic tests. A closer collaboration between clinicians and the laboratory has a high priority.

The implementation of quantitative tests could shed further light on the relation between bacterial load and the seriousness of the disease (236), produce useful prognostic information, and help in the differentiation between colonization and infection. More information could be gathered on the length of the postinfection carrier state as well as on the importance of subclinical infections and how prone these are for spreading infection.

Several other agents responsible for respiratory infections should be considered separately because of the specific clinical picture for which they are responsible: *Chlamydia psittaci*, *B. parapertussis*, *Coxiella burnetii*, and *Pneumocystis jirovecci*.

The importance of LRTI viruses in chronic respiratory diseases such as chronic obstructive pulmonary disease and cystic fibrosis should also be better evaluated.

The need to detect an ever expanding number of infectious agents will exceed the possibility of mixed real-time NAATs. The task will be taken over by the next generation of diagnostics, the array technology that opens a wide access to the infectious agents (237–239).

The rapid molecular characterization of the previously unknown SARS-CoV within a few weeks after the appearance of the disease and the discovery of bocavirus and recently MERS-CoV illustrate the potency of NAATs for broadening the knowledge on "hidden" viruses remaining to be discovered.

In the very near future, NAATs will probably not be done at the point of care, but this must remain an objective for further development of the technology.

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Molecular and Mass Spectrometry Detection and Identification of Causative Agents of Bloodstream Infections

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26

INTRODUCTION

Bloodstream Infections

Bloodstream infections (BSIs) are severe diseases associated with a high morbidity and mortality, which increases with the delay until administration of the first appropriate antibiotic (1–8). For this reason, empiric treatments made of broad-range anti-infectious compounds or made of a combination of antimicrobials are started immediately after the sampling of blood bottles. BSIs can be caused by various microorganisms. In the absence of microbiological documentation, physicians suspect a BSI on the basis of clinical symptoms, which trigger the start of empirical treatments. The clinical presentations are multiple and include fever or hypothermia, increases in heart rate, change in inflammatory variable (C-reactive protein, procalcitonin, and white blood cell count increase), and organ failure (2, 9). These symptoms are generally nonspecific and only suggest bloodstream dissemination. Empirical treatments are made of broad-spectrum antibiotics on the basis of the clinical and epidemiological data, but this does not exclude any risk of inappropriate initial treatment.

Similar to delayed introduction of the first antibiotic treatment, an inadequate treatment is associated with a significant increase of mortality (10, 11). In a recent study there was a nearly 3-fold increase in the risk of mortality when the antimicrobial treatment was inappropriate (12). *Pseudomonas aeruginosa* or *Acinetobacter baumannii*, which are associated with frequent multidrug-resistant profiles, are often the microorganisms for which empirical antibiotic coverage is inappropriate. *Enterobacteriaceae* producing extended-spectrum beta-lactamase (ESBL), such as *Escherichia coli*, or producing inducible cephalosporinase, such as *Enterobacter cloacae*, are also commonly associated with an inadequate empirical treatment (13). The mortality rate of patients suffering from a methicillin-resistant *Staphylococcus aureus* (MRSA)-associated BSI was significantly higher among patients receiving an inappropriate empirical antibiotic treatment (168/342, 49.1%) than among patients receiving an appropriate empirical antibiotic treatment (56/168, 33.3%) (14, 15). Inadequate treatments can also occur for organisms such as *Enterococcus faecalis* that are intrinsically resistant to some commonly administered first-line antibiotics such as ceftriaxone (11). Broad-spectrum molecules also have a detrimental impact on beneficial bacteria that constitute the protective flora and contribute to the emergence of multidrug-resistant strains. Moreover, some of these antimicrobial molecules can also have a toxic effect for the patient (10, 11, 16). The rapid identification of the causative agent of the BSI thus allows the adjustment of the anti-infectious therapy or the reduction of the spectrum (de-escalation) with significant clinical benefits.

BSIs are characterized by a low quantity of circulating microbes. On the basis of plating methods, the bacterial load has been estimated to be around 1 to 10 CFU/ml of blood in adults (17–21). This method is efficient for determining the number of bacteria that survive the plating protocol; hence, the true number of circulating bacteria is likely underestimated. DNA copies present in the circulation should be even higher because they also include DNA released by dead bacteria or clumping bacteria as well as DNA from bacteria engulfed in phagocytes. On the basis of quantitative PCR, Bacconi et al. recently estimated that during bacteremia, the blood contains 10^3 to 10^4 bacterial cells/ml (22), which is higher than the analytic sensitivity of most of the available molecular methods.

Hence, the ideal diagnostic method for BSI should (i) be able to identify a broad range of pathogens, (ii) have an analytic sensitivity lower than 10 to 100 CFU/ml, (iii) be rapid, (iv) be quantitative to give an idea of the severity of the infection, (v) give an antibiotic susceptibility profile, (vi) be associated with a short hands-on time, and (vii) be automated (23). These are all potential characteristics that molecular diagnosis methods may fulfill, at least to some extent.

Molecular methods for the diagnosis of BSI refer to nucleic acid-based methods but also to non-nucleic acid-based methods. PCR-based methods that constitute the nucleic acid amplification-based methods can be applied both on positive blood culture and on whole blood. Indeed, the PCR step increases the sensitivity of the detection, which makes it suitable for paucibacterial samples such as blood (Fig. 1) (24). Non-amplification-based methods such as fluorescent *in situ* hybridization (FISH) or microarray have limited sensitivity. Hence, they are

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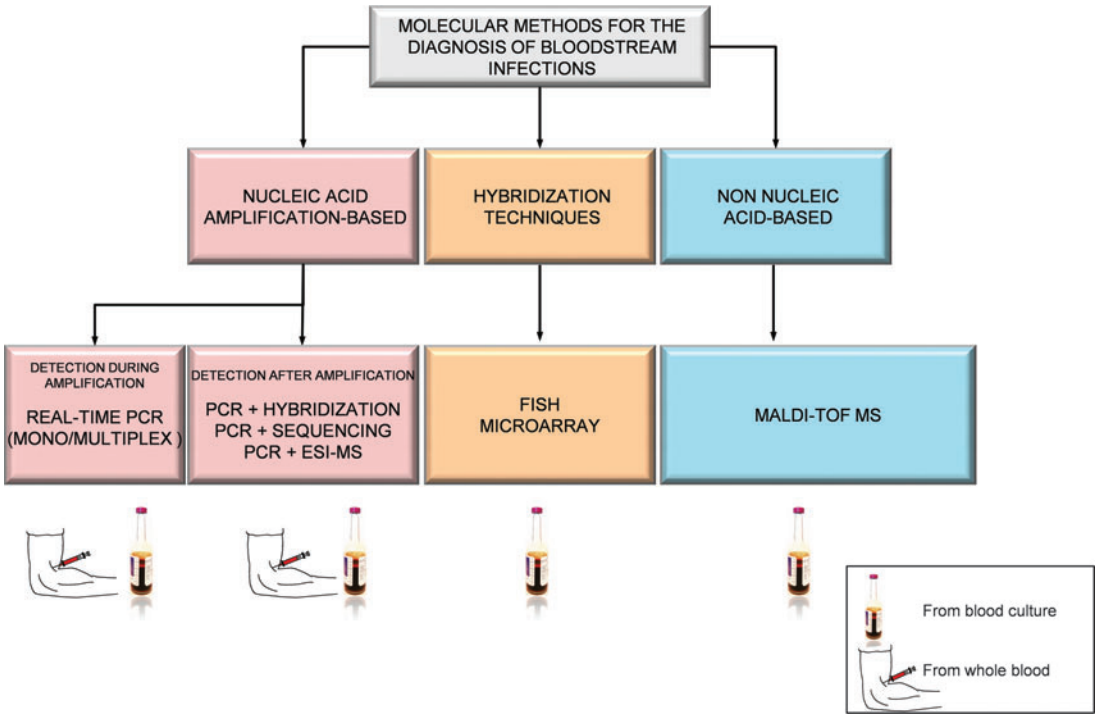

FIGURE 1 Different molecular technologies used for the detection and identification of microbes during bloodstream infections. Molecular methods for the diagnosis of BSI include nucleic acid-based methods and non-nucleic acid-based methods. Nucleic acid amplification-based techniques can be applied on positive blood cultures or used directly on blood, whereas non-nucleic acid amplification-based techniques such as FISH and microarray or non-nucleic acid-based methods such as MALDI-TOF MS can be used only on positive blood culture. Adapted from reference 150.

restricted to positive blood culture. For the same reason, matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), a non-nucleic acid-based method that analyzes microbial protein mass spectrum, is suitable for diagnosis from positive blood culture.

In this chapter we present the molecular methods available for the microbial diagnosis of BSI. We report and discuss the performance as well as the advantages and disadvantages of these methods.

Blood Culture-Based Diagnosis of BSIs

Blood culture is currently the "gold standard" to establish the etiology of BSI. Blood bottles contain specific liquid broth for the growth of bacteria or fungi present in blood. Distinct media are available to grow different microbes (24). Blood culture-based diagnosis has been improved by the use automated incubators that can detect bacterial or fungal growth in blood bottles thanks to associated physicochemical variations. When the automated system detects the growth, visualization of the microorganism is possible using standard Gram or fluorescent staining (Fig. 2). The Gram staining can give a first presumptive etiology of the BSI. However, only the final identification of the pathogen and its antibiotic susceptibility testing can ensure the adequacy of the ongoing antibiotic treatment. Thus, when a blood culture is detected as positive, diagnostic laboratories have to identify the microbe rapidly and with the highest sensitivity and specificity. However, the time to positivity of the blood culture bottles can vary from a few hours to 24 to 48 h for fast-growing bacteria, and even more for slow-growing bacteria or fungi. In addition, it is generally admitted that more than 50% of BSIs occur with negative blood culture (25, 26). When the blood culture becomes positive, using conventional methods, one or more days can be necessary to identify the pathogen and to determine the exact etiology of the BSI (Fig. 2). These methods include phenotypic characterization using biochemical and enzymatic tests. Most of these phenotypic characterizations require a subculture in liquid media or a subculture on solid media to obtain isolated colonies. Finally, BSIs involving multiple organisms may also further increase the time to identification of most detection methods.

Inside positive blood culture, bacteria concentration reaches 1.10^6 to 2.10^8 for Gram-negative cocci and 2.10^7 to 1.10^9 for Gram-negative bacilli (27, 28). These concentrations allow the use of amplification-based methods such as PCR and real-time PCR as well as non-amplification-based methods such hybridization or MALDI-TOF MS (Fig. 1).

Blood is a challenging sample for the detection and identification of pathogens since it contains a low number of microorganisms in comparison to human components (DNA, proteins, and cells) (29). These compounds can interfere with detection or identification by leading to false-positive or false-negative (inhibition) results. All these components are transferred into the blood culture at the time of sampling the blood. MALDI-TOF MS is a non-nucleic acid-based molecular method that has considerably accelerated and simplified the identification of pathogens from positive culture. It is based on the analysis of the mass spectrum generated by bacterial proteins, mainly

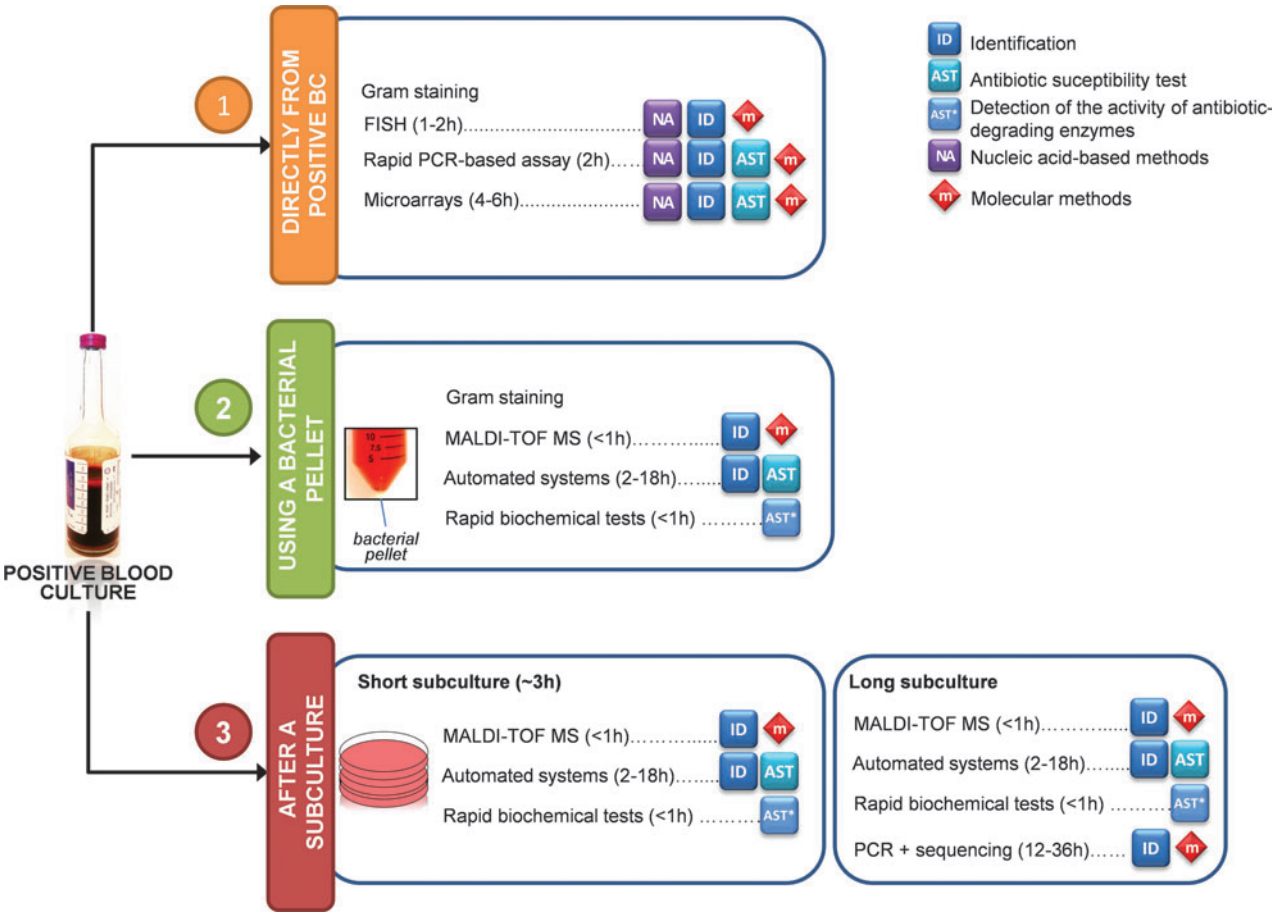

FIGURE 2 Conventional and molecular methods for the identification of microorganisms from positive blood culture. When a blood culture is detected as positive, several strategies are available to identify the incriminated microorganism after the initial Gram staining, as follows. (1) Identification directly from the positive blood culture using nucleic acid-based methods. (2) Identification after the microbe's enrichment, namely a purified bacterial pellet suitable for MALDI-TOF MS analysis and some automated identification/antibiotic susceptibility testing approaches such as Vitek2 and Phoenix and rapid biochemical tests HMRZ, based on the chromogenic cephalosporin HMRZ-86 {(7R)-7-[2-(aminothiazol-4-yl)-(z)-2-(1-carboxy-1-ethylethoxyimino) acetamido]-3-(2,4-dinitrostyryl)-3-cephem-4-carboxylic acid trifluoroacetate, E-isomer}, and ESBLNDP (ESBL Nordmann-Poirel-Dortet). (3) Identification after a subculture. To date, the automated system Vitek2 has been validated on short subcultures as well as the rapid biochemical tests HMRZ and CARNP (carbapenemase test Nordmann-Poirel); long subcultures are suitable for any type of analysis including phenotypic characterization; automated systems such as Vitek2, Phoenix, and MicroScan WalkAway; as well as PCR followed by sequencing. Adapted from reference 24.

housekeeping proteins (Fig. 3). This technique is now applicable on positive blood culture but still requires a sample preparation step to discard blood and other non-bacterial components.

Microbial Diagnosis of BSI Directly from Blood

The diagnosis of BSI directly from whole blood has been a major concern for medical diagnostic microbiologists. Indeed, blood cultures appear to have some intrinsic limitations: (i) approximately 50% of BSIs are blood culture negative (25, 26); (ii) in the case of positive blood culture, the time to positivity can vary from hours to days; (iii) blood culture requires a large quantity of blood, which is difficult to obtain from some patients such as pediatric patients; (iv) an antibiotic treatment is often initiated prior to blood culture. Many technical improvements have been made to increase the performance of detection methods from whole blood, but some limitations remained. Nucleic acid-based methods were limited by the need for a large volume of blood due to the low number of CFU per milliliter associated with the presence of human DNA in excess, mainly due to white blood cell DNA (30). In addition, these methods are also sensitive to contaminants—bacterial or fungal DNA—or to the presence of DNA from dead organisms that could lead to false-positive results (31–34). Quantitative analyses are a more powerful way to interpret such positive results.

To improve the sensitivity of nucleic acid-based methods, the excess of human DNA should be removed. This could be achieved by removing white blood cells before nucleic acid extraction or by selective removal of human DNA after extraction. Nucleic acid-based methods, in particular PCR, are sensitive to various inhibition mechanisms. Some of these mechanisms have been characterized, but many of them remain unknown (29, 35–37). Inhibitory compounds can be contained in the sample or they can be the result of the sample preparation process. The blood contains some well-known PCR inhibitors such as hemoglobin, bile salts, and heme found in erythrocytes (30, 38, 39), as well as in lactoferrin found in leukocytes

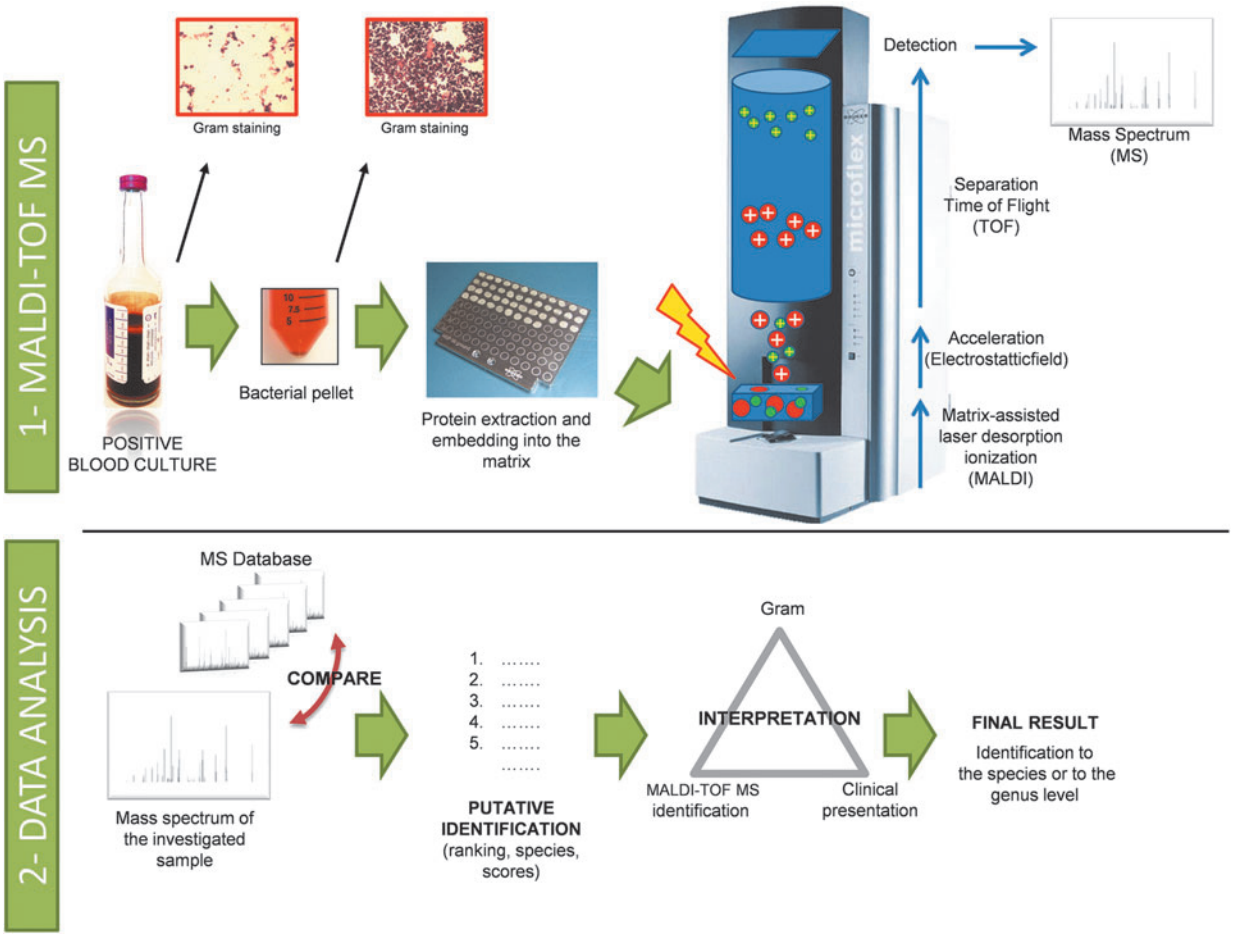

FIGURE 3 Microbial identification from positive blood culture using MALDI-TOF MS. The sample that is deposited on the MALDI-TOF multiwell plate can be a bacterial pellet obtained by centrifugation and erythrocyte lysis. Alternatively, the sample can be bacteria obtained after a subculture. Proteins are then extracted and embedded in a matrix directly on the multiwell conductive metal plate and submitted to the MALDI-TOF MS, which separates the proteins according to their molecular weights and their charges. This generates a mass spectrum (MS), which in a second stage is compared to a database of spectra. This analysis provides the identification of the microorganism with a confidence score, which allows acceptance at the species or at the genus level. The identification is interpreted according to the Gram staining and to the clinical presentation. Adapted with permission from reference 110.

(30). The inhibition of PCR-based methods by these compounds is due to the release of iron (from heme), which is known to interfere with DNA synthesis (40). Immunoglobulin present in the bloodstream, in particular IgG, can inhibit PCR by binding single-stranded DNA (41). Red blood cells present in blood bottles can also impair non-nucleic acid-based methods such as MALDI-TOF MS because they bring a large amount of proteins that would interfere with the quality of the protein mass spectrum. Several protocols are now available for their removal prior to MALDI-TOF MS analysis from positive blood bottles (24, 42, 43).

At present, each available molecular method is associated with a specific sample preparation. For the detection of a large number of organisms, the limited sensitivity of broad-range PCR targeting housekeeping genes has been overcome by the use of multiplex PCR or multiple real-time PCR. A recent technology, PCR/ESI-MS (PCR and electrospray ionization mass spectrometry), is a method that associates PCR and ESI-MS. The PCR allows the specific detection of small amounts of bacterial, fungal, or viral DNA from whole blood. This technology is associated with the analysis of PCR amplicons by ESI-MS, which determines the base composition rather than the nucleotide sequence. This has been shown to be sufficient for organism identification at the species level. Methods have been developed to remove the excess of nonbacterial DNA and/or inhibitors (30, 35, 37, 44). Most of these methods are time-consuming, require intensive labor, and are specific for one type of sample, one type of analysis, or one type of device.

MOLECULAR METHODS FOR THE IDENTIFICATION OF PATHOGENS FROM POSITIVE BLOOD CULTURES

Blood culture is currently the reference method for the identification of pathogens involved in BSIs because it is easy to perform and sensitive due to the large volume of blood that can be analyzed when multiple blood bottles are collected (24). When a blood culture is positive, conventional methods including phenotypic characterization

and rRNA gene sequencing have an extremely high specificity. However, these methods are time-consuming because they require an additional subculture and are challenged by a broad variety of molecular methods. FISH, the first molecular method to be developed, is based on the binding of specific probes on a pathogen's DNA. FISH is a rapid, sensitive, and specific method but is dependent on the choice of the probes to be tested. Microarrays allow the detection and identification of pathogens as well as resistance genes. PCR-based methods allow the rapid detection of a single organism (specific real-time PCR) or multiple organisms (broad-range PCR or multiplex PCR). These methods are highly sensitive to contaminating DNA or PCR inhibitors. MALDI-TOF MS, one of the latest commercialized technologies, is presented as a revolution for clinical microbiology laboratories (45). Indeed, MALDI-TOF MS can identify in less than 1 h most bacterial and fungal agents generally recovered from blood cultures.

Fluorescent *In Situ* Hybridization (FISH)

FISH is based on the specific binding of fluorescent peptide nucleic acid probes (PNAs) to the rRNA—16S rRNA for bacteria and 18S rRNA for fungi and other eukaryotes. Basically, slides are prepared from a positive blood culture, the fluorescent PNAs are applied, and unbound PNAs are removed by a washing step. Bound fluorescent PNA is generally observed by a fluorescent microscope because flow cytometry detection still needs further investigation and development (46). FISH requires a high number of living cells, which prevents its use directly on whole blood. Multiple probes that recognize microorganisms at the genus level or at the species level are available. All the solutions are based on multiple probes that detect and distinguish two or three organisms. The first kit of the PNA-FISH system (AdvanDx, Wolburn, MA) allowed the detection of *S. aureus* and coagulase-negative staphylococci (47–51). The second kit allows the detection of *E. faecalis* versus *Enterococcus faecium* and other *Enterococcus* spp. (52). *E. coli* and *P. aeruginosa* can be detected by using a PNA mix or a mix of three probes that also include *Klebsiella pneumoniae* (53–55). *Candida albicans*, *Candida glabrata*, *Candida parapsilosis*, and *Candida krusei* can also be detected (56–60).

The QuickFISH system is a more rapid version of the PNA-FISH system. The turnaround time is shortened by reducing the hybridization phases to 15 min and by removing the wash step. At present, the QuickFISH system provides kits that detect and distinguish *S. aureus*/coagulase-negative staphylococci, *E. faecalis*/*E. faecium*, and *E. coli*/*P. aeruginosa* (61–63). The diversity of microorganisms that are currently detected is still limited, but these bacterial species represent about 95% of the pathogens involved in BSIs. The choice of the probe to be tested will depend on the Gram result, the clinical presentation, and the local epidemiology. The sensitivity of the QuickFISH system is 99.5% for the detection of *S. aureus* and 98.8% for coagulase-
negative staphylococci, with a combined specificity of 89.5% (62). Martinez et al. reported 97.9% concordance with conventional detection methods for Gram-positive bacteria and 95.7% for Gram-negative bacteria (61). Yeast (*C. albicans*, *C. parapsilosis*, *Candida tropicalis*, *C. glabrata*, and *C. krusei*) can also be identified using probes targeting 26S rRNA sequences (60). Commercial methods give a result in 1.5 to 3 h with high sensitivity and specificity but cannot replace other detection methods because these probes do not detect many significant pathogens (Table 1) (52, 61, 64, 65).

Microarray

Microarrays are based on species-specific or genus-specific DNA probes immobilized on a chip on which the microorganism's DNA will specifically hybridize. The high density of the chips allows the use of sequences specific for different pathogens and probes for the detection of virulence factors and/or resistance genes. Species identification is determined by the pattern of hybridization and the intensity of the signal (66–69). The analytic sensitivity of microarrays ranges between 10 and 10^5 CFU/ml depending on the pathogen, which allows their use from positive blood culture but not directly from blood (67). The Verigene Gram-Positive Blood Culture system and the Verigene Gram-Negative Blood Culture system (Northbrook, IL) can detect Gram-positive or Gram-negative microorganisms, respectively, as well as associated resistance genes. The time to identification is approximately 2.5 h from positive blood culture bottles (Table 1). After being extracted from 350 μl of blood culture from a positive vial, the bacterial DNA is suspended in a specific buffer and hybridized on specific synthetic oligonucleotides followed by a second hybridization step involving gold particles. The signal is further amplified via a silver staining process which increases the sensitivity of the system. The reading is automated.

The Verigene Gram-Positive Blood Culture system can detect nine organisms at the species level (*S. aureus*, *Staphylococcus epidermidis*, *Staphylococcus lugdunensis*, *Streptococcus anginosus* group, *Streptococcus agalactiae*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *E. faecalis*, and *E. faecium*), four genera (*Staphylococcus* spp., *Streptococcus* spp., *Micrococcus* spp., and *Listeria* spp.), and three resistance genes (*mecA* [methicillin], *vanA* [vancomycin], and *vanB* [vancomycin]). Studies performed on both adult and pediatric patients reported 89.7 to 99% concordance for the detection from positive blood culture between the Verigene Gram-Positive Blood Culture system and the traditional method (61, 70–75). Misidentifications have been reported for *Streptococcus* spp. In particular, group mitis *Streptococcus* spp. have been misidentified as *S. pneumoniae*, and *Streptococcus oralis* has been misidentified as *S. anginosus* (74). Performance of the Verigene system is higher on monobacterial samples. Mixed culture can prevent the identification of one of the pathogens. Buchan et al. reported 98.6% sensitivity and 94.3% specificity for the detection of the *mecA* gene in 5,999 staphylococci cultures and 100% sensitivity and specificity for the detection of the *vanA* gene in 81 cultures containing *E. faecalis* and *E. faecium* (76). Samuel et al. reported 91% concordance for *mecA* and 100% for *vanA* detection compared to routine methods (74). In the case of mixed populations of staphylococci, the resistance gene cannot be associated with a specific pathogen (76).

The Verigene Gram-Negative system can detect five pathogens at the species level (*E. coli*, *K. pneumoniae*, *Klebsiella oxytoca*, *P. aeruginosa*, and *Serratia marcescens*) and four genera (*Acinetobacter* spp., *Citrobacter* spp., *Enterobacter* spp., and *Proteus* spp.). The Verigene Gram Negative BC assay displays 91 to 100% agreement for the detection of Gram-negative pathogens from both adult and pediatric patients (77–81). Among the false-negative results, mixed blood cultures have been incriminated, but some of these negative or noncalled results involved bacte-

TABLE 1 Commercially available molecular systems for the microbial identification of pathogens during BSI[a]

System (manufacturer)	Method	Time to result	Microorganism coverage	Resistance and virulence markers	Sensitivity, specificity, and correlation with conventional methods[b]	References
Identification from positive blood culture						
PNA FISH and QuickFISH (AdvanDx, Wolburn, MA)	FISH	<1–3 h	4 Gram-positive 4 Gram-negative 5 fungi	None	97–100% 90–100% 96–99%	54, 62–64, 243–247
AccuProbe (Gen-Probe, San Diego, CA)	FISH	<1 hour	*Staphylococcus aureus* *Enterococcus* spp. *Streptococcus pneumoniae* *Streptococcus* group A *Streptococcus* group B	None	80.8–100% 98.7–100% NR	248, 249
Verigene (Nanosphere, Northbrook, IL)	Microarray	2.5 h	12 Gram-positive 9 Gram-negative	*mecA*, *van*A/B, bla_{KPC}, bla_{NDM}, CTX-M,VIM, IMP, OXA12	81–100% 98–100% NR	70, 72, 73, 75–77, 79–82
Prove-it Sepsis (Mobidiag, Finland)	Microarray	3.5 h	60 bacteria 13 fungi	*mecA*	95% 99% NR	250
FilmArray (Idaho Technology, Salt Lake City, UT)	Multiplex PCR	1 hour	8 Gram-positive 11 Gram-negative 5 fungi	*mecA*, *van*A/B, bla_{KPC}	97–95% 91–98% NR	90, 92, 93, 224
Xpert MRSA/SA BC (Cepheid, Sunnyvale, CA)	Real-time PCR	1 hour	*S. aureus*	*mecA*	100% 99–100% NR	103, 104, 216–218
StaphSR assay (BD GeneOhm, San Diego, CA)	Multiplex PCR	1–2 h	*S. aureus*	*mecA*	96–100% 95–98% NR	219, 220
StaphPlex (Genaco Biomedical Products, Huntsville, AL)	Multiplex PCR + Microarray	5 h	*S. aureus*	*mecA* (+ PVL)	100% 95–100% 92%	107
MALDI-TOF MS Bruker Daltonics (Bremen, Germany) or bioMérieux (Marcy l'Etoile, France)	Mass spectrometry	<1 hour	<1,000[c]	Not in routine	–- – 76–99%	27, 61, 110, 115, 120, 251, 252

(*Continued on next page*)

TABLE 1 Commercially available molecular systems for the microbial identification of pathogens during BSI[a] (*Continued*)

System (manufacturer)	Method	Time to result	Microorganism coverage	Resistance and virulence markers	Sensitivity, specificity, and correlation with conventional methods[b]	References
Identification from whole blood						
SepsiTest (Molzym, Bremen, Germany)	Broad-range PCR + sequencing	6 h (1–10[d] ml)	>345 bacteria and fungi	None	21–87% 85–96% NR	142, 144–148
SeptiFast (Roche Molecular System, Germany)	Multiple broad-range real-time PCR	3.5–5 h (1.5 ml)	6 Gram-positive 8 Gram-negative 5 fungi	*mecA*[d]	43–95% 60–100% 43–83%	144, 155–158, 160, 161, 165, 167, 168, 238, 253–261
MagicPlex (Seegene, Seoul, Korea)	Multiple PCR+ multiplex real-time PCR	3–5 h (1 ml)	21 bacteria at species level (90 at genus level) 6 fungi	*mecA*, *vanA/B*	37–65% 77–92% 73%	146, 172
VYOO (SIRS-Lab, Jena, Germany)	Multiplex PCR + electrophoresis	8 h (5 ml)	14 Gram-positive 18 Gram-negative 7 fungi	None	NR NR 70%	145, 152, 262
PLEX-ID (Abbott Molecular, Des Plaines, IL)	Multiplex broad-range PCR + ESI-MS	6 h (1.25–5[e] ml)	Up to 800 (Gram-positive, Gram-negative, fungi)	*mecA*, bla_{KPC}, *vanA/B*	50–91%[d] 98–99% 79–97%	22, 212, 263, 264

[a]Adapted from references 24 and 29.
[b]NR, not reported.
[c]Dependent on the mass-spectrum database.
[d]With an additional kit.
[e]For the latest version.
[f]Depending on the volume.

ria covered by the chip (77). As for many molecular assays, it is not possible to distinguish *E. coli* and *Shigella* spp. (*Shigella dysenteriae*, *Shigella flexneri*, *Shigella boydii*, and *Shigella sonnei*). This distinction should be achieved with enzymatic analysis. The Verigene Gram-Negative assay can detect six resistance genes: the extended-spectrum beta-lactamase CTX-M and the carbapenemases KPC, NDM, VIM, IMP, and OXA groups with 92.3 to 100% concordance with routine methods (77, 82). Other microarrays for the detection of resistance genes are currently being developed (83).

The performances of microarrays are extremely high for the detection of both pathogens and resistance cassettes when applied to positive blood cultures, but this is supported by a limited number of studies.

Nucleic Acid Amplification-Based Methods

PCR provides a rapid and specific technology for pathogen identification from positive blood culture (84). The specificity provided initially by the use of specific primers can be increased in the case of real-time PCR by the use of specific probes. The use of PCR on blood or on hemorrhagic samples was limited for a long time by the fact that PCR is sensitive to the presence of inhibitory compounds such as hemoglobin contained in erythrocytes (30, 38, 39), lactoferrin contained in leukocytes (30), or immunoglobulin (41). To avoid inhibition, recently developed methods use new or improved nucleic acid extraction and/or amplification techniques.

Multiplex-PCR increases the time to result because it interrogates several targets (pathogens or resistance genes) at the same time. In addition, it can increase both the specificity and the sensitivity of an analysis thanks to the use of multiple targets for the same organism (85, 86). Multiplexing is achieved through the use of several specific primer pairs in the same reaction. Different methods can be applied to identify the amplified sequence(s): (i) the use of specific probes labeled with distinct fluorochromes, (ii) the analysis of the probe or the amplicon melting curves (87–89), (iii) the amplicon size (electrophoreses), and (iv) the sequencing of the amplification product. The latest technology, PCR/ESI-MS, is based on the analysis of the amplicon by MS (Fig. 4). The latter technique will be discussed in depth in the paragraph on pathogen detection from blood.

Multiplex PCR

The FilmArray system (Idaho Technology, Salt Lake City, UT) is a solution that allows the identification of more than 25 pathogens and four antibiotic resistance genes from positive blood culture in 1 h (90). The FilmArray is a closed system that uses multiple-PCR and is expected to identify 90 to 95% of the pathogens involved in BSIs as well as the resistance genes *mecA*, *vanA* and *vanB*, and bla_{KPC}. A kit for the identification of potential bioterrorism agents (*Bacillus anthracis*, *Francisella tularensis*, and *Yersinia pestis*) is also available (91).

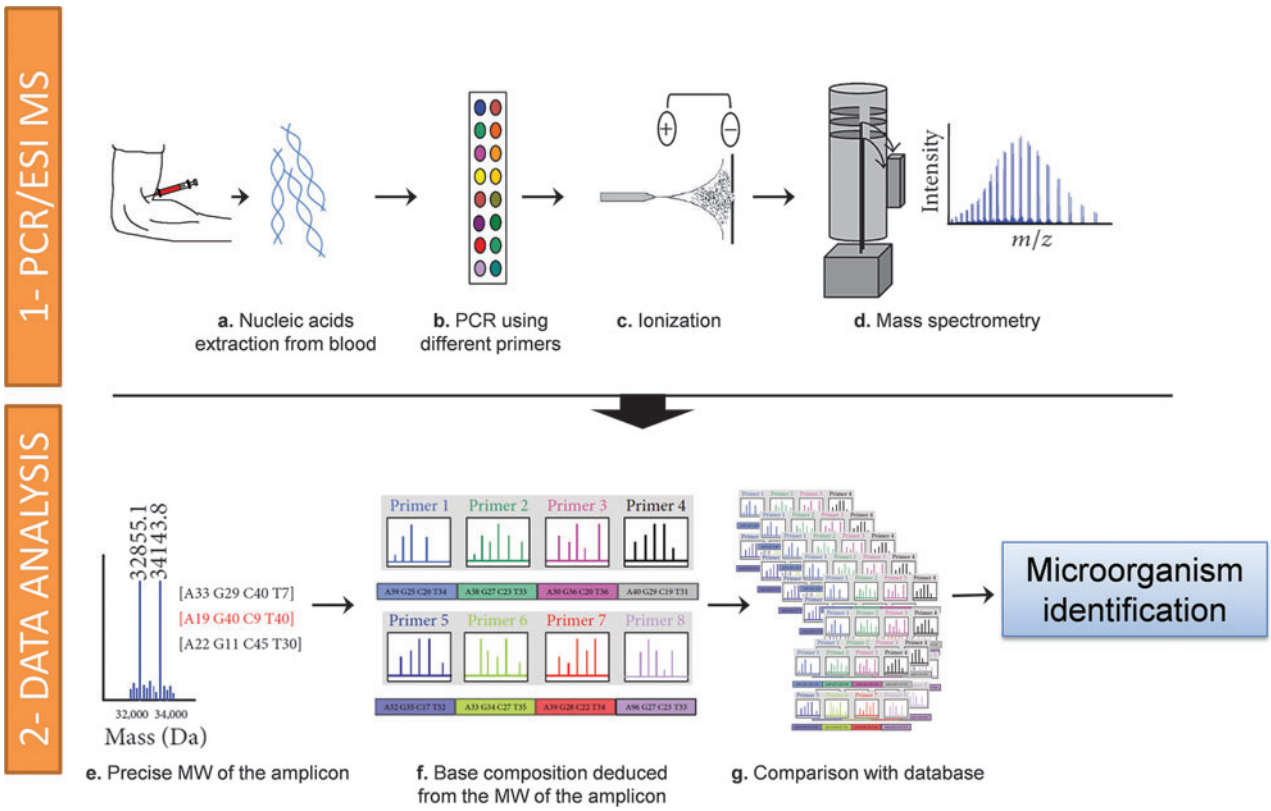

FIGURE 4 Schematic workflow of PCR/ESI-MS. (a) Nucleic acids are extracted from the sample, directly from whole blood, and (b) amplified by multiple PCR using multiple pairs of primers; each color represents a different primer. After amplification, the molecular mass of the amplicon(s) is precisely determined using ESI-MS (c, d, and e), from which (f) the base composition of the amplicon(s) is deduced. Finally, (g) information—base composition—obtained from one or more amplicon(s) is compared with a database, which provides the identification with a confidence score. Adapted from reference 123.

In a prospective study on 102 blood cultures, the FilmArray system displayed 91% sensitivity and 77% specificity when compared to conventional identification methods for the detection of pathogens present in the FilmArray panel only (90). The detection of resistance genes revealed 100% specificity and 100% sensitivity in this study. In another study, FilmArray displayed 98.5% sensitivity and 100% specificity for the identification of Gram-negative bacteria, 96.7% sensitivity and 93.7% specificity for Gram-positive bacteria, and 96% sensitivity and 98.9% specificity for the detection of the *mec*A gene (92). Another study, performed on 118 monobacterial cultures, reported 92% correct identification of the FilmArray system, but mixed cultures gave no results (79). The identification of *C. albicans* and *C. glabrata* reached 100% sensitivity and 99.5% specificity (92). This is consistent with other studies showing good performance of FilmArray for the identification *C. albicans* and *C. glabrata* (93). The FilmArray system can also be applied on other sterile samples such as cerebrospinal fluid (94).

Rapid PCR-Based Systems

The clinical and epidemiological impact of pathogens such as MRSA or rifampin-resistant *Mycobacterium tuberculosis* has contributed to the development of methods for the rapid detection of resistance. *S. aureus* is a significant agent of community-acquired and nosocomial infections (95). The mortality rate is increased in patients infected with MRSA in comparison to patients infected with MSSA (methicillin-sensitive *S. aureus*) (96–98). The increased percentage of MRSA in comparison to MSSA has highlighted the importance of rapid detection methods for MRSA (99–102). Several methods based on multiplex real-time PCR are available: the GeneXpert MRSA/SA BC Assay (Cepheid, Sunnyvale, CA) (103, 104), the StaphSR assay (BD GeneOhm, San Diego, CA) (105, 106), and the StaphPlex (Genaco Biomedical Products, Huntsville, AL) (107). The GeneXpert and StaphSR assays are rapid PCR-based systems developed for detecting *S. aureus* in only approximately 1.5 h. They require only limited hands-on time and skills. The application of the GeneXpert MRSA assay on positive blood culture was shown to have an impact on anti-infectious treatments by reducing the use of glycopeptides (108). Alternatively, the StaphPlex system can identify several staphylococci at the species level. The StaphPlex system is based on several PCRs (18 target genes) and microarray analysis for the identification of staphylococci at the species level and the detection of resistance genes and the Panton-Valentine leukocidin (PVL). The results are obtained within 5 h (107). Because these methods are largely aimed at detecting resistance genes, they will be discussed in the section dedicated to resistance detection.

Matrix-Assisted Laser Desorption Ionization-Time of Flight Mass Spectrometry (MALDI-TOF MS)

Principle of MALDI-TOF MS

MALDI-TOF MS is a method that allows the identification of a broad range of microbes at the species or genus level from positive culture. The method was first developed to be used on colonies before its application on blood culture. Microbe identification using MALDI-TOF MS is based on the analysis of the mass spectrum generated by the bacterial or fungal component, mainly housekeeping proteins (the ribosomal proteins which are basic are specifically extracted using acidic matrix). This spectrum is a unique fingerprint for each microorganism but displays features shared between genetically related bacteria (109). The comparison of the spectrum with a database of spectra obtained for characterized organisms allows microbe identification at the species or at the genus level depending on a score assigned by the identification software (110).

MALDI-TOF MS was first used for bacterial identification in 1975 (111). The routine use of mass spectrometry for the identification of bacteria in the diagnostic laboratory was proposed in 1996 when correct identification had been achieved directly from whole bacteria coming from single colonies (109, 112). Identification of bacteria using MALDI-TOF MS is divided into three steps: (i) ionization of the sample, (ii) separation of proteins in a flight tube, and (iii) generation of a mass spectrum by determining the mass of the proteins (Fig. 3). The sample preparation consists of mixing the cells from a bacterial colony with a crystallizing matrix that triggers the ionization of the sample achieved by a laser (MALDI). Protein accessibility can be facilitated by a quick formic acid extraction. Ionized proteins are accelerated by an electrostatic field and separated along a flight tube. The separation is due to the time of flight (TOF), a function of the mass and of the charge of the proteins. Protein detections will generate a mass spectrum unique for a defined bacterial strain corresponding to a mass-to-charge ratio (m/z) between 1,000 and 20,000 kDa. However, several peaks are shared between bacteria from the same genus or species and serve as biomarkers for bacterial identification. The identification is performed by comparison of the mass spectrum with a database of spectra obtained from characterized bacterial strains. The software that performed the spectral analysis and the comparison with the database assigns a score for the identification. Depending on the threshold recommended by the provider and the in-house algorithm, the identification can be rejected or accepted either at the species level or only at the genus level.

Application of MALDI-TOF MS on Positive Blood Culture Bottle

In clinical microbiology, MALDI-TOF MS was first used on pure bacterial colonies before its application on positive blood culture for the diagnosis of BSI (Fig. 3) (110). The initial analysis on a positive blood culture bottle is Gram staining. The Gram staining is especially recommended (i) to confirm that the detection is truly due to the presence of a microorganism, (ii) to give a presumptive etiology of the BSI, and (iii) to disclose mixed bacteremia. In positive blood cultures, the bacterial concentration ranges from 10^6 to 10^9 CFU/ml, which in theory is sufficient for MALDI-TOF MS identification. However, the presence of large amounts of nonbacterial material (erythrocytes, nutrient from the growth media) impairs the direct identification from the blood vial.

Application of MALDI-TOF MS After a Short Subculture

A short subculture (2 to 3 h) on agar plates can be performed from the positive blood vial to obtain a thin layer of bacteria (24). From this thin layer, the MALDI-TOF MS analysis can be performed (Fig. 2) (113, 114). Short subcultures are also adequate for antibiotic susceptibility testing using automated systems (113). However, this procedure is mainly suitable for fast-growing bacteria.

Application of MALDI-TOF MS Directly from the Blood Culture Vial via a Bacterial Pellet

The more rapid method is to perform the identification directly from the positive blood culture (Fig. 2 and 3). The goal is to get rid of nonbacterial components present in the bottles, such as red blood cells, and to concentrate the bacteria. Bacterial concentration can be achieved by centrifugation and erythrocyte lysis by ammonium chloride (43, 115). This generates a pellet that can be identified by MALDI-TOF MS (Fig. 3). This method allowed the identification of 78.7% of the samples obtained. Moreover, 99% of the MALDI-TOF MS identifications were correct at the species level (115). Alternative erythrocyte lysis techniques can be achieved with formic acid (116). Any methods applying mild detergent that would solubilize the erythrocyte membrane but not the microbe membrane lead to similar results (117–119). Alternatively, gel-based separator tubes have proven efficient in concentrating bacteria and removing red blood cells (120).

MALDI-TOF MS has a major impact on the time to result since it can be achieved directly from positive blood culture, without subculture (121). Sample processing and identification using MALDI-TOF MS takes approximately 1 hour, which makes it time-efficient for fast-growing bacteria as well (115, 116).

The accuracy of the MALDI-TOF MS identification is dependent on an extended and correct spectrum database. This database can be implemented with the spectrum obtained from clinical isolates. This is applicable for strains for which the MS identification failed and that were identified without any doubt with other methods such as gene sequencing or enzymatic assay. This is also applicable for rare pathogens that can be identified faster and more accurately using MALDI-TOF MS (122, 123). However, some closely related bacterial species such as the different *Streptococcus* species remain difficult to distinguish using MALDI-TOF MS. For instance, group mitis *Streptococcus* is often misidentified as *S. pneumoniae*. *S. pneumoniae* identification should thus be confirmed by phenotypic tests such as optochine susceptibility (75, 120, 124). Similarly, the distinction of *E. coli* and *Shigella* spp. is impossible using routine MALDI-TOF MS procedures and requires additional phenotypic confirmation (125, 126). Recently, a new approach based on the analysis of biomarker peaks was proposed to differentiate these two closely related pathogens (127).

Efficiency and Reproducibility of MALDI-TOF MS Identification

Even if a mass spectrum can be obtained from a single colony, this biomass represents a significant amount of bacteria. MALDI-TOF MS identification is therefore not applicable directly from blood but is dependent on microorganisms' proliferation in blood culture and on an additional concentration step from the positive blood culture (24). The requirement of a culture limits the use of MALDI-TOF for the detection of noncultivable bacteria.

Proteins used as biomarkers for MALDI-TOF MS identification of bacteria are mainly involved in housekeeping functions (128). Ribosomal proteins contribute to approximately half of the peaks present in the mass spectrum (129, 130). This makes it reproducible and robust because several peaks would be conserved in genetically related bacteria. Nevertheless, experimental conditions can impact the presence and the relative abundance of detected peaks. This is true for both routine identification and for the implementation of the peaks' pattern into the in-house database. The culture media, the growth conditions, and the age of the colony may impact the spectrum (131). The presence of agar residue or blood proteins in the sample may impact the spectrum quality (110). The quality of the matrix may also influence the spectrum (128).

Importance of Gram Staining and the Subculture

Gram staining on positive blood culture is still mandatory (i) to confirm the presence of a microbe in the bottles, (ii) to validate the identification of the MALDI-TOF MS, which should be congruent with the characteristic provided by the Gram staining (132), and (iii) to disclose polymicrobial infections, because MALDI-TOF MS poorly identifies mixed bacterial samples. Mixed infection may also be detected upon subculture, which remains essential for antimicrobial susceptibility testing (see below).

MOLECULAR METHODS FOR THE DETECTION OF PATHOGENS DIRECTLY FROM BLOOD

BSI is initially suspected by clinicians on the basis of clinical signs and symptoms. At this stage the etiological agent is, however, difficult to determine because the clinical presentation is generally similar. To accelerate the time to result of microbial diagnosis, molecular methods that can be used directly on whole blood have been proposed. Nevertheless, blood as a sample presented many technical limitations: (i) the low quantity of circulating microbes during BSI (1 to 10 CFU/ml), (ii) the presence of PCR inhibitors in blood that are not completely removed by current nucleic acid extraction methods (see "Introduction"), (iii) the sensitivity of PCR to contamination, which implies the use of pure (nucleic acid free) material and ideally performance of quantitative analyses (133), (iv) the ability of PCRs to detect the presence of DNA from both living and dead microbes, which makes the interpretation of positive results difficult (BSI versus DNAemia), and (v) the presence of an excess of human DNA.

Different PCR technologies are now available. Broad-range PCRs could be useful, especially when the etiological agent is not suspected, but they are often limited by lower sensitivity. Pathogen-specific PCRs display higher sensitivity. Real-time PCRs can be multiplexed to detect several targets at the same time with good sensitivity. A recent innovation that can be used directly on whole blood is PCR-ESI-MS, which is presented as a universal and fast method.

Broad-Range PCR

The need for a PCR-based universal detection method is stressed by the importance of rapid diagnosis for critically ill patients, especially since the etiology of the BSI is difficult to establish on the basis of the clinical presentations. This is the case for neonate patients, with the additional difficulty that only small volumes of blood are available, which significantly decreases the sensitivity of blood culture. Alternatively, such universal and sensitive methods may be used to screen blood samples taken from neutropenic patients or other high-risk subjects for early detection of the etiological agent of a BSI.

The 16S rRNA gene that displays a sufficient level of conservation among bacterial species is the target of in-house PCRs used in many molecular diagnostic laboratories (134–138). In many organisms, the ribosomal operon

is present in multiple copies, which increases the sensitivity of PCRs targeting this genomic region. The intergenic spacer is more polymorphic and more species specific. Specific PCR primers are designed on conserved regions that surround highly variable regions. Thus, the sequencing of the amplicon allows the identification of the microbe at the genus or species level. Most of these PCRs cannot be used directly on blood because of their limited sensitivity and specificity. However, some studies have reported sensitivity of PCR targeting the 16S rRNA gene ranging from 10 to $2.5.10^2$ CFU per reaction directly from blood (139, 140). Gaibani et al. have developed a broad-range, real-time PCR that targets a 97-base-pair sequence of the 23S rRNA gene. This PCR is expected to detect 90% of the bacteria involved in BSIs but does not give any identification, since the short amplified fragments do not allow discrimination upon sequencing (141). This real-time PCR can be used on whole blood (100 µl). Its sensitivity ranges from 10 to 10^3 CFU per reaction for *E. coli* and *S. aureus*, depending on the extraction method.

The SepsiTest system (Molzym, Bremen, Germany) is based on broad-range PCR using universal primers that target the 16S rRNA gene for bacterial identification and the 18S rRNA for fungal identification. The amplicons are sequenced and analyzed by BLAST to make identifications at the species level (≥99% identity) or genus level (≥97%) (142, 143). Of course, this cutoff does not apply to all bacterial species and should be used with caution. The analysis is achieved from 1 ml of whole blood. After lysis of human cells, the human DNA is degraded by a DNase. The PCR is achieved using the reagent provided by the manufacturer and leads to amplicons of about 450 base pairs. The time to result is approximately 6 h. Depending on the study, the sensitivity and specificity are variable when compared to blood culture. A first study performed in critically ill patients led to 28.6% sensitivity and 85.3% specificity of the SepsiTest when blood culture is used as the gold standard (144, 145).

In a second study, pathogens could be detected in 26% (13/50) of the critically ill patients, while SepsiTest could detect pathogens in only 12% of the patients (145). In a third study, still using blood culture as the gold standard, SepsiTest sensitivity was 21% and specificity was 96% (146). In contrast, high sensitivity and acceptable specificity (87% and 85.5%) were monitored in a multicenter study involving 342 blood samples from 187 patients (142). In a study performed on patients supported by extracorporeal membrane oxygenation, the sensitivity and specificity were 78.6% and 88.4%, respectively, when compared to blood culture, with 97.7% concordance of positive results (147, 148). In this study, SepsiTest could detect at least a pathogen in 25% of patients with negative blood culture. For patients with suspected endocarditis, Kühn et al. reported higher performances of SepsiTest over blood culture (143). The variable sensitivity and specificity of the SepsiTest compared to blood culture needs to be investigated. One specific feature of SepsiTest is that it can be used on sterile samples other than blood with the potential to replace home-made broad-range PCR (148, 149). A limitation of this method is that the extraction is not automated (150). Another important limitation of all these molecular tests is that they do not provide strains for detailed antibiotic susceptibility testing.

The VYOO system (SIRS-Lab, Jena, Germany) is based on multiplex PCRs that can detect 34 bacteria and seven fungi from 5 ml of whole blood. The system contains several steps, including nonautomated steps. Nucleic acid extraction is assisted by magnetic beads. Then there is a step of enrichment in microbial DNA based on (i) the methylation difference between microbial (bacteria and fungi) DNA and the human DNA and (ii) the use of chromatography affinity (44). The PCR is achieved on DNA, the concentration of which has to be manually adjusted to 1 µg in 25 µl. Two PCRs with a specific pool of primers are required. The amplicons are applied to electrophoresis in an ethidium bromide-stained agarose gel. Pathogen identification is determined on the basis of the band pattern.

In a study performed on 311 blood samples from 245 patients in an intensive care unit of a university hospital analyzed in parallel with blood culture and with the VYOO system, the VYOO system gave 30.1% positive samples, with a median time to result of 24.2 h; blood culture bottle gave 14.5% positive samples, with a median time to positivity of 68 h, with only a 40% correlation between the two methods (151). Positive PCR samples correlated well with the level of procalcitonin. Additionally, the PCR results from blood were usually confirmed by the pathogen identified in the suspected site of infection. Importantly, for 34% of the patients with positive VYOO tests, the anti-infectious therapy was inadequate, including infections that involved vancomycin-resistant enterococci, multidrug-resistant staphylococci, and fungi (151). In another study, the VYOO system gave positive results in 51.4% of samples from patients with BSIs, whereas blood culture gave 27.7% positive results (152).

These studies suggest that VYOO is more sensitive than blood culture, which could be explained by the association of a large starting volume (5 ml) and the enrichment in microbial DNA before amplification. More studies are required, but these preliminary performances are promising despite the fact that many steps are not yet automated.

Real-Time PCR and Multiplex PCR

The multiplexing of real-time PCRs allows the simultaneous detection of several microbes with good sensitivity and specificity. This is the technology used for the LightCycler SeptiFast system (Roche Molecular System, Germany) and the MagicPlex Sepsis system (Seegene, Seoul, Korea).

Principle of the LightCycler SeptiFast System

The LightCycler SeptiFast system is based on broad-range, real-time PCR that can identify 19 pathogens (8 Gram-negative bacteria, 6 Gram-positive bacteria, and 5 fungi) representing approximately 90% of the pathogens responsible for BSIs. SeptiFast can be used directly from whole blood with an overall time to result of about 5 h (3.5 when associated with an automated extraction system). The targets of the multiplexed broad-range, real-time PCR are the internal transcribed spacer between the 16S and the 23S ribosomal DNA for bacteria and the internal transcribed spacer between the 18S and the 5.8S ribosomal DNA for fungi. Identification at the species level is determined by the distinct melting curves of the specific probes. The LightCycler can detect eight Gram-negative bacteria: *E. coli*, *K. pneumoniae/K. oxytoca*, *Serratia marcescens*, *E. cloacae/E. aerogenes*, *Proteus mirabilis*, *P. aeruginosa*, *A. baumannii*, and *Stenotrophomonas maltophilia*. Six Gram-positive bacteria can be recognized: *S. aureus*, coagulase-negative staphylococci, *S. pneumoniae*, *Streptococcus*, spp., *E. faecium*, and *E. faecalis*. Six fungi can be detected and

identified at the species level: *C. albicans*, *C. tropicalis*, *C. parapsilosis*, *C. glabrata*, *C. krusei*, and *Aspergillus fumigatus*. Basically, the assay consists of three separated reactions that contain distinct primer and probe mixes to detect, respectively, Gram-negative bacteria, Gram-positive bacteria, and fungi. The LightCycler SeptiFast system is designed for the analysis of 1.5 ml of blood on which mechanical cell lysis is achieved with the SeptiFast Lys KIT MGRADE and the MagNALyser from Roche Diagnostics prior to DNA extraction using a commercial kit provided by the same manufacturer. The assay contains an internal control that consists of a synthetic double-stranded DNA, similar to the expected amplicon but with a distinct probe binding site.

The analytic sensitivity ranges from 3 to 30 CFU/ml for bacteria and is 100 CFU/ml for fungi (153, 154). The experimental sensitivity (42.9 to 95%) and specificity (60 to 100%) of SeptiFast are variable depending on the study and on the patient characteristics (Table 1) (32, 155).

Diagnosis of Infection in Febrile Neutropenia Using the LightCycler SeptiFast System

From a group of 86 febrile neutropenic patients representing 141 episodes of fever, blood culture and SeptiFast detected approximately the same number of microorganisms: 44/141 (31.2%) and 42/141 (29.8%), respectively. However, the association of blood culture and SeptiFast increases the rate of documentation from about 30 to 43% (61/141), which might be due to the fact that only 12 organisms were detected by both blood culture and SeptiFast (156). A similar observation was made by Mancini et al. in a study performed on 103 samples from neutropenic patients with hematological diseases in which 20.4% of the samples were found positive with blood culture and 33% were found positive using SeptiFast, with only 83% correlation between the two methods (157). For Bravo et al., the agreement between blood culture and SeptiFast was 69% for neutropenic patients and 75% for patients from the intensive care unit (158). All these studies suggest that SeptiFast cannot replace blood culture but that the two techniques could be complementary (156, 157, 159, 160).

Lamoth and colleagues suggested that the low sensitivity of SeptiFast was due (i) to organisms absent from the SeptiFast analytic spectrum (40% of false negatives) and (ii) to the cutoff that decreases the sensitivity for the detection of coagulase-negative staphylococci or streptococci and increases the rate of false-negative results (156). False-negative results obtained for Gram-negative cocci might also be explained by the inefficient lysis of these microorganisms (161) or to PCR inhibition in the case of high bacterial load. The adjustment of the experimental procedure and an adequate cutoff might increase the performance of the SeptiFast assay for neutropenic patients (156, 161). In a context of persistent fever, SeptiFast identified new pathogens in 89% of the cases, whereas blood culture identified 8% of the pathogens (156). Similar results were obtained by von Lilienfeld-Toal et al. (162). In this study all the patients with a probable fungal infection had positive SeptiFast results for A. *fumigatus*. This suggests a potential added value of the SeptiFast assay for the detection of fungemia in neutropenic patients (155–157, 162, 163).

Diagnosis of Infectious Endocarditis

A study performed on 63 patients with suspected endocarditis revealed a low sensitivity of SeptiFast when compared to blood culture (153). Among 19 patients with positive blood culture at admission, SeptiFast detected eight bacteria (41% sensitivity). Before admission, 22 patients had positive blood culture, but because of efficient antibiotic treatment they had negative blood culture at the time of this study. Among them, SeptiFast detected only three bacteria (3/22) (153). In this study, SeptiFast did not detect any microbes for patients without any etiology (100% specificity) (153). Because the low sensitivity of SeptiFast was not due to pathogens that are not detected by this system, it could be due, as discussed below, to a low bacterial load. In another study, involving 20 patients with endocarditis, SeptiFast performed on excised cardiac valves displayed a higher sensitivity (95%) and specificity (100%) than culture (sensitivity 15% and specificity 100%) (164, 165). In patients with suspicion of *Candida* spp. endocarditis, SeptiFast was as sensitive as blood culture to detect fungal infection (166).

Diagnosis of Neonatal Sepsis

In a study performed on 1,673 pediatric samples (803 children), the detection rate of infection was higher with SeptiFast (14.6%) than with blood culture (10.3%), which corresponded to a sensitivity of 85.0% and a specificity of 93.5% of the SeptiFast results (167). The cumulative positive rate of blood culture and SeptiFast was 16.5%. Another study confirmed a higher sensitivity but a lower specificity of SeptiFast for the detection of late-onset neonatal sepsis (168).

Advantages and Disadvantages of the LightCycler SeptiFast System

The performance of the SeptiFast system is variable depending on the study. The overall sensitivity of SeptiFast is not higher than the sensitivity of blood culture, but together SeptiFast and blood culture increase the rate of microbial documentation of BSI. This suggests that SeptiFast cannot replace blood cultures but that these two methods are complementary. One of the major advantages of SeptiFast is the time to result (<5 h). In a study performed on 114 consecutive patients with clinical evidence of sepsis the mean time to results for SeptiFast was less than 8 h, whereas the blood culture mean time to positive results was 3.5 days and for negative results was 5 days (169). In a study performed on emergency department patients with suspected sepsis, Schaub et al. reported a median time to positivity of blood culture of 16 h (without organism identification) (170). SeptiFast might be useful in cases of a persistent bacteremia, for instance, in a neutropenic patient with persistent fever, for which SeptiFast often identified additional pathogens. Many studies have been conducted on the performance of SeptiFast. However, there is a need for interventional studies to clearly determine the clinical impact of this new technology.

One of the limitations of SeptiFast is the absence of exact quantification. The cutoff can be used for the interpretation of streptococci- and coagulase-negative staphylococci-positive results in particular. This is expected to reduce the number of false-positive results but might prevent the detection of low-grade infections. In some studies performed on neutropenic patients, the sensitivity of SeptiFast for the detection of coagulase-negative streptococci is decreased, which could be because the cutoffs were established for nonneutropenic patients. In contrast, in pediatric studies SeptiFast leads to increasing numbers of false positives due to coagulase streptococci. Adding quantitative data to SeptiFast has the potential to make it possible to predict the severity of the sepsis (171).

The HACEK group of fastidious bacteria (*Haemophilus*, *Actinobacillus*, *Cardiobacterium*, *Eikenella*, and *Kingella*), which is involved in a significant number of blood culture-negative bacteremias, is not covered by SeptiFast. For this reason SeptiFast is not sufficient by itself to diagnose all types of bacteremia, and alternative methods are required to cover the entire bacterial kingdom. Finally, in the context of an increasing number of multidrug-resistant organisms, SeptiFast is limited in that it does not provide any data on the resistance profile of identified organisms, except for MRSA with the use of an additional kit (Table 1).

The MagicPlex Sepsis System

The MagicPlex Sepsis system is based on multiplexed real-time PCRs that can detect 90 pathogens at the genus level, 25 at the species level (19 bacteria and 6 fungi), and the resistance genes *mecA*, *vanA*, and *vanB* directly from whole blood (1 ml). A specific nucleic acid extraction kit is used for enrichment in microbial DNA. A first amplification is a screening that provides either (i) an amplicon bank that consists of Gram-negative bacteria and fungi or (ii) an amplicon bank that consists of Gram-negative bacteria (n = 73) and resistance genes conferring resistance to methicillin (*mecA*) or vancomycin (*vanA* and *vanB*). A second step is a screening for bacteria and fungi at the genus level and for the presence of resistance genes (time to result 5 h).

The performance of the MagicPlex system was compared to blood culture on 267 patients from the intensive care unit, from the hematology department, and from the emergency department of a tertiary hospital, which revealed a 73% agreement between the two methods, with no statistical difference between their sensitivity (172). For patients with antibiotic treatment, the sensitivity of MagicPlex (65%) was lower than the sensitivity of blood culture (71%); specificity was, respectively, 92% and 88% for the two methods. Another study, performed on 140 patients with suspected BSI, reported 37% sensitivity and 77% specificity of the MagicPlex system when considering blood culture as the gold standard (146).

Additional studies are required to determine the exact potential of this device. This method is limited by the number of pathogens detected at the species level and by the absence of quantification. Another limitation is the absence of automation and the need for a specific extraction method, which might make it difficult to integrate in an automated molecular diagnostic laboratory.

PCR-Electrospray Ionization Mass Spectrometry (PCR/ESI-MS)

Principle, Characteristics, and Performance of PCR/ESI-MS

PCR/ESI-MS is a technology that was initially developed to face bioterrorism threats or to run public health investigations. The aim was to provide a rapid method for the detection and identification of pathogens from various types of samples. The technology had to detect low quantities of a given pathogen even in polymicrobial samples and should include the detection of noncultivable or fastidious microorganisms. In addition, the technology should be able to detect known microorganisms as well as unknown pathogens. Molecular diagnosis was chosen because it is fast and broad range.

The PCR/ESI-MS approach is based (i) on the amplification of a microorganism's DNA by multiple PCRs and (ii) on the identification of the organism(s) at the species or genus level through the analysis of the amplicon by ESI-MS (23, 173). Basically, PCR/ESI-MS consists of five steps: (i) extraction of the microorganism or sample DNA, (ii) amplification of the DNA using multiple pairs of primers, (iii) precise determination of the molecular mass of the amplicon(s) using ESI-MS, (iv) deduction of the base composition of the amplicon(s) from the exact molecular weight of the amplicon, and (v) identification of the pathogen(s) by integrating the information obtained from several amplicon(s) (Fig. 4) (173, 174).

Before being applied to blood (see next paragraph) PCR/ESI-MS was first applied to bacterial colonies and environmental samples. DNA amplification is based on multiple broad-range PCRs, which is more sensitive than a single broad-range PCR based on degenerated primers. The primers target a conserved genomic region surrounding polymorphic regions (for instance DNA regions encoding for the 16S or the 23S rRNA). Moreover, these PCRs are devoid of fluorescent dye or probes, which allows a high level of multiplexing. DNA amplification may be performed in a 96-well plate via the following steps: (i) 95°C for 10 min; (ii) 8 cycles of 95°C for 30 s, 48°C increasing by 0.9°C at each cycle for 30 s, and 72°C for 30 s; (iii) 37 cycles of 95°C for 15 s, 56°C for 20 s, and 72°C for 20 s; and (iv) final extension of 2 min at 72°C and holding at 4°C (175). The major innovation of PCR/ESI-MS is that the pathogen's identification relies only on the base composition (A, C, G, and T) of the amplicons (23, 173). The base composition is obtained by the integration of the exact mass of the amplicon, the length of the amplicon, the mass of each base, and the complementarity rules of DNA (Fig. 4). The choice of the PCR targets makes the base composition of one or more amplicon(s) sufficient for pathogen identification. The absence of Sanger sequencing dramatically reduces the time to result(s). Thus, ESI-MS provides the base composition of an amplicon in about 1 min, the mass of the amplicon being determined by the TOF. The base composition of the amplicon(s) is compared to a database that provides the identification at the genus or species level with a score of probability (Q-score) inferred by ESI-MS triangulation software that relies on multiple amplified regions. The Q-score integrates multiple parameters such as the number of primer pairs that gave an amplicon, the number of potential microorganisms, and the proximity of the base compositions to reference matches in the database.

A set of nine pairs of primers is used for the coverage of the bacteria kingdom. Four pairs are necessary for *Candida* spp. Four additional pairs of primers have been designed to detect the resistance cassettes *mecA*, bla_{KPC}, *vanA*, and *vanB*. With clinical samples (up to 1.25 ml) other than blood, PCR/ESI-MS displays good sensitivity and specificity. This includes environmental samples as well as clinical samples such as cerebrospinal fluid (176–178) and respiratory tract samples (179–181). Compared to culture, the PCR/ESI-MS correctly identified 95.6% and 81.3% of the strains at genus level and species levels, respectively. Among 395 respiratory samples, PCR/ESI-MS displayed 67.6% of agreement at the genus level and 66.6% at the species level with culture. PCR/ESI-MS was able to identify fungi in 20.3% (35/172) of the respiratory specimens with a negative culture, suggesting a better sensitivity than culture (182). A commercial kit available for the detection of viruses (183–191) has been shown to display better performance than real-time PCR and microarrays (183).

PCR/ESI-MS has also been successful at identifying *Mycobacterium* spp. at the species level and at determining associated resistance genes using a panel of eight PCRs (192–194).

Thus, PCR/ESI-MS represents a universal method that can be applied to bacteria, viruses, and fungi and that is expected also to identify any unknown species. Indeed, when primers are designed to identify all known members of characterized groups, they allow the detection of unknown or new emerging pathogens from these groups (193, 195, 196). This provides the potential for the rapid detection of emerging pathogens. In addition, PCR/ESI-MS can detect and identify all of the pathogens present in polymicrobial samples with quantitative results. This method can also be used on formalin- or paraffin-embedded tissue (197).

Finally, because of its accuracy, PCR/ESI-MS has been extensively used for genotyping and serotyping of bacteria and viruses to detect single nucleotide variations, which represents a tool for health care epidemiological investigation or outbreak follow-up (181, 194, 198–208). This accuracy of the PCR/ESI-MS method naturally relies on a representative database and on its maintenance.

Application of PCR/ESI-MS to the Diagnosis of BSI Directly from Blood

The first PCR/ESI-MS instrument developed was TIGER (Triangulation Identification for the Genetic Evaluation of Risk), which allowed the detection of specific organisms even in polymicrobial samples (196). The Ibis T5000 instrument, the first commercial version of TIGER, could identify up to 800 pathogens from whole blood (23, 209). When compared to blood culture, PCR/ESI-MS applied on 1 ml of whole blood displayed 50% efficiency. A second commercial version, the PLEX-ID (Abbott), using up to 1.25 ml of blood, displays good sensitivity and specificity on most samples tested, which includes environmental samples as well as clinical samples such as cerebrospinal fluid and sputum (178, 179, 184, 193, 210). The first procedure of the PLEX-ID for whole blood consisted of mechanical cell lysis, using beads, facilitated by the addition of proteinase K, SDS, and heating at 56°C. DNA purification was then achieved with magnetic silica beads and eluted in 250 µl of water at 70°C (211). The PCR is based on nine pairs of primers for bacteria detection, four pairs for *Candida* species, and four pairs for detecting resistance cassettes (*mecA*, bla_{KPC}, *vanA*, *vanB*). One additional pair of primers corresponds to the extraction control. The PLEX-ID gave 78.6% agreement with blood culture over 906 specimens taken from adult and pediatric patients (464 with positive blood cultures and 442 with negative blood cultures). While 33 negative culture specimens were found positive by PLEX-ID, 97 positive culture specimens were found negative by PLEX-ID (211). In this study the estimated sensitivity of PCR/ESI-MS was 85.9%, whereas the estimated sensitivity of blood culture was 41.2%.

To further increase the sensitivity of detection directly from whole blood, a second version of the PLEX-ID that could analyze up to 5 ml of blood has been developed. In this version, the increased sample volume was associated with a DNA extraction method and PCR conditions optimized for whole-blood samples (22). In this version, 5 ml of whole blood was lysed in the presence of 665 µl of a commercial buffer (100 mM Tris solution containing guanidinium thiocyanate and detergent), 145 µl of bovine serum albumin 10% containing a pumpkin DNA extraction control, and yttria-stabilized zirconium oxide beads (212). After the removal of lysed red blood cells by centrifugation, the nucleic acid was extracted from the supernatant by means of silica-coated magnetic beads. The PCR used 30 µl of the eluate with 25 µl of PCR master mix containing the pool of primer pairs (211). Rather than trying to remove the excess of human DNA, PCR conditions were defined in the context of high concentrations of human DNA. This was achieved by testing multiple PCR conditions by modulating Mg^{2+}, primers, and polymerase concentration and annealing temperature in the presence of 12 µg of human DNA (22, 175). In this background, a high primer concentration (750 µM) together with a high polymerase concentration (2.2 units per reaction) resulted in a PCR yield of 86% of the yield when 1 µg human DNA was present. Modulating Mg^{2+} concentration or the annealing temperature had only a weak impact on the PCR yield. This procedure was then evaluated in a prospective study involving 331 patients with suspected BSI (22). For each patient two blood bottles (one aerobic and one anaerobic) were inoculated with 5 ml of whole blood, and an additional blood sample was collected for PCR/ESI-MS analysis from the same venipuncture (211). The PCR/ESI-MS displayed 83% sensitivity and 94% specificity compared to blood culture. Interestingly, this corresponded to 35 positive specimens (10.6%) by PCR/ESI-MS and 18 positive by culture (5.4%). In the absence of any method to investigate the discrepant results, a second aliquot was analyzed using PCR/ESI-MS, which confirmed almost all the identifications and increased the sensitivity of PCR/ESI-MS to 91% and the specificity to 99% (22).

The limit of detection of the PCR/ESI-MS method developed by Bacconi et al. is 16 CFU/ml for *S. aureus*, *K. pneumoniae*, and *E. faecium*, and 4 CFU/ml for *C. albicans*. It is generally admitted, based on plating methods, that the number of bacteria circulating during BSI is between 1 and 10 CFU/ml (17–21). The analysis of the data from the literature performed by Bacconi and colleagues estimated that the amount of bacterial DNA in blood during a BSI varies between 10^3 to 10^4 genome copies per ml (22). This difference could be due to the fact that plating methods reflect the number of cells that survive the procedure rather than the number of circulating cells. Molecular diagnosis directly from blood could also detect free DNA, DNA resulting from dead bacterial cells, and bacterial DNA present within phagocytes, which may explain the high performance of the PLEX-ID on whole blood. However, this extremely high sensitivity may lead to contamination by DNA. For this reason, the use of ultra-clean reagents associated with molecular biology laboratory practices is mandatory.

PCR/ESI-MS is also a promising tool for the diagnosis of blood culture-negative BSI. Indeed, among 464 patients with positive blood cultures and 442 patients with negative blood culture, 33 culture-negative cases were detected as positive by PCR/ESI-MS, and these cases were demonstrated to be true BSIs based on the analysis of the clinical presentation (211). Using primers and probes specific for *Ehrlichia chaffeensis* targeting the 16S gene and a genus-specific set of primers and probes, Eshoo and colleagues reported 18.8% of PCR/ESI-MS positive whole blood specimens among 213 blood samples from patients with suspicion of ehrlichiosis (175). From the same pool of specimens, PCR/ESI-MS identified *Rickettsia rickettsii* from four samples and *Neisseria meningitidis* from one sample.

PCR/ESI-MS was designed to detect all pathogens present in a mixed microorganism population (213, 214). This is also true for polymicrobial blood culture since PCR/ESI-MS could identify 29 bottles with mixed populations out of 234 positive blood culture bottles (215). A quantitative analysis performed using an internal control helps with (i) interpreting results for polymicrobial samples and (ii) identifying true positive versus contaminating organisms (211). Further studies are required to determine if the quantification could also help define the severity of the infection.

In conclusion, the PLeX-ID (now commercialized by Abbott under the name Iridica), which provides reliable results in less than 6 h, is a versatile system that may be directly used on blood starting from 5 ml of samples and that represents a complementary approach to blood cultures.

MOLECULAR DETECTION OF ANTIMICROBIAL RESISTANCE DURING BSIS

BSI is characterized by a high level of morbidity and mortality that increases with the delay of the introduction of an efficient anti-infectious therapy (1–8). The rapid identification of the pathogen gives a first indication of the effectiveness of the empiric therapy. However, rapid information on the resistance profile of the agent of a BSI would make it possible to better adjust the empirical treatment.

Rapid PCR-Based Methods for Resistance Detection from Positive Blood Cultures

Detection of Resistance Mechanisms of Gram-Positive Bacteria

S. aureus is a significant agent of both community-acquired infections and nosocomial infections (95). In recent years, an alarming increase has been observed in the percentage of MRSA, a drug-resistant pathogen associated with significant increases in mortality (96–100), which explains why many rapid methods are now available to simultaneously identify *S. aureus* and detect the *mecA* gene associated with methicillin resistance (Table 1) (101, 102).

The GeneXpert MRSA/SA BC Assay is a good example of such a dual detection method based on real-time PCR for the identification of *S. aureus* and the detection of the *mecA* gene. The analysis can be performed directly from positive blood culture in about 1 hour and was validated in both adult and pediatric patients with blood culture positive for Gram-negative cocci in clusters with 100% sensitivity and 99.5 to 100% specificity (103, 104). The GeneXpert MRSA/SA BC Assay dramatically reduced the time to detection of *S. aureus* and had an impact on the detection of MRSA (Fig. 2) (104, 216–218).

Another rapid PCR-based assay based on real-time PCR, the StaphSR assay, can identify and differentiate MSSA and MRSA from positive blood cultures in about 1.5 h with an analytical sensitivity of 15 DNA copies per reaction mixture, which corresponds to 10^3 DNA copies per ml (105, 106). The assay was validated on blood culture with a predominance of Gram-negative cocci in clusters showing 95.6 to 100% sensitivity and 95.3 to 98.4% specificity for the detection of MRSA (105, 216, 219, 220). Discrepant results are mainly explained by mixed culture or inhibition of the PCR. Negative MRSA detection is also due to MREJ (SCCmec right-extremity junction) variants that contain the staphylococcal cassette chromosome *mec* (SCCmec) without the *mecA* gene (221).

The StaphPlex system is based on multiple PCRs (18 target genes) and microarray analysis for the identification of staphylococci at the species level, for the detection of resistance genes, and for the detection of the PVL, with a time to result of about 5 h. The overall accuracy for the detection of staphylococci at the species level from monobacterial or polybacterial culture was 91.7% compared to conventional methods (107). The StaphPlex system exhibited 100% sensitivity and 95.5 to 100.0% specificity for the detection of the *mecA* gene. Similar results were obtained for the detection of the PVL gene (107).

The FilmArray is another PCR-based system that can detect the *S. aureus mecA* gene as well as the *vanA* and *vanB* genes implicated in vancomycin resistance in *Enterococcus* spp. (222, 223). The FilmArray system exhibits high sensitivity and specificity (100%) for the detection of the *mecA* gene from *S. aureus*-positive culture (90, 222, 224). Another study reported 96% sensitivity and 98.9% specificity for the detection of the *mecA* gene (92). For the detection of the *vanA* and *vanB* genes in *Enterococcus* spp., Blaschke et al. reported only 85% specificity of the FilmArray system compared to blood culture (90). In this study, the FilmArray system detected a *van* gene in two *Enterococcus* strains that were found to be vancomycin sensitive by culture-based methods. The second strain was an *Enterococcus casseliflavus* strain bearing the *vanC* gene. During the development of the system, more resistance markers have been successfully tested (90).

The microarray of the Verigene Gram-negative system includes probes for the detection of the *mecA* gene with 98.6% sensitivity and 94.3% specificity (76). This system also contains probes for the detection of vancomycin-resistant enterococci with 100% sensitivity and specificity in a prospective study from positive blood culture (76).

The major weakness of the nucleic acid methods is that they cannot assign the resistance gene to the correct microorganism in the case of mixed cultures. This is particularly limiting for the detection of the *mecA* gene in a mixed culture of *S. aureus* that, for instance, would contain contaminating coagulase-negative staphylococci (76).

Detection of Resistance Mechanisms of Gram-Negative Bacteria

Gram-negative bacteria can exhibit resistance mechanisms to multiple antibiotics, representing a major problem for the treatment of infections. In particular, *Enterobacteriaceae* have developed multiple resistance mechanisms to cephalosporins, such as extended-spectrum β-lactamase. This presented carbapenem as an alternative for the treatment of severe infections involving *Enterobacteriaceae* and nonfermentative bacteria such as *P. aeruginosa* and *A. baumannii*. However, resistance to carbapenem may also occur. It might be caused by reduced permeability with loss of porins, by the overexpression of efflux pumps, or alternatively, by the production of carbapenem-degrading enzymes, namely carbapenemases. The emergence of carbapenem-resistant strains and their spread worldwide are alarming both from a therapeutic point of view and from an epidemiological point of view. Nucleic acid detection systems are focusing on the bla_{KPC} gene, which encodes for *K. pneumoniae* carbapenemase. This is the case for the FilmArray system, which includes primers targeting the bla_{KPC} gene (79, 90, 92, 224), and the Verigene Gram-negative system, which contains probes for the detection of the bla_{KPC} gene (79). More studies involving KPC-positive strains are needed to determine the sensitivity and specificity of

these systems. Moreover, future tools should aim at detecting a broader range of genes encoding ESBL and/or carbapenemases.

Resistance Detection from Positive Blood Culture Using MALDI-TOF MS

The use of the MALDI-TOF MS for the detection of resistant strains was first proposed to distinguish between MRSA and MSSA colonies (225). However, recent developments are mainly directed toward Gram-negative bacteria in which MALDI-TOF MS may detect antibiotic-degrading proteins such as carbapenemases. A bacterial inoculum is incubated with ertapenem. The MALDI-TOF MS analysis performed on the supernatant is used to determine the shift of two specific peaks associated with the degradation of the carbapenem. This method has been validated on bacterial colonies with 100% sensitivity and 100% specificity for the detection of carbapenemase-producing strains (226). Carvalhaes and colleagues have proposed a method to be used directly from bacterial pellets obtained from positive blood culture, which is able to identify 72.4% (21/29) of the carbapenemase-producing isolates after 4 h of incubation (227). MALDI-TOF MS represents an alternative for the detection of carbapenemase from positive blood culture that has the advantage, over PCR, of providing a phenotypic result.

Resistance Detection Directly from Whole Blood

As stated above, the detection of resistance genes directly from blood would considerably impact the choice of therapy. Four primer sets of the PLEX-ID system are used to detect the resistance cassettes *mecA*, *vanA*, *vanB*, and bla_{KPC} (228, 229). The PLEX-ID system can also detect the presence of the *S. aureus* PVL (228). PCR/ESI-MS was also shown to be successful at identifying the mutations in *gyrA* and *parC* genes involved in *A. baumannii* resistance to quinolones (230, 231). In the case of a mixed population, quantitative analysis could help associate a resistance gene to the corresponding pathogen. The MagicPlex system can detect three resistance markers (*mecA*, *vanA*, *vanB*) directly from blood (146). From their study, Carrara et al. failed to detect 6 MRSA strains out of 10 using the MagicPlex system (172). The VYOO system can detect five resistance genes: *mecA*, *vanA*, *vanB*, β-lactamase bla_{SHV}, and β-lactamase $bla_{CTX\text{-}M}$. However, there is a lack of studies reporting the sensitivity of this method. Finally, the SeptiFast system contains primers for the detection of *mecA* only (Table 1).

Although molecular methods have the ability to detect some resistance genes, more studies are required to assess their performances and their clinical impact. In addition, increasing the number of available resistance markers would be an advantage.

CONCLUSIONS AND FUTURE DIRECTIONS

Nucleic acid methods have succeeded in overcoming most of the obstacles that limited the sensitivity of the detection of pathogens from blood-containing samples. The sensitivity and specificity of these methods are now adequate for use in diagnostic laboratories.

One major problem in assessing the performance of new molecular methods is that blood culture associated with traditional identification methods remains the gold standard. In this context, many assays display extremely high analytic sensitivity but limited apparent specificity due to a limited correlation with blood culture. This could be overcome by the use of other evaluation methods to challenge blood culture. In their study, Bacconi and colleagues showed that 16S sequencing is not an appropriate method to be used as reference for the evaluation of PCR/ESI-MS. Indeed, only 2 of the 35 samples that tested positive using PCR/ESI-MS were found to be positive by 16S sequencing when the sequencing was performed on the same material. Thus, one of the limits to developing new methods for the diagnosis of BSI is that blood culture remains the gold standard (22). To compare the performance of blood culture and other methods, clinical presentations and local epidemiology should be taken into account to properly investigate discrepant results (22, 211). Nevertheless, the poor correlation between conventional blood culture-based methods and some new molecular methods suggests that different pathogens are detected by these two methods and that these diagnostic tools should be complementary. Moreover, blood culture cannot be replaced since the availability of a strain in pure culture is mandatory to precisely test the antibiotic susceptibility of bacteria.

Although new molecular methods are very appealing, further studies are needed to determine their impact on the management of patients with BSIs (29). For instance, there is a lack of studies that address the impact on antibiotic stewardship. While most of the studies try to measure with precision the performance of the new molecular methods, it is not yet clear how these methods can be integrated in a molecular laboratory. This is particularly true because none of the methods would replace blood culture. Some practical details have to be investigated. Thus, the number of samples, the number of venipunctures, and the frequency of sampling for molecular diagnostics has to be addressed. Otherwise, information (routinely provided by the blood culture) on the likelihood of infection (such as a possible catheter infection) versus contamination would be missing.

Molecular methods can also be helpful for organisms that are phenotypically closely related or for rare organisms. However, because some genetically closely related organisms cannot be distinguished on the basis of molecular methods, such as *E. coli/Shigella* (125, 126) or group mitis streptococci/*S. pneumoniae* (75, 120, 124), phenotypic distinction methods remain crucial. In the same manner, the accuracy of many molecular methods is based on their databases, which have to be maintained by adding nucleic acid sequences or obtained by mass spectrum for new clinical isolates or rare organisms. For nucleic acid methods, it is also crucial to conduct a follow-up of the emergence of single nucleotide polymorphisms or mutations (insertion/deletion) that could affect the hybridization of specific primers and probes. One solution would be to rely on multiple targets (sequence or gene) for the identification of a given organism (86, 105).

As stressed earlier, Gram staining remains mandatory for blood culture-based methods since (i) it can help reveal polymicrobial infection, (ii) it serves as a quality control, and (iii) it sometimes helps with identification because of specific phenotypic traits. Nevertheless, the presence of a single morphotype on the Gram stain does not exclude the presence of multiple organisms, since *Enterobacteriaceae* often exhibit a similar Gram staining morphotype.

Regarding polymicrobial identification, the quantification provided by some nucleic acid-based methods can be

helpful at determining the significance of the respective organisms. Another research focus is the attempt to provide clinical scores that would predict the severity of bacteremia (35, 232). The time to positivity of blood culture has been proposed as a criterion for BSI severity (6, 233, 234). Similarly, quantification could provide some insight on the severity of the BSI. Thus, analysis of 250 whole-blood samples from 20 adult patients (13 survivors and 7 nonsurvivors) with culture-proven MRSA showed that the levels of *mecA* DNA were higher in the nonsurvivors (5.48 copies/ml) than in the survivors (4.58 log copies/ml; P = 0.003, two-tailed Mann-Whitney U test). This suggested that the level of *mecA* DNA in blood could potentially be used to monitor MRSA bacteremia and evaluate responses to therapy (235). Similarly, a positive correlation has been found between the level of *S. pneumoniae* DNA monitored by real-time PCR and the need for mechanical ventilation and the risk of septic shock and death (236, 237). Another study performed with SeptiFast on 94 patients reported that the median cycle threshold value was 16.9 for patients with severe septic shock and 20.9 for patients with non-severe sepsis and that a cycle threshold value <17.5 was correlated with more positive blood culture and longer hospital stays (171, 238).

Quantification can also help identify the presence of a contaminant. In this context, the threshold of the quantitative results becomes an important parameter to limit the number of false-positive and false-negative results. Molecular solutions devoid of quantitative or at least semiquantitative analysis will render the interpretation of the positive results difficult. The interpretation of polymicrobial as well as monobacterial detection is facilitated by the fact that PCR/ESI-MS gives a quantitative analysis performed using an internal control. This can also help identify true positive versus contaminating organisms (211) and determine the relevance of each pathogen for mixed infections (176). Further studies are required to determine if the quantification could also help define the severity of the infection.

Regarding persisting bacteremia in the context of an ongoing treatment, molecular methods have been able to rapidly detect new organisms that blood culture could not detect. However, nucleic acid methods are not adequate for the follow-up of persistent infections because they detect the persistence of DNA from dead organisms rather than a true persisting organism. Therefore, many methods are now being developed to detect only DNA from living organisms (239, 240). This could be of particular interest to monitor the efficiency of an antibiotic treatment (241, 242).

Indicating the presence of resistance genes is an important added value of some molecular methods that are especially helpful at identifying risks of treatment failure. Development should focus on the integration of more molecular markers. Molecular diagnosis may also detect the presence of virulence genes, which helps predict the severity of infection. Thus, the StaphPlex system helps identify staphylococci at the species level and detect resistance genes as well as the PVL with excellent sensitivity (100%) and specificity (>95.5%) (107).

In conclusion, molecular diagnostics has significantly improved the diagnosis of BSI due to the reduction of the time to result and to the high sensitivity and specificity. In particular, MALDI-TOF MS is a revolution for the diagnosis of BSI from positive blood culture. To improve the management of patients suffering from BSIs, microbiologists and clinicians should imagine new laboratories and algorithms that associate these new culture-independent and culture-dependent molecular methods with conventional methods, aiming to benefit from both diagnosis methods.

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Molecular Diagnosis of Gastrointestinal Infections

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27

Enteric infections cause significant morbidity, mortality, and financial cost worldwide. This chapter discusses the molecular techniques for laboratory detection of the bacteria, viruses, and parasites most commonly responsible for infection of the lower gastrointestinal tract.

BACTERIAL GASTROINTESTINAL DISEASE

Bacterial agents of gastroenteritis are responsible for overwhelming morbidity and mortality worldwide, with an estimated 76 million cases and 5,000 deaths annually (1). Interestingly, the etiologic agents of community-acquired diarrheal diseases are limited to a short list of Gram-negative organisms (Table 1). Without exception, all of these pathogens are transmitted through consumption of contaminated food and water or direct fecal-oral inoculation. Laboratory workup of enteric pathogens consists of fecal culture on a selective medium followed by biochemical and serotypic identification. Not only is conventional culture slow (1 to 5 days), but phenotypic identification can be inaccurate due to immense variability in the biochemical properties of pathogenic bacteria (1, 2).

Numerous studies have reported on laboratory-developed PCR assays for the detection of enteric pathogens (discussed in detail below). Overall, multiplex, real-time PCR assay is favored because of its speed and elimination of postamplification carryover. With the recent availability of commercial, Food and Drug Administration (FDA)-approved multiplex PCR assays for syndromic diagnosis of gastrointestinal infections, the application of molecular tests for the detection of enteric bacterial pathogens in the routine microbiology laboratory is going to become more common. The commercial PCR assays provide comprehensive coverage of the pathogens listed in Table 1. Combined with their ease of use, they make possible rapid and accurate detection of pathogens in the routine laboratory. Here we summarize the advances achieved thus far for the detection of enteric bacterial pathogens by molecular methods and discuss the remaining challenges. We will also discuss the special case of hospital-acquired *Clostridium difficile* infection and the role of molecular methods in its diagnosis.

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Community-Acquired Bacterial Pathogens

Enterohemorrhagic *Escherichia coli*

Shiga toxin-producing *E. coli* (STEC), also known as enterohemorrhagic *E. coli* (EHEC), is the etiologic agent of sporadic and epidemic diarrhea, hemorrhagic colitis, and hemolytic uremic syndrome (HUS) (3, 4). Among hundreds of serotypes, O157:H7 has emerged as the most important cause of HUS in North America and Europe, causing 73,000 diarrhea cases in the United States annually (1). Non-O157 serotypes, which now exceed O157:H7 in incidence in several states (5), also cause both sporadic cases and community outbreaks of diarrhea and can be equally pathogenic (6–8). Most notably, in May and June 2011, a large outbreak of *E. coli* O104:H4-associated diarrheal illness in Germany led to HUS in over 800 patients, many of whom were adults, and ultimately resulted in 54 deaths (9). STEC expresses many virulence determinants including the Shiga toxins, or verocytotoxins Stx1 and Stx2, and adhesin intimin (Eae) (10). Stx1 and Stx2 share considerable homology with the Shiga toxin produced by *Shigella dysenteriae*, with Stx1 being nearly identical (11).

Timely laboratory identification of STEC has important implications for outbreak containment and patient management, including prompt parenteral hydration, monitoring for development of HUS, and avoidance of antibiotics and antidiarrheal agents, which can exacerbate disease (3). Laboratory isolation of STEC is based on active screening of diarrheal stool on O157:H7 selective agar such as sorbitol-MacConkey or O157:H7 chromogenic agar and is confirmed with biochemical and serotypic tests. Because selective agar does not detect non-O157 STEC (12), the CDC has issued recommendations to test simultaneously for O157 and non-O157 STEC in all stool specimens from patients with acute community-acquired diarrhea using enzyme immunoassays (EIAs) for Shiga toxin antigens and nucleic acid-based methods for detection of the corresponding genes (*stx1* and *stx2*) (13). EIA and PCR can be done directly on stool samples or after overnight enrichment in a selective broth. A number of EIAs have been approved by the FDA for the diagnosis of human STEC infections and have the ability to detect non-O157 STEC in addition to O157, but EIAs can have relatively high

TABLE 1 Enteric bacterial pathogens identified in the microbiology laboratory

Pathogen	Disease	Detection assay[a]
Escherichia coli O157:H7 and non-O157:H7	Hemorrhagic colitis, hemolytic uremic syndrome	SA, BC, AG
Shigella spp.	Dysentery or shigellosis	SA, BC, AG
Salmonella enterica	Gastroenteritis, typhoid fever	SA, BC, AG
Yersinia enterocolitica	Enterocolitis	SA, BC
Campylobacter spp.	Gastroenteritis	SA, BC
Vibrio spp.	Cholera, gastroenteritis	SA, BC
Aeromonas spp.	Gastroenteritis	BC
Plesiomonas shigelloides	Gastroenteritis	BC

[a]SA, selective agar; BC, biochemicals; AG, serologic agglutination.

false-positive rates and have been reported to miss *E. coli* O157 (13).

Various laboratory-developed and commercial PCR assays have been developed for the detection of STEC from stool. Although multiplex PCR assays identifying O157:H7 and non-O157:H7 serotypes have been reported (14), multiplex, real-time PCR assays have also been developed to detect all STEC serotypes by targeting *stx1* and *stx2* gene sequences (5, 15–17). There are currently two commercial FDA-approved PCR assays available for detection of STEC, and several more are in late-stage development (18). These assays were shown to be highly sensitive and specific when tested on patient and animal stool samples. Table 2 lists the primers used to detect *stx1* and *stx2* in human fecal specimens. A limitation to this approach, however, is the finding that 5% of HUS-causing EHEC are *stx*-negative and therefore would not be detected by assays that target Shiga toxins (19). It is possible that targeting of other virulence factors conserved among EHEC, such as *eaeA*, may further increase the sensitivity of detection of *stx*-negative EHEC (20, 21).

Campylobacter Species

Campylobacter jejuni and *Campylobacter coli* are the most common causes of gastroenteritis globally and account for more than 1.4 million cases in the United States annually (1, 22). Sporadic cases and outbreaks of diarrheal diseases caused by other *Campylobacter* species such as *Campylobacter lari* and *Campylobacter upsaliensis* also occur (23, 24). The majority of *Campylobacter* infections result from consumption of undercooked poultry and other meats (25). Furthermore, invasive disease and immune-mediated complications can follow *Campylobacter* enteritis (26). Laboratory diagnosis of *C. jejuni* and *C. coli* is done by culture on

TABLE 2 Gene targets and multiplex, real-time primers for the detection of STEC

Target	Primers/probes	Sequence	Reference
stx1	1Slt224	ATGTCAGAGGGATAGATCCA	15
	1Slt385	TATAGCTACTGTCACCAGACAAT	
	1SltB1FAM	GCGAGGCGCTTTGCTGATTTTTCACATGTTACCCCTCGC	
stx2	2Slt537	AGTTCTGCGTTTTGTCACTGTC	
	2Slt678b	CGGAAGCACATTGCTGATT	
	2SltB1TET	GCGAGGCACTGTCTGAAACTGCTCCTGTCCTCGC	
stx1	Forward	TCTCGACTGCAAAGACGTATGTAGA	21
	Reverse	TCCTGATGAAATAGTCTGTAATGGAGTAC	
	Probe FAM	FAM-TCGCTGAATGTCATTCGCTCTGCAATA-TAMRA	
stx2	Forward	ACCCCACCGGGCAGTT	
	Reverse	GGTCAAAACGCGCCTGATA	
	Probe FAM	FAM-TTTTGCTGTGGATATACGAGGGCTTGATGT-TAMRA	
eaeA	Forward	TGTTGCTTTGTTTAATTC(T/C)GATAAGC	
	Reverse	GGAATCGGAGTATAGTTTACACCAA	
	Probe FAM	FAM-AGTCGAATCCTGGTGCGGC-TAMRA	
stx1	FWD 03	GCGGTTACATTGTCTGGTGA	5
	REV 03	AGAACGYCCACTGAGATCAT	
stx2	FWD 01	CATGACAACGGACAGCAGTT	
	REV 02	CGGAAGCACATTGCTGATT	
stx1	FWD 02	TCGCTTTGCTGATTTTTCACA	5
	REV 02	ATGGCGATTTATCTGCATCC	
stx2	FWD 03	TCATCATATCTGGCGTTAATGG	
	REV 01	GACAGTGCCWGACGAAATTCTC	

selective agar under microaerophilic conditions at 42°C, though other pathogenic *Campylobacter* species may not grow under these conditions. Cultures are incubated for 48 to 72 hours before being evaluated. Species determination is done by biochemical tests, but the accuracy of these assays is suboptimal, and most laboratories do not routinely distinguish between C. *jejuni* and C. *coli* (27). However, differentiating these species may have some clinical value because C. *coli* has been shown to be more resistant to macrolide antibiotics (28).

To improve the accuracy and speed of identification, a number of PCR assays have been developed for the detection and identification of C. *jejuni* and C. *coli* directly from fecal specimens (29). In addition, rapid, sensitive, and specific real-time PCR assays have been developed for the detection of C. *jejuni* and C. *coli* from culture and fecal samples spiked with organisms (30, 31). The primer sequences and targets described are shown in Table 3. Validation of PCR assays on infected human fecal specimens shows increased sensitivity over conventional methods. Schuurman et al. applied primers developed by Best et al. and others in a monoplex, real-time PCR to identify C. *jejuni* in patient stool specimens. They showed that real-time PCR had superior sensitivity (98%) for the detection of C. *jejuni* when compared to conventional culture (78%) (32). The "gold standard" was defined as all culture-positive and all PCR-positive specimens confirmed by a secondary PCR of an independent target. In another study, Ghosh and colleagues showed enhanced sensitivity of PCR over culture (33). The performance of multiplex assays for C. *jejuni* and C. *coli* identification in human stool specimens has also shown increased sensitivity compared to conventional methods (33, 34). However, similar to existing laboratory practices, these assays do not detect non-C. *jejuni* and non-C. *coli* species, which are known to cause enteric disease in humans. This issue was partially addressed in a real-time PCR assay and a hybridization assay developed to detect C. *jejuni*, C. *coli*, C. *lari*, and C. *upsaliensis* in pig fecal samples compared to culture (35). Others have used *Campylobacter* species-specific primers (36). Another issue that needs to be addressed is the decreased sensitivity of culture due to the delay in media inoculation when PCR results are used to guide decisions on whether to culture for antibiotic susceptibility testing (32).

Salmonella, *Shigella*, *Yersinia enterocolitica*, and *Plesiomonas shigelloides*

The *Enterobacteriaceae* family includes a number of pathogens that cause intestinal infections. Except for *P. shigelloides*, for which causality has not been proven, *Salmonella*, *Shigella*, and *Y. enterocolitica* (STEC is discussed above) are intrinsic enteric pathogens in humans. While *Shigella* is spread through person-to-person transmission, *Salmonella*, *Yersinia*, and *P. shigelloides* are transmitted from animals to humans through consumption of contaminated water and food products. The genus *Shigella* consists for four species or subgroups, all of which can cause dysentery in humans. There are about 450,000 cases of shigellosis in the United States each year (1). The genus *Salmonella* consists of two species, *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies, I to VI. Up to 99% of *Salmonella* isolates from clinical specimens belong to *S. enterica* subsp. *enterica* (subsp. I) (37).

TABLE 3 Gene targets and multiplex, real-time PCR primers for the detection of C. *jejuni* and C. *coli*

Target	Primers/probes	Sequence	Reference
hipO	C. *jejuni*-F1	TGCTAGTGAGGTTGCAAAAGAATT	31
	C. *jejuni*-R1	TCATTTCGCAAAAAAATCCAAA	
	C. *jejuni*-FAM	ACGATGATTAAATTCACAATTTTTTTCGCCAAA	
glyA	C. *coli*-F1	CATATTGTAAAACCAAAGCTTATCGG	
	C. *coli*-R1	AGTCCAGCAATGTGTGCAATG	
	C. *coli*-VIC	TAAGCTCCAACTTCATCCGCAATCTCTCTAAATTT	
mapA	C. *jejuni*-forward	CTGGTGGTTTTGAAGCAAAGATT	30
	C. *jejuni*-reverse	CAATACCAGTGTCTAAAGTGCGTTTAT	
	C. *jejuni*-probe-FAM	TTGAATTCCAACATCGCTAATGTATAAAAGCCCTTT	
ceuE	C. *coli*-forward	AAGCTCTTATTGTTCTAACCAATTCTAACA	
	C. *coli*-reverse	TCATCCACAGCATTGATTCCTAA	
	Cc-probe-VIC	TTGGACCTCAATCTCGCTTTGGAATCATT	
glyA	C. *jejuni*-F	TAATGTTCAGCCTAATTCAGGTTCTC	35
	C. *jejuni*-R	GAAGAACTTACTTTTGCACCATGAGT	
	C. *jejuni*-FAM	AATCAAAGCCGCATAAACACCTTGATTAGC	
glyA	C. *coli*-F	GTTGGAGCTTATCTTTTTGCAGACA	
	C. *coli*-R	TGAGGAAATGGACTTGGATGCT	
	C. *coli*-TET	TGCTACAACAAGTCCAGCAATGTGTGCA	
glyA	C. *lari*-F	CAGGCTTGGTTGTAGCAGGTG	
	C. *lari*-R	ACCCCTTGGACCTCTTAAAGTTTT	
	C. *lari*-TET	CATCCTAGTCCATTCCCTTATGCTCATGTT	
glyA	C. *upsaliensis*-F	TCGTAGCTGGTGAGCATCCTAG	
	C. *upsaliensis*-R	GGTTTTGTGTGTGGTTGAGCTT	
	C. *upsaliensis*-FAM	CCTTTCCCTCACGCACACATCG	

There are 1.4 million cases of diarrhea due to *Salmonella* each year in the United States (1). Animals are the main reservoir of salmonella, and food products derived from them are the main source of infections in humans. *Y. enterocolitica* is among one of three *Yersinia* species that cause disease in humans. Unique to *Y. enterocolitica* is the enhanced ability to replicate at lower temperature, which explains its geographic distribution in the cooler regions of North America and Europe and the association with septic transfusions of blood products stored in the refrigerator (38). Although many animal species carry nonpathogenic *Y. enterocolitica* in their gastrointestinal tracts, pathogenic strains are more commonly acquired through consumption of contaminated swine products (39). Diarrheal disease due to *P. shigelloides* occurs most frequently in humans residing in or visiting tropical countries and is associated with consumption of seafood (40). Laboratory diagnosis of *Salmonella*, *Shigella*, and *Y. enterocolitica* is done by culture on selective agar followed by biochemical and serotypic identification. *P. shigelloides* is cultured on nonselective blood agar and is similarly identified by biochemical tests. For *Y. enterocolitica*, cultures are incubated at room temperature for optimal growth.

A number of groups have developed real-time PCR assays to detect pathogenic *Enterobacteriaceae*. Schuurman et al. showed increased sensitivity (100%) of monoplex, real-time PCR for the detection of *S. enterica* from diarrheal stool over routine culture (87%) (32). Iijima et al. also showed increased sensitivity over culture of multiplex, real-time PCR targeting two regions of the invasion (*inv*A) gene for detection of *S. enterica* and other enteric bacterial pathogens (21). The primer sequences used in these studies are shown in Table 4. Detection of *Shigella* species by PCR is challenging because *Shigella* cannot be easily distinguished from enteroinvasive *E. coli*. However, both pathogens have a similar clinical presentation and management, thus perhaps negating the need to distinguish them from each other (41). Nearly all PCR assays for *Shigella* and enteroinvasive *E. coli* target the invasion plasmid antigen H (*ipaH*) gene (42). Souza et al. showed increased sensitivity of multiplex, real-time PCR compared to culture for the detection of *Shigella*/enteroinvasive *E. coli* from diarrheal stool of children (43). Identification of pathogenic *Yersinia* is facilitated through detection of sequences encoded on a virulence plasmid (44). This is useful given that many species of nonpathogenic *Yersinia* exist. Khare et al. showed 100% sensitivity for detection of *Y. enterocolitica* from human stool samples in Cary-Blair medium (45). Unlike other enteric pathogens, real-time PCR assays for the detection of *P. shigelloides* have been minimally tested in the clinical laboratory (46–48).

Vibrio Species

The *Vibrio* genus is made up of 82 species of aquatic bacteria (www.bacterio.net), 12 of which cause disease in humans. Members of this genus are responsible for both sporadic and epidemic enteric infections, with toxigenic *Vibrio cholerae* accounting for the majority of cases globally. In the developed world, *Vibrio parahaemolyticus* and *Vibrio vulnificus* infections associated with seafood consumption account for the majority of reported cases of gastroenteritis (49). Laboratory isolation and identification of *Vibrio* species from stool is performed on thiosulfate-citrate-bile salts-sucrose agar. Pure cultures are subsequently identified by biochemical assays, which have limited accuracy for species identification (2, 50), or by matrix-assisted laser desorption ionization-time of flight mass spectrometry, which has great accuracy (51). Several groups have designed sensitive and specific gel-based multiplex PCR assays for the identification of enteropathogenic *Vibrio* species (52–54). A multiplex, real-time PCR assay for the identification of *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus* has also been reported (55). The gene targets from these assays are shown in Table 5. Although these assays outperformed conventional methods, only one was validated on stool specimens (52).

Hospital-Acquired Bacterial Pathogens: *C. difficile*

C. difficile is an anaerobic, Gram-positive, spore-forming rod that is the etiologic agent of *C. difficile*-associated diarrhea (CDAD), pseudomembranous colitis, and toxic megacolon (56, 57). CDAD is a significant problem in health care facilities, where conditions predispose to *C. difficile* infection. Health care costs attributed to *C. difficile* exceed $1 billion annually in the United States (58, 59), and over the past decade we have witnessed the emergence of a more virulent and resistant *C. difficile* clone (BI/NAP1) (60, 61). CDAD is thought to occur when the gut microbiota is

TABLE 4 Gene targets and real-time PCR primers for the detection of *Salmonella*

Target	Primers/probes	Sequence	Reference
*inv*A	Styinva-JHO-2-left	TCGTCATTCCATTACCTACC	365
	Styinva-JHO-2-right	AAACGTTGAAAAACTGAGGA	
	Target probe	FAM-TCTGGTTGATTTCCTGATCGCA-TAMRA	
*ttr*BCA	ttr-6 (forward)	CTCACCAGGAGATTACAACATGG	366
	ttr-4 (reverse)	AGCTCAGACCAAAAGTGACCATC	
	Target probe (ttr-5)	FAM-CACCGACGGCGAGACCGACTTT-Dark Quencher	
*inv*A	Forward-1	TTCCATTACCTACCTATCTGGTTGATT	21
	Reverse-1	GAACGACCCCATAAACACCAA	
	Probe	FAM-CCTGATCGCACTGAATATCGTACTGGCG-TAMRA	
	Forward-2	AGCGCCGCCAAACCTAA	
	Reverse-2	AACGACGACCCTTCTTTTTCC	
	Probe	FAM-AGCAAAGGCGAGCAGCCGCTTAGT-TAMRA	

TABLE 5 Gene targets used for identification of *Vibrio* species

Target	Species	Assay	Reference
dnaJ	*V. cholerae*	Conventional, multiplex PCR	52
	V. mimicus		
	V. parahaemolyticus		
	V. vulnificus		
	V. alginolyticus		
sodB	*V. cholerae*	Conventional, multiplex PCR	53
	V. mimicus		
flaE	*V. parahaemolyticus*		
hsp60	*V. vulnificus*		
toxR	*V. cholerae*	Conventional, multiplex PCR	54
	V. vulnificus		
	V. parahaemolyticus		
ftsZ	*V. cholerae*	Real-time, multiplex PCR	55
	V. vulnificus		
	V. parahaemolyticus		

altered in favor of C. *difficile* due to antibiotic treatment and/or other medical and surgical interventions (62–64). The pathology and symptoms that follow are mediated by three secreted toxins, known as toxin A (TcdA), toxin B (TcdB), and the binary toxin (65–68). Except for TcdB, which is usually present in toxinogenic strains, the other two toxins may be absent in some clinical isolates (69–72). As with any life-threatening infection, rapid diagnosis of CDAD is of primary importance for execution of appropriate medical and/or surgical intervention, as well as for containing the spread of infection to other hospitalized patients. The presence of C. *difficile* in the stool should be interpreted cautiously, though, because 50% of neonates, 2 to 6% of healthy adults, and 8 to 20% of hospitalized patients are colonized with C. *difficile* (65, 73–80). Thus, diagnosis of C. *difficile* should only be investigated in patients who have been hospitalized for ≥72 hours and are producing at least three loose stools per day (69, 81). Furthermore, due to the lack of a test of cure and risk of false-positive results, repeat testing on a routine basis is not recommended (82, 83).

Choosing an optimal diagnostic assay for rapid and sensitive detection of C. *difficile* in stool requires awareness of the existing assays. The gold standard for the diagnosis of CDAD is the cytotoxin assay, which detects the presence of TcdB in stool filtrate by assaying for cytopathic effect on cultured mammalian cells (84). A high degree of specificity is achieved by testing for this cytopathic effect in the presence and absence of an anti-TcdB neutralizing antibody. Although the cytotoxin assay is considered to be the gold standard, studies on hospitalized patients with diarrhea have shown its sensitivity to range from 36 to 77% when compared to toxinogenic culture (81, 85, 86). This may be due, in part, to the low stability of TcdB in the stool or the nonspecificity of toxinogenic culture. The cytotoxin detection assay is of limited clinical utility because it has a long turnaround time (24 to 72 hours) and its accuracy depends heavily on the technical expertise of trained technologists. Anaerobic culture is a more sensitive method of detecting C. *difficile* in stool, but it requires confirmation of toxin production because nontoxinogenic strains are commonly isolated (81, 85, 86). In addition, clinical decision-making based on anaerobic culture is difficult, given a similarly long turnaround time. For these reasons, many microbiology laboratories have implemented EIA for rapid detection of C. *difficile* in stool specimens (87).

Glutamate dehydrogenase, a sensitive (>90%) but nonspecific target (also present in nontoxinogenic strains), is used for C. *difficile* screening (88, 89). When glutamate dehydrogenase is positive, toxin production is confirmed via EIA or the cell culture cytotoxicity assay (90, 91). Discordant results, which occur in about 15% of specimens, have to be resolved with a more sensitive test such as toxinogenic culture or PCR (89, 92). Although EIA is rapid and glutamate dehydrogenase has a high negative predictive value for ruling out CDAD (88), EIA for TcdA and/or TcdB was shown to have a sensitivity of 46 to 95% (85, 93, 94). Thus, EIA lacks the sensitivity to serve as a stand-alone test for accurate diagnosis of CDAD.

Over the last decade many clinical laboratories in North America have adopted FDA-approved, qualitative, real-time PCR assays for rapid detection of toxin gene-encoding C. *difficile* in stool (95). Some commercial assays allow on-demand, near-patient testing and resulting, thus making possible timely therapeutic, infection-prevention decision making. Molecular assays target the conserved regions of *tcdB* and *tcdA* genes. The primer sequences for several laboratory-developed tests are shown in Table 6. Studies indicate that real-time PCR provides the best analytical sensitivity (pooled 90%), specificity (pooled 96%), and speed when compared to cell culture cytotoxicity, EIA, and anaerobic culture (Table 7) (96). A cost-benefit analysis of all testing modalities identified stand-alone on-demand PCR as the most cost-effective method for screening of CDAD in hospital settings (97). However, there are conflicting data regarding which test better approximates CDAD versus colonization. While several studies suggest that detection of toxin is a better predictor of CDAD (94, 98), other studies have not been able to reproduce these findings and recommend PCR for sensitive detection of C. *difficile* (99, 100).

VIRAL GASTROINTESTINAL DISEASE

Acute viral gastroenteritis is a significant cause of morbidity and mortality worldwide. These infections result in a dehydrating diarrhea that is often effectively treated with either oral or intravenous fluid replacement. However, rapid identification of a viral etiology in both children and adults with diarrhea may allow the discontinuation of unnecessary antibacterial and antiparasitic agents, thus reducing medication side effects and the development of resistance. Furthermore, the prompt identification of viral causes of gastroenteritis is crucial for the epidemiologic investigation of gastroenteritis outbreaks and also plays an important role in infection control.

The unique nature of viruses presents certain challenges to the molecular virologist, though these challenges appear to have been met with determination and creativity. While viruses lack a universal identifier, such as the 16S ribosomal gene used extensively in bacterial molecular diagnostics, this potential detection issue simply requires assay designers to perform more detailed sequence analyses to identify appropriate genomic targets. Consistent with this, accurate and easily accessible sequence databases are critical for the

TABLE 6 Published primers and probes for detection of *Clostridium difficile*

Target	Primers/probes	Sequence	Reference
tcdA	tcdA441	TCTACCACTGAAGCATTAC	367
	tcdA579	TAGGTACTGTAGGTTTATTG	
	tcdAB1FAM (beacon)	CACGCGGATTTTGAATCTCTTCCTCTAGTAGCGCGTG	
tcdB	tcdB2667	ATATCAGAGACTGATGAG	
	tcdB2746	TAGCATATTCAGAGAATATTGT	
	tcdBB2TET (beacon)	CACGCCTGGAGAATCTATATTTGTAGA AACTGGCGTG	
tcdB	398CLDs	GAAAGTCCAAGTTTACGCTCAAT	368
	399CLDas	GCTGCACCTAAACTTACACCA	
	551CLD (TaqMan)	FAM-ACAGATGCAGCCAAAGTTGTTGAATT-TAMRA	
tcdB	*tcdB*-1F	GAAGGTGGTTCAGGTCATAC	85
	tcdB-1R	CATTTTCTAAGCTTCTTAAACCTG	
	SYBR Green		
tcdB	*tcdB* torward	TTGGAAGTACAAAAAGAAGAACTTGA	
	tcdB reverse	TTTTAATGTTGTTATTAAAGCATCAGC	369

development of virus detection protocols. For example, within-species genomic variability found in some viruses may require continuous refinement of primers, probes, and reaction conditions to ensure comprehensive coverage of clinically relevant variants. Many enteric viral pathogens have RNA genomes and therefore require an additional reverse transcription (RT) step prior to amplification. While this was once a significant concern, HIV diagnostics have driven the development of effective RT reagents. As current technologies and reagents continue to improve, and as new advances are applied to the critical questions of clinical virology, the range, accuracy, and availability of molecular assays for enteric viral pathogen detection are certain to benefit.

The following sections detail the molecular strategies for the clinical identification of the most common and important causes of viral gastroenteritis: rotavirus, calicivirus (norovirus and sapovirus), astrovirus, and adenovirus (Table 8).

Rotavirus

Rotaviruses are the most important human pathogens in the family *Reoviridae*, and each year, acute rotaviral gastroenteritis is responsible for over 500,000 deaths worldwide (101). In the winter months, approximately 50% of hospitalizations and emergency department visits and 30% of outpatient visits for acute gastroenteritis among U.S. children aged <3 years are caused by rotavirus (102). It is estimated that annual rotavirus-related health costs in the United States exceed $1 billion (101).

Given the significant morbidity, mortality, and cost associated with rotavirus infection and the protective immunity observed after primary infection, the last decade has seen the development of several oral, live-attenuated rotavirus vaccines. The first of these vaccines, RotaShield, was associated with an increased risk of intussusception, a life-threatening blockage of the intestine, and in 1999 was withdrawn from the U.S. market (103). However, in 2006, two new rotavirus vaccines, Rotarix and RotaTeq, became available. Phase III clinical trials revealed that the vaccines were tolerated well, showed no increased risk of intussusception, and reduced rotavirus-associated hospital admission by 85 to 96% and hospital admissions for all causes of gastroenteritis by 42 to 59% (104, 105). In February 2006, RotaTeq was recommended for routine use among U.S. infants (106). The results of the CDC analysis of the 2007–2008 rotavirus season indicated that, when compared with the 15 previous seasons (1991–2006), rotavirus activity appeared delayed in onset by 2 to 4 months and diminished in magnitude by >50% (102). Subsequent analysis of data from 2007–2011 confirmed significantly reduced rates of rotavirus-coded hospitalizations compared to the prevaccine years 2001–2006 (107). Numerous cost-benefit analyses have been performed to evaluate the cost-effectiveness of routine rotavirus vaccination in both developed and developing countries, though results have been mixed (108).

Rotaviruses have an 11-segment, double-stranded RNA genome with a total genome size of 18.5 kb. The genome encodes 12 proteins, 6 of which are the structural viral proteins (VPs). The structural proteins form the triple-layered, icosahedral protein capsid of this ~75-nm, unenveloped virus. Interestingly, it was the wheel-like appearance of the capsid detected by electron microscopy that gave rotavirus its name (109). More importantly, the structural viral proteins account for the current rotavirus classification scheme (110). Based on the antigenic characteristics of VP6, which forms the middle layer of the

TABLE 7 Performance of real-time PCR for detection of *Clostridium difficile*-associated diarrhea

Gold standard	Sensitivity	Specificity	PPV[a]	NPV[b]	Reference
Direct cytotoxicity	97%	100%	100%	96%	367
Direct cytotoxicity	87%	96%	57%	99%	368
Direct cytotoxicity	100%	94%	55%	100%	370
Direct cytotoxicity	87%[c]	97%	60%[c]	99%	81
Anaerobic culture	93%	97%	76%	99%	85

[a]PPV, positive predictive value.
[b]NPV, negative predictive value.
[c]Underestimated given that cytotoxicity assay had a sensitivity of 36% compared to toxinogenic culture.

TABLE 8 Enteric viral pathogens

Virus	Family	Nucleic acid	Subtypes causing human disease	Common gene targets
Rotavirus	*Reoviridae*	dsRNA	Groups A and C	VP4, VP6, VP7, VP2, NSP3
Norovirus	*Caliciviridae*	ssRNA	Genogroups I, II, IV	ORF1-ORF2 junction
Sapovirus	*Caliciviridae*	ssRNA	Genogroups I, II, IV, V	Pol-capsid junction
Astrovirus	*Astroviridae*	ssRNA	Serotypes I–VIII	ORF1a, ORF1b, ORF2, 3′ UTR
Adenovirus	*Adenoviridae*	dsDNA	Group F Serotypes 40 and 41	Fiber, hexon, E1A, E1B

capsid, rotaviruses are divided into seven serogroups, A through G, though the International Committee on the Taxonomy of Viruses recognizes five rotavirus species, A though E (111). Groups A, B, and C are known to cause human gastroenteritis, though the group A serotype is most commonly identified in clinical specimens. Group A rotaviruses are further classified based on the outer capsid proteins VP4 (protease sensitive or P protein) and VP7 (glycoprotein or G protein). There are at least 24 VP4 or P types and 15 VP7 or G types. Because the gene segments that encode these proteins can segregate independently, a dual P and G typing strategy is used. Because the variation in these proteins results in epitopes that elicit characteristic neutralizing antibodies, the classification was initially serologic. However, sequence analysis revealed good genotype-serotype correlation, and P/G genotyping is now standard practice. Though >70 P/G genotypes have been associated with human gastroenteritis, five combinations (P[8]G1, P[4]G2, P[8]G3, P[8]G4, and P[8]G9) account for a majority of infections worldwide (112, 113).

Most clinical microbiology laboratories use one of a wide variety of commercially available immunoassays for routine surveillance and clinical diagnosis of rotavirus infection in stool. The antibodies used in these assays generally cross-react with VP6 antigens common to group A rotavirus. A number of formats are available including enzyme immunoassays, latex agglutination tests, and immunochromatographic assays (114–117). These assays are rapid, inexpensive, easy to use, and, given the high concentration of rotavirus shed in the stool of infected individuals, relatively sensitive. However, RT-PCR assays show superior analytical and clinical sensitivity when compared to immunoassays (118–120).

A number of rotavirus RT-PCR assays using traditional gel electrophoresis as well as real-time strategies have been developed. Though seminal work using electron microscopy, electropherotyping, and hybridization probes provided the foundation for rotavirus identification and characterization, rotavirus molecular diagnostics began with the work of Gouvea and colleagues (121) in the late 1980s. Their sequential, two-reaction, RT-PCR assay targets the VP7 gene for both rotavirus identification and typing. The first reaction contains primers that recognize conserved VP7 sequences specific for group A rotavirus. If the expected, full-length VP7 gene product is amplified, the second multiplex typing reaction is performed using six internal serotype-specific forward primers and a common reverse primer. Analysis of amplicon size via gel electrophoresis allows the G genotype (G1-4, G8-9) to be determined.

Subsequently, Gentsch and colleagues devised a similar sequential, two-reaction, RT-PCR strategy targeting the VP4 gene for detection of group A rotavirus and P genotyping (122). The first reaction contains primers that recognize conserved VP4 sequences, while the second multiplex typing reaction uses four internal serotype-specific reverse primers and a common forward primer to identify the P genotype. These primer sets are often used in conjunction with the VP7 primers described above to provide full P and G genotyping (123). Numerous other groups have designed additional primers targeting VP4 and VP7 to further extend identification and genotyping (124–129).

This nested typing strategy in its current format is not well suited for routine use in the clinical microbiology lab. Given the specimen handling required for sequential reaction setup and analysis by gel electrophoresis, there is significant potential for amplicon contamination and the introduction of error. In addition, from a clinical perspective, there is no evidence that genotyping provides prognostic information or guides treatment decisions. However, P/G genotyping plays an important role in epidemiologic studies as well as vaccine development and surveillance. The current rotavirus vaccines induce immunity against the most common rotavirus genotypes but do not protect against infection with less common strains. Continued monitoring will be crucial to identify and respond to emerging genotypes, escape variants, and novel rotavirus reassortments that may evolve under the selection pressure of incomplete vaccination.

While these typing strategies may be appropriate for state and county public health laboratories, the simplicity and superior sensitivity of real-time RT-PCR may allow these assays to challenge the antigen immunoassay for routine clinical use. One set of real-time assays employs SYBR Green analysis for detection and uses primers targeting group A rotavirus-specific sequences in either VP4 (130) or VP6 (131, 132). Interestingly, Kang et al. took advantage of their semiquantitative assay to correlate disease severity with the estimated stool viral load, suggesting that rotavirus quantitation may be a useful clinical marker. A second series of real-time RT-PCR assays uses the hydrolysis probe methodology with primer and probe sets targeting structural VP2 (123), VP4 (133), VP6 (134), VP7 (133), or nonstructural protein 3 (NSP3) (135–140). When compared to latex agglutination immunoassay, the real-time RT-PCR described by Logan et al. (134) increased rotavirus detection by 111%.

Caliciviruses

The *Caliciviridae* family of small, nonenveloped, positive-stranded RNA viruses comprises five genera, including *Norovirus* and *Sapovirus* (141). The *Norovirus* and *Sapovirus* genera contain the human enteric viruses of the same names, as well as a number of viruses that cause primarily enteric diseases in other animals. Norovirus, previously known as the Norwalk-like virus, was identified in stool specimens from an outbreak of gastroenteritis in Norwalk, Ohio, and was the first viral agent shown to cause

gastroenteritis (142). Subsequently, sapovirus was similarly identified from an outbreak in Sapporo, Japan (143).

Noroviruses are the leading cause of epidemic gastroenteritis in all age groups and have been associated with high-profile outbreaks in hospitals, nursing homes, cruise ships, and the military (144, 145). It is estimated that each year noroviruses are responsible for 64,000 diarrheal episodes requiring hospitalization, 900,000 clinic visits among children in industrialized nations, and ~200,000 deaths of children <5 years old in the developing world (145, 146). The morbidity, mortality, and health care costs due to sapovirus gastroenteritis have not yet been determined, though the current impact is likely to be less than that of norovirus.

The caliciviruses are composed of a linear, positive-sense RNA genome that is 7.3 to 8.5 kb in length and packaged in a nonenveloped, icosahedral protein capsid 27 to 40 nm in diameter (141). Both the noroviruses and sapoviruses show a high level of genetic diversity and are classified into genogroups (GI to GVI) based on the sequence analysis of the capsid VP1 (147–149). Each genogroup can be further subdivided into numerous genotypes. Human norovirus infections are caused, in decreasing order of frequency, by GII (mostly GII.4), GI, and to a very limited extent, GIV (some genotypes of which also infect pigs) (146, 150, 151). While GI, GII, GIV, and GV sapoviruses have been reported in human infections, the molecular epidemiology of this virus is less well understood (152). Neither norovirus nor sapovirus vaccines are currently available.

Unlike most other viral pathogens, the development of molecular assays for the identification and typing of norovirus and sapovirus has outpaced antigen detection and serologic characterization. This is due, at least in part, to the lack of a culture system to propagate these viruses *in vitro*. Though norovirus immunoassays are commercially available, they do not yet have the performance characteristics required to replace RT-PCR as the method of choice for norovirus detection (153–156). These findings are not entirely surprising given that the significant genetic diversity of norovirus strains underlies significant antigenic diversity. Sapovirus immunoassays are not commercially available, and their development faces similar challenges (157).

As indicated above, RT-PCR is the gold standard for the detection and typing of norovirus and sapovirus. The norovirus diagnostics field is more advanced than the sapovirus field, so we will discuss the numerous conventional and real-time norovirus RT-PCR assays first.

The first-generation norovirus assays utilized a variety of primers based solely on the first described Norwalk virus genome and required RT in a separate tube prior to PCR (158–162). These assays underestimated norovirus genetic diversity and therefore did not perform well when applied to clinical specimens. The second-generation assays took advantage of sequences from additional norovirus strains and for the most part used primers directed at conserved regions of the viral polymerase (163–166). Importantly, these assays required post-PCR analysis via hybridization probes or sequencing to improve sensitivity and specificity. The difficulty of designing broadly reactive primers to accommodate norovirus diversity was illustrated in a study comparing a set of five additional second-generation, conventional RT-PCR assays tested against a panel of stool specimens selected to cover a range of norovirus genogroups/genotypes (167). Though 84% of the specimens were detected by at least one assay, the sensitivity of individual assays ranged from just 52 to 73%. These conventional assays were optimized in a variety of ways to improve detection (168–173); however, there remained issues of assay complexity and postamplification specimen handling.

These limitations were addressed with the development of real-time RT-PCR norovirus diagnostics. These assays used numerous detection methods, including SYBR Green (174–177), hydrolysis (178–188), and hybridization probes (189, 190). While many of these assays were directed at the viral polymerase gene, further sequence analysis revealed that a conserved region at the ORF1-ORF2 polymerase-capsid junction could also be used as an effective diagnostic target. Kageyama et al. described the first of these junction-targeting assays, which used two reactions to detect both GI and GII noroviruses (182). Similarly, Höhne and Schreier designed a two-reaction, real-time assay for the GI and GII genogroups using their own ORF1-ORF2 primer-probe sets (191). Importantly, this assay did not require a separate RT reaction.

To further minimize reaction setup time and the potential for carryover contamination, GI and GII ORF1-ORF2 primer-hydrolysis probe sets were optimized for use in a single multiplex reaction (179, 181). In these assays, the probes were differentially fluorescently labeled to allow simultaneous detection and genogrouping. This multiplex, multiprobe approach directed at ORF1-ORF2 has also been used with GII/GIV hydrolysis probes (183), GI/GII/GIII hydrolysis probes (192), GI/GII/GIV hydrolysis probes (193), and GI/GII hybridization probes (190). It remains unclear, however, whether immediate norovirus genogrouping is important for outbreak control or clinical management. Simple, broadly reactive assays (160, 194) may be more important in the acute setting. Alternatively, the routine use of assays that detect and type simultaneously may allow laboratories to more rapidly monitor epidemiological patterns and highlight geographic regions or communities requiring further investigation (195).

Given the numerous real-time RT-PCR assays available for norovirus detection and genotyping, well-controlled, comparative studies similar to the earlier work of Vinjé et al. (167) are required to accurately determine the relative performance characteristics of these assays. Vainio and Myrmel (196) provided the first of these studies looking at two assays described above (180, 197), as well as two assays initially designed for screening shellfish (198, 199). After a detailed analysis, the duplex, GI/GII, real-time RT-PCR assay designed by Jothikumar et al. (198) was selected for in-house use. Compared to a conventional nested approach (196, 200), this assay, which employs modifications of the Kageyama primer-probe sets (182), had a clinical sensitivity of 91%. These results were superior to those of the Richards SYBR Green assay (80%) and slightly inferior to those of the Höhne assay (93%), but with the advantage of detection of GI and GII noroviruses in a single reaction. This study was performed on specimens from norovirus outbreaks in Norway, so additional work will be required to account for norovirus diversity in different populations. Furthermore, this assay was selected based on both clinical and practical considerations, yet it was the only assay evaluated as capable of single-tube identification of multiple genogroups. Future work is required to provide a more comprehensive analysis of the numerous real-time norovirus assays now available with these characteristics.

A number of conventional RT-PCR assays for sapovirus have also been developed (201–204), as well as several

multiplex, real-time, hydrolysis probe-based assays (183, 205–207). Interestingly, the most conserved region of the sapovirus genome also appears to be the polymerase-capsid junction, and this region is targeted in several assays (206, 207).

Several commercial RT-PCR reagents are available for norovirus RNA detection, though comparisons with one another or with the large number of lab-developed norovirus RT-PCRs have not been performed. The Argene Calicivirus/Astrovirus consensus test (bioMérieux, Marcy l'Etoile, France) is a second-generation RT-PCR assay that requires a detection step via microplate hybridization using biotinylated probes. This test can identify caliciviruses and astroviruses but cannot distinguish between noroviruses and sapoviruses (208). Kele et al. used selected samples from sporadic cases of gastroenteritis in Hungary to evaluate the performance of the Argene Calicivirus/Astrovirus consensus and SmartNorovirus (Cepheid, Sunnyvale, CA), a set of primers and differentially labeled probes that allow real-time PCR detection and differentiation of GI and GII noroviruses by real-time RT-PCR (209). When true positives were defined as at least one positive RT-PCR result, the sensitivities of Argene consensus and SmartNorovirus were 92.8% and 91.2%, respectively.

Butot and colleagues (210) compared a laboratory-developed GI/GII real-time RT-PCR (211) based on previously described primer/probe sets (182, 199, 212) with commercial assays from Generon (Castelnuovo Rangone, Italy) and AnDiaTec (Kornwestheim, Germany). When tested against a comprehensive panel of human stool specimens containing previously genotyped GI and GII noroviruses, the laboratory-developed test detected 100% (59/59) of samples, whereas Generon detected 49% (29/59) and AnDiaTec, 71% (43/59).

R-Biopharm (Darmstadt, Germany) offers two internally controlled, norovirus real-time PCR assays: RIDAGENE Norovirus for qualitative detection of GI and GII noroviruses and RIDAGENE Norovirus I & II, which both detect and differentiate GI and GII noroviruses in a single reaction. When the AccuPower Norovirus real-time PCR assay (Bioneer Co., Daejeon, South Korea), another internally controlled assay for the detection of G1 and GII noroviruses, was compared with RIDAGENE Norovirus, there was 99.0% (96/97) positive percent agreement and 95.1% (175/184) negative percent agreement (213). Similarly, comparison of the RIDAGENE Norovirus I & II assay with conventional RT-PCR on stool specimens from distinct outbreaks in Victoria, Australia, in 2012 and 2013 revealed 98% sensitivity (85% GI [11/13]; 100% GII [87/87]) and 98% (98/100) specificity (214).

Future comparative studies with large, globally distributed sets of stool samples from sporadic gastroenteritis cases and outbreak settings will be required to better define the performance characteristics of these commercial reagents.

In addition to the wide variety of RT-PCR-based amplification assays, isothermal PCR alternatives have also been developed for norovirus detection. Several groups have designed nucleic acid sequence-based amplification (NASBA) strategies using previously published primer pairs modified for NASBA compatibility (215–218). A small study by Houde et al. (217) determined that NASBA and RT-PCR using the Kageyama GII primer sets (182) showed equivalent analytic sensitivity, but the NASBA assay provided less consistent signals. Many NASBA formats, including those referenced above, require an additional product detection step, often hybridization of an electrochemiluminescent probe. Other groups have designed NASBA assays using molecular beacon probes that allow real-time detection (219–221). The real-time NASBA designed by Patterson et al. was 88% sensitive when compared to conventional RT-PCR, suggesting that this assay requires further optimization (219). A norovirus NASBA assay, Swiftgene Norovirus GI/GII, is commercially available in Japan (Kainos Laboratories, Tokyo, Japan).

Another real-time isothermal amplification strategy, transcription reverse-transcription concerted reaction coupled with intercalation-activating fluorescent probes, is also being commercially developed in Japan (Tosoh Bioscience, Tokyo, Japan) (222). When compared to nested RT-PCR with commercial primers (Nanogen Advanced Diagnostics, Turin, Italy) on 387 stool samples, reverse-transcription concerted reaction demonstrated 96.6% sensitivity and 81% specificity (223).

The final real-time, non-PCR nucleic acid-based approach for norovirus diagnosis is reverse-transcription, loop-mediated isothermal amplification (RT-LAMP) (224–226). Fukuda et al. (224) described a two-reaction, GI and GII genogroup-specific RT-LAMP assay using primers directed at the ORF1-ORF2 junction. When compared to conventional RT-PCR (168), the RT-LAMP assay had 100% clinical sensitivity. Based on this work, a commercialized assay, LoopampÆ Norovirus GI and GII, was developed (Eiken Chemical, Tokyo, Japan) (225, 227). One advantage of this approach is that detection is via inexpensive, real-time turbidimetry or simple, endpoint, visual examination. Iturriza-Gómara et al. (226) compared the Loopamp assay to genogroup-specific real-time RT-PCR modified from Kageyama and colleagues (182). Using 510 human stool samples, the Loopamp assay showed 83.3% sensitivity for GI strains, 97.4% sensitivity for GII strains, and 100% specificity.

Astrovirus

The family *Astroviridae* contains the human enteric pathogen astrovirus, another cause of acute pediatric gastroenteritis as well as sporadic diarrhea and outbreaks in adults (228). Astroviruses were first identified by electron microscopy of stool specimens and received their name based on a distinctive star-like appearance (229, 230). The overall severity of astrovirus infection is the lowest of the common viral causes of gastroenteritis (rotavirus, norovirus, adenovirus), and deaths due to astrovirus are exceedingly rare, consistent with the limited impact this virus has on global public health (228). Furthermore, the high incidence of asymptomatic viral shedding and the frequent coinfection with other viral and bacterial enteric pathogens make the isolated finding of astrovirus in stool of questionable utility without further clinical and laboratory evaluation.

Similar to the caliciviruses, the astroviruses are nonenveloped, with an icosahedral protein capsid 28 to 30 nm in diameter and a single-stranded, positive-sense RNA genome 6.4 to 7.3 kb in length. However, human astroviruses can be cultured, and sera raised against culture-adapted strains have allowed these viruses to be grouped into eight serotypes. A commercial enzyme immunoassay is available for astrovirus antigen detection (OXOID, Cambridge, UK), but its performance was widely variable when compared to RT-PCR (231, 232). Importantly, the sequence of the viral capsid protein encoded by ORF2 correlates directly with the serotype (233, 234), making

RT-PCR the most common technique for astrovirus detection and typing.

Several conventional, nested RT-PCR assays with type-specific primers directed at ORF2 have been developed for astrovirus typing (235, 236), while numerous conventional assays targeting a variety of conserved genomic regions (ORF1a and ORF1b encoding the nonstructural proteins, ORF2, 3′-UTR) are available for astrovirus detection (234, 237–241). A detailed discussion of these conventional RT-PCR strategies can be found in a review from Guix et al. (242). Only a few real-time astrovirus assays have been reported for clinical use (243–246). Royuela et al. (245) developed a SYBR Green-based assay using a single primer set targeting a conserved region of ORF1a, while Dai et al. (246) utilized a primer-hydrolysis probe set targeting the ORF1b-ORF2 junction. Grimm et al. (244) described a multiplex, hydrolysis probe strategy for astrovirus identification directed at viral capsid sequences (244). Finally, Tai et al. developed a conventional NASBA assay (243) using previously published primers to ORF1a (239).

Few commercial assays for astrovirus RNA detection are currently available. In combination with the norovirus/sapovirus endpoint RT-PCR assay described above, Argene Biosoft (North Massapequa, NY) offers astrovirus detection that similarly requires postamplification microplate hybridization for product confirmation.

Adenovirus

Perhaps best known for its roles in upper respiratory infections and conjunctivitis, adenoviruses, family *Adenoviridae*, are also an important cause of acute pediatric gastroenteritis. Initially, epidemiologic studies could not identify a causal relationship between stool cultures positive for adenovirus and clinical diarrheal illness. However, examination of stool from an outbreak by electron microscopy revealed adenovirus particles that, at the time, could not be cultured (247). Further characterization of these fastidious, enteric adenoviruses showed them to be the etiologic agents of diarrhea and classified them as species or group F, serotypes 40 and 41 (141). The prevalence of the enteric adenoviruses in stool samples from children with gastroenteritis is relatively low, ranging from 1 to 11% worldwide, though the infections are of significant severity and clinically indistinguishable from infection with rotavirus or norovirus (141). There is a live, oral vaccine protective against adenovirus serotypes 4 and 7 that is used in the military to reduce the incidence of acute respiratory disease common among new military recruits (248). However, this vaccine is not available to the general population and does not protect against the enteric adenoviruses.

The adenoviruses are nonenveloped, with an icosahedral protein capsid ~90 nm in diameter and a linear, double-stranded DNA genome ~36 kb in length. The human adenoviruses are classified into 7 species or groups (111), A through G, encompassing at least 56 serotypes (141). The epitopes that determine adenoviral serotypes are found on the virion hexon protein and the terminal knob portion of the fiber protein (141). Analysis of variable regions within the genes encoding these proteins allows molecular typing that also correlates with the serotype (249, 250). There are a variety of commercially available broadly reactive immunoassays for adenovirus antigen detection from clinical specimens, including stool, as well as an immunoassay specific for the enteric adenoviruses, Premier Adenoclone Type 40/41 (Meridian Biosciences, Cincinnati, OH).

Numerous PCR-based assays for both adenovirus detection and typing have been developed (251–271), though this discussion will focus on the assays that are able to specifically identify the group F adenoviruses responsible for gastroenteritis. Because other adenovirus groups that do not cause diarrhea are often shed in the stool of symptomatic patients, these enteric adenovirus-specific assays are best suited for the evaluation of viral gastroenteritis. However, broadly reactive adenovirus PCR assays are more appropriate for use in the setting of transplant or other immunocompromise, where detection of any type will likely result in treatment with an antiviral nucleoside analog, such as cidofovir (272).

Initial work from Allard et al. detailed the design of several enteric adenovirus-specific primer sets targeting the early viral genes, E1A and E1B, for use in conventional PCR (273, 274). Subsequently, Hussain et al. (275) modified previously reported enteric-specific primers directed at the E1B gene (276) and showed that their assay had 99% sensitivity when compared to immunoelectron microscopy. Tiemessen and Nel also developed a conventional PCR assay for enteric adenoviruses, but instead targeted group F-specific sequences in the fiber gene (277). More recently, Jothikumar et al. reported a quantitative, real-time PCR fluorescence resonance energy transfer assay using a primer-probe set to the fiber gene that could differentiate adenovirus 40 from 41 by melting curve analysis (278). In contrast, Logan et al. described a real-time PCR assay with primers and hydrolysis probes targeting the hexon gene that detected both group F viruses but did not distinguish between them (134). Assays for group F-specific viral DNA detection have not yet been commercialized.

Multiplex Approaches to the Diagnosis of Viral Gastroenteritis

In addition to the analytic sensitivity and specificity achieved with nucleic acid-based testing, these methods also make possible the simultaneous detection of all viral agents of infectious gastroenteritis in a single reaction. While many of the assays described in the sections on rotavirus, calicivirus, astrovirus, and adenovirus apply multiplexing to the identification of multiple genotypes of a given pathogen, several assays have extended this approach to cover more than one virus family (279–282). One of the first of these assays, a conventional RT-PCR developed by O'Neill et al., was able to identify group A rotavirus, norovirus, and enteric adenovirus using primers to the VP7, viral polymerase, and hexon genes, respectively (279). Though this assay was 100% sensitive when compared to electron microscopy (rotavirus, adenovirus) and monoplex PCR (norovirus), it required a second, nested reaction and subsequent gel electrophoresis for product identification. Similarly, Yan et al. (282) designed a conventional multiplex, RT-PCR assay requiring a separate RT step, using four sets of primers targeting the capsid regions of the norovirus (GI and GII genogroups), sapovirus, and astrovirus genomes. Rohayem et al. (281) compared a norovirus (GI and GII genogroups), astrovirus, and adenovirus (A–F), single-tube, multiplex RT-PCR against previously published conventional monoplex assays (167, 239, 273, 283) and showed equivalent performance. However, many of the advantages of multiplexing

are lost in these assays because they require endpoint product detection.

More recently, real-time assays have been developed for multiplex diagnosis of the most common enteric viral pathogens (284–288). For example, Jiang et al. (285) compared a single-tube, multiplex real-time PCR for norovirus GI and GII; sapovirus GI, GII, GIV, and GV; human rotavirus A; adenoviruses 40 and 41; and astrovirus with the individual single-target assays using 812 clinical stool specimens and demonstrated sensitivities and specificities that ranged from 75 to 100% and from 99 to 100%, respectively. Despite the large number of stool samples, this sample set contained few positives for norovirus GI, sapovirus, and astrovirus. For this assay, and others, the advantages of multiplexing will need to be weighed against decreased clinical sensitivity for some targets.

PARASITIC GASTROINTESTINAL DISEASE

Intestinal protozoan infections are a common cause of diarrheal disease worldwide. Like the bacterial and viral agents of gastroenteritis, infections in children, pregnant women, and the immunocompromised are associated with significant morbidity and mortality. It is estimated that there are more than 58 million cases of childhood protozoal diarrhea per year, resulting in direct costs of approximately $150 million (289). Three groups of intestinal protozoa are responsible for the majority of human disease. These include the amoeba, flagellates, and coccidia (Table 9). The molecular approaches for the diagnosis of important pathogens in each of these groups are in various stages of development, and overall, the work is at a similar stage to that of the enteric bacteria but lags behind the progress made in the design of assays for detection of enteric viruses. The major target for molecular diagnostics is the small subunit rRNA gene, which in eukaryotic organisms encodes the 18S rRNA. While there was initial concern that protozoan cysts would be resistant to fecal nucleic acid extraction techniques, this potential limitation is no longer an issue because there are numerous extraction protocols and commercial kits capable of efficient parasite DNA extraction from stool. These techniques are only starting to be applied to the detection of nematode and cestode eggs in stool, so molecular diagnostics for the clinical diagnosis of parasitic worms will not be covered in the following sections.

Amoebae

The human intestinal protozoan parasite *Entamoeba histolytica* is the etiologic agent of amoebic dysentery and invasive extraintestinal amoebiasis, which commonly manifests as amoebic liver abscesses. *E. histolytica* is responsible for significant morbidity and mortality, with about 50 million people infected worldwide, resulting in approximately 40,000 to 100,000 deaths each year (290–292). Several other *Entamoeba* species inhabit the intestinal tract of humans, including *Entamoeba dispar*, *Entamoeba moshkovskii*, *Entamoeba polecki*, *Entamoeba coli*, and *Entamoeba hartmanii*, though only *E. histolytica* has been clearly shown to be pathogenic. Other enteric amoebae, such as *Endolimax nana* and *Iodamoeba butschlii*, are also considered nonpathogenic. *E. histolytica* vaccines are in development but are not commercially available (293).

In patients with suspected infectious colitis, it is critical to properly identify *E. histolytica* to facilitate prompt treatment with the appropriate antiparasitic agents, usually the nitroimidizole derivative metronidazole plus a luminal agent such as paromomycin. This treatment strategy attempts to eliminate the invading organism and eradicate intestinal colonization, thus reducing both the risk of the development of invasive disease and the danger to public health. The traditional laboratory diagnosis of *Entamoeba* involves the careful microscopic examination of the stool. Not only is this approach complicated by a confusing array of fecal artifacts and often unreliable microscopic criteria for differentiating among the *Entamoeba*, *E. histolytica*, *E. dispar*, and *E. moshkovskii* are morphologically indistinguishable. As far back as 1925, Brumpt hypothesized that amoebic infections were caused by morphologically identical species with different pathogenic potentials (294). This hypothesis was borne out by genetic analysis over 60 years later, demonstrating that these amoebae are separate species (295).

The routine laboratory diagnosis of *Entamoeba* infection continues to be predominantly microscopy-based, and therefore these species' morpho-copies are not commonly differentiated. Though a number of antigen detection kits are commercially available for *Entamoeba* identification, few immunoassays, such as the TechLab *E. histolytica* II (TechLab, Inc., Blacksburg, VA) and *Entamoeba* CELISA PATH (Cellabs, Brookvale, Australia), are designed to specifically identify *E. histolytica* antigens in stool. When compared to PCR, these assays show good specificity but

TABLE 9 Pathogenic enteric protozoa and fungi

Organism	Species	Disease	Common gene targets
Amoeba	*Entamoeba histolytica*	Amoebic dysentery, amoebic liver abscesses	Episomal 18S rRNA
Flagellates	*Giardia intestinalis*	Epidemic and sporadic diarrhea	18S rRNA
	Dientamoeba fragilis	Chronic diarrhea	18S, 5.8S rRNA
Coccidia	*Cryptosporidium parvum* *Cryptosporidium hominis* *Cyclospora cayetanensis* *Isospora belli*	Severe, intractable diarrhea in the immunocompromised	18S rRNA, rDNA internally transcribed spacer, DNAJ-like, *Cryptosporidium* oocyte wall protein
Microsporidia	*Enterocytozoon bieneusi* *Encephalitozoon intestinalis*	Severe, intractable diarrhea in the immunocompromised	18S rRNA

widely variable clinical sensitivity, ranging from 0 to 79% (296–298). The diagnostic utility of these *E. histolytica*-specific immunoassays is therefore controversial, and some recommend that all detection and differentiation of *Entamoeba* species should be performed by PCR (298).

Numerous conventional and real-time PCR assays have been developed for the detection of *Entamoeba* species (reviewed extensively in reference 299). Given the clear clinical importance of distinguishing pathogenic from nonpathogenic amoebae, the following discussion will focus on real-time, *E. histolytica*-specific detection. Whole-genome sequencing of *E. histolytica* revealed a genome 23.7 Mb in size encoding >8,000 genes (300–302). The most common assay target is the gene encoding the 18S small subunit rRNA (303). The rRNA genes in *E. histolytica* are carried exclusively on a large number of 24-kb circular episomes that are estimated to make up ~20% of the total cellular DNA (300, 304). The species specificity of rRNA loci coupled with this natural gene amplification makes these genes attractive diagnostic targets. Not surprisingly, several groups have developed primer-probe sets directed at the *E. histolytica* 18S rRNA gene for use in SYBR Green (305), fluorescence resonance energy transfer (306), molecular beacon (296), and hydrolysis probe-based (307–309) real-time assays. Qvarnstrom et al. (305) compared three of these assays (305–307) as well as a hydrolysis probe-based real-time assay targeting episomally encoded repeat sequences (307). When these assays were tested against a small, well-characterized panel of stool specimens, the hydrolysis probe-based 18S rRNA assay showed superior performance characteristics and was recommended for the specific and quantitative diagnosis of amebiasis.

Flagellates

As their name suggests, the flagellates are typically characterized by the presence of flagella, a specialized organelle of locomotion. Human enteric flagellates include *Giardia intestinalis*, *Dientamoeba fragilis*, *Chilomastix mesnili*, and *Trichomonas hominis*, though only *G. intestinalis* and *D. fragilis* are considered pathogenic. These two organisms will be considered in turn.

G. intestinalis, synonymous with *Giardia lamblia* and *Giardia duodenale*, was first observed in 1681 by the microscopist Van Leeuwenhoek, reportedly during the examination of his own loose stool. The prevalence of *G. intestinalis* in specimens submitted for ova and parasite analysis is estimated to be 2 to 5% in the developed world and 20 to 30% in the developing world (310). *G. intestinalis* causes both epidemic and sporadic disease and is an important cause of water- and foodborne diarrhea, day care center outbreaks, and diarrhea in men who have sex with men. Perhaps because mortality due to *Giardia* is uncommon, a vaccine for human use has not yet been developed. However, accurate diagnosis of *G. intestinalis* infection is important, because effective antiprotozoal agents are widely available.

Stool microscopy remains the primary technique for the routine diagnosis of *G. intestinalis* infection in the clinical microbiology laboratory, though this evaluation is often done in concert with any one of a large number of commercially available immunoassays. *Giardia* establishes residence in the proximal small intestine, so occasionally more invasive duodenal sampling techniques (string test, duodenal aspirate, or duodenal biopsy) are required to make the diagnosis.

The *G. intestinalis* genome is approximately 11.7 Mb in size and is composed of ~6,500 genes (311). Molecular assays for *Giardia* identification frequently target the small subunit 18S rRNA gene (312–315). For example, Verweij et al. (314) designed a real-time, 18S rRNA hydrolysis probe-based PCR assay and compared its performance to a commercial stool antigen assay and traditional stool microscopy. When a positive result in two out of three methods was used as the criterion for true positive cases, both immunoassay and real-time PCR had a sensitivity of 98%, whereas microscopy had a sensitivity of 89%. Schuurman et al. (315) essentially recapitulated these results testing the same real-time assay against a different immunoassay, with the rapid antigen detection kit achieving higher specificity.

Given the apparent high clinical sensitivity and specificity of *Giardia* antigen tests, PCR-based assays will likely be useful in multiplex assays targeting multiple enteric pathogens. It is estimated that PCR can detect the DNA of less than 1 cyst per reaction (314), suggesting that these molecular methods may be best employed in microscopy-negative, antigen-negative cases to perhaps obviate the need for more invasive duodenal sampling.

The other human pathogen in this category is *D. fragilis*. This aflagellate organism morphologically resembles the amoeba, but sequence analysis suggests it is more closely related to the flagellates. *D. fragilis* has only relatively recently been recognized as a cause of human chronic diarrhea, and therefore the worldwide disease burden is not well defined (316). Though the *D. fragilis* life cycle, mode of transmission, and complete genome sequence are not known, infections can be effectively treated with a variety of antiprotozoal agents (317–319).

The identification of *D. fragilis* via the microscopic examination of fecal specimens is difficult, and permanently stained smears are required. Furthermore, no stool antigen immunoassays are available to aid in diagnosis. To address these diagnostic issues conventional and real-time PCR assays targeting the rRNA genes of *D. fragilis* have been developed (320–324). These assays are both sensitive and specific when compared to microscopy, indicating that molecular approaches will likely be useful in further characterizing the epidemiology of this poorly understood infection.

Coccidia

The major pathogenic intestinal coccidia include *Cryptosporidium parvum*, *Cryptosporidium hominis*, *Cyclospora cayetanensis*, and *Cystoisospora (Isospora) belli*, with *Cryptosporidium* species being the most common (325–327). These obligate intracellular parasites invade the intestinal epithelia and generally cause water- and foodborne, self-limited diarrheal disease in immunocompetent hosts. However, in the immunocompromised, these pathogens can also cause severe, prolonged gastroenteritis with life-threatening, intractable diarrhea. Rapid, accurate identification and differentiation of the intestinal coccidia is particularly important in immunocompromised patients, because effective treatments are available for *Cyclospora* and *Isospora*, but not *Cryptosporidium* (328). For patients with HIV or patients on immunosuppressive therapy, the identification of these pathogens may also result in the adjustment of medication regimens (antiretrovirals or immunosuppressants, respectively) to promote host immune system recovery.

As with the other intestinal protozoa, stool microscopy remains the primary diagnostic technique for the identification of coccidia infection. However, these organisms are often missed on routine stool analysis, and the use of modified acid-fast stains, differential interference contrast microscopy,

or fluorescence microscopy improves detection. While a number of antigen immunoassays are commercially available for the clinical detection of *Cryptosporidium* in stool, these assays have not yet been developed for *Cyclospora* or *Isospora*. Similarly, of these enteric coccidia, only *C. parvum* has had its complete genome sequenced (9.1 Mbp encoding ~3,800 genes) (329). Consequently, numerous conventional and real-time PCR assays have been designed to identify and differentiate clinical *Cryptosporidium* species isolates (330–342), whereas few *Cyclospora* (343, 344) and *Isospora* (345, 346) assays have been reported. In the case of *Cryptosporidium*, one study (340) suggests that the sensitivity of real-time PCR is equivalent to direct fluorescent antibody testing (~89%). However, additional studies are required to compare the test characteristics of *Cryptosporidium* PCR with antigen immunoassays, as well as to determine the clinical utility of speciation. In addition, strategies for the molecular identification of *Cyclospora* and *Isospora* require continued evaluation to clearly define their role in the laboratory diagnosis of intestinal protozoal infection.

Multiplex Approaches to the Diagnosis of Parasitic Gastroenteritis

To take advantage of the multiplexing capabilities of PCR-based techniques, several groups developed real-time assays for the simultaneous detection of the most common causes of parasitic diarrhea: *E. histolytica*, *G. intestinalis*, and *C. parvum*/*C. hominis* (307, 347–349). Verweij et al. (307) designed an assay using primer-hydrolysis probe sets targeting the *E. histolytica* and *G. intestinalis* 18S rRNA genes and the *C. parvum*/*C. hominis* DNAJ-like protein gene. While this report only tested the assay against control samples, a subsequent study by ten Hove et al. (347) evaluated assay performance on a large number of stool specimens gathered in The Netherlands from patients consulting their general practitioner about gastrointestinal complaints. This work confirmed that many patients with *G. intestinalis* and *C. parvum*/*C. hominis* infections remain undiagnosed via microscopy and argued for the routine use of multiplex PCR for the detection of diarrhea-causing protozoa. Similarly, Haque et al. (348) developed a multiplex assay using primer-hydrolysis probe sets directed at the *E. histolytica* and *G. intestinalis* 18S rRNA genes, as well as the *C. parvum*/*C. hominis* oocyte wall protein gene. Though there were no differences in the analytic detection limits between the multiplex assay and each respective monoplex assay, the multiplex assay showed slightly decreased clinical sensitivity and specificity when tested against a well-characterized panel of stool specimens.

Microsporidia

The microsporidia are composed of more than 1,000 species of small, spore-forming, obligate intracellular organisms originally classified as protozoa but now considered fungi. Of the 14 species currently known to infect humans, the most common are *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis*, two enteric pathogens that can cause severe, persistent diarrhea, particularly in the immunocompromised (350, 351). The prevalence of *E. bieneusi* and *Encephalitozoon* species, including *E. intestinalis*, in patients with HIV is as high as 50% in some regions, with the overall prevalence estimated to be ~15% (352). It is critical to identify and differentiate these species because infections with *Encephalitozoon* species are effectively treated with the common antiparasitic agent albendazole, whereas *E. bieneusi* infections require fumagillin, an antibiotic with significant systemic toxicity (353, 354).

The diagnosis of microsporidiosis is made through the microscopic examination of stool specimens and requires modified trichrome or chemofluorescent stains to distinguish these small organisms from other elements in the stool (350, 355). While antibody reagents for species-specific immunofluorescent microscopy have been commercialized to a limited extent (for example, Bordier Affinity Products, Lausanne, Switzerland), identification to the species level is not routinely done. Furthermore, neither broadly reactive nor species-specific stool antigen immunoassays are widely available.

A number of conventional (reviewed extensively in reference 350) and real-time (356–359) PCR assays have been developed for the identification of pathogenic microsporidia in stool. The complete genome sequence of *E. intestinalis* is available, revealing an extremely compact genome of 2.3 Mb encoding ~1,800 genes (360). However, the majority of assays for microsporidia target the rRNA genes. For example, Verweij et al. (359) designed a multiplex assay with differentially labeled primer-hydrolysis probe sets directed at the *E. bieneusi* rRNA region internally transcribed spacer and *Encephalitozoon* species small-subunit rRNA gene. Though this assay performed well when tested against a limited set of known stool specimens, further com-

TABLE 10 Targets included in syndromic multiplex panels[a]

Targets	xTAG GPP	FilmArray
Aeromonas	NA	✓ (IUO)
Campylobacter	✓	✓
Clostridium difficile, toxin A/B	✓	✓
Plesiomonas shigelloides	NA	✓
Salmonella	✓	✓
Yersinia enterocolitica	✓ (RUO)	✓
Vibrio	NA	✓
Vibrio cholerae	✓ (RUO)	✓
Enteroaggregative *E. coli*	NA	✓
Enteropathogenic *E. coli*	NA	✓
Enterotoxigenic *E. coli*	✓	✓
Shiga-like toxin-producing *E. coli*	✓	✓
E. coli O157	✓	✓
Shigella/enteroinvasive *E. coli*[b]	✓	✓
Cryptosporidium	✓	✓
Cyclospora cayetanensis	NA	✓
Entamoeba histolytica	✓ (RUO)	✓
Giardia	✓	✓
Adenovirus F 40/41	✓ (RUO)	✓
Astrovirus	NA	✓
Norovirus GI/GII	✓	✓
Rotavirus A	✓	✓
Sapovirus	NA	✓
Astrovirus	NA	✓

[a]xTAG GPP, Luminex xTagÆ gastrointestinal pathogen panel (Luminex Corporation, Toronto, Canada); FilmArray, FilmArrayTM Gastrointestinal Panel (BioFire Diagnostics, Salt Lake City, UT); IUO, investigational use only; RUO, research use only.

[b]The xTAG GPP primers do not cross-react with enteroinvasive *E. coli*.

parative studies are necessary to better characterize the assay's clinical sensitivity and specificity. The importance of proper identification for optimal treatment provides a clear clinical rationale for the continued development of microsporidia diagnostics.

SYNDROMIC ASSAYS

Recent advancements in molecular diagnostic technologies with multiplexing capabilities have made possible syndromic nucleic acid amplification tests (NAATs) for the diagnosis of all causes (bacterial, viral, and parasitic) of gastrointestinal infections in a single test (Table 10). Two such assays have recently been approved by the FDA, and several more are in development or undergoing clinical trials (18, 361). Some of these assays allow on-demand near-patient testing, thus making syndromic testing possible in community hospitals, where technical resources are more limited. Accuracy studies indicate that their sensitivities and specificities are superior or comparable to conventional methods and NAATs (45, 361–364). However, larger studies are needed to determine the sensitivity and specificity of all pathogens targeted in these assays. Compared to conventional NAATs, the syndromic assays offer several advantages. First, they provide comprehensive coverage of many bacterial, viral, and parasitic etiologies of gastroenteritis. Second, they allow detection of co-infections. Third, they allow consolidation of multiple laboratory tests that are currently used to diagnose gastroenteritis. Given these advantages, syndromic tests are believed to improve efficiency, turnaround times, and laboratory costs (361).

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Diagnostic Approaches to Genitourinary Tract Infections

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28

The accurate diagnosis of urethral and cervico-vaginal infections can be complex, requiring multiple, sophisticated laboratory tests, without which the causative organism may remain unknown. Failure to diagnose and cure people with urethral and vaginal infections is associated with patient morbidity and mortality and can facilitate the continued transmission of infections to partners and from mother to infant during birth (1).

Urethral and cervico-vaginal infections are related to many adverse health outcomes. In women, infections of *Chlamydia trachomatis* and *Neisseria gonorrhoeae* are related to infertility, pelvic inflammatory disease, chronic pelvic pain, and ectopic pregnancy (1). Herpes simplex virus (HSV) and syphilis infections may cause genital ulcerations that interfere with the natural mucosal and epithelial barriers, with resultant inflammation that enhances the acquisition and transmission of HIV (2). Bacterial vaginosis increases the risk of the acquisition and transmission of infections including HIV, HSV type 2 (HSV-2), *Trichomonas vaginalis*, *N. gonorrhoeae*, and *C. trachomatis* (3–6). In addition, in women, cervico-vaginal infections are associated with adverse outcomes of pregnancy.

A medical history alone is insufficient for the accurate diagnosis of genitourinary tract infections. A careful physical examination and laboratory testing are essential to determine the etiology of urethral and vaginal symptoms. Many sexually transmitted infections (STIs) can be asymptomatic and can have long-term adverse effects if left untreated but are not diagnosable without screening tests. Therefore, although the current World Health Organization guidelines recommend syndromic management (7), syndromic management is unlikely to enhance STI control (8). The early identification and subsequent treatment of STIs and other genital infections are paramount to achieving an effective reduction in the disease burden (9).

Nucleic acid amplification tests (NAATs) have been available since the early 1990s and are the newest generation of non-culture-based diagnostic tests for infectious diseases. NAATs detect and amplify bacterial, viral, or parasitic nucleic acid sequences specific to the targeted organism, and different NAATs may use different methods of target capture and amplification. Common, currently commercially available, and U.S. Federal Drug Administration (FDA)-cleared NAATs for sexually transmitted infections include PCR (Roche cobas Amplicor, Cepheid Gene Xpert, Abbott RealTime), strand displacement amplification (BD ProbeTec), and transcription-mediated amplification (TMA) (Hologic). NAATs offer many advantages compared to bacterial culture, including ease of specimen collection and transport and markedly improved sensitivity. That superior sensitivity has revolutionized the ability to diagnose certain infections. NAATs have essentially replaced bacterial culture for the diagnosis of gonococcal and chlamydial infections because of their high sensitivity and specificity. Many NAATs have been optimized for use with several specimen types including noninvasive specimens such as urine and patient self-collected specimens (such as vaginal and rectal swabs) (10–12). Those factors make NAATs ideal for a range of settings. An additional advantage of NAATs is the advent of multiplex assays that simultaneously detect multiple targets to diagnose multiple pathogens or strains of organisms.

The development of NAATs has also improved our understanding of the epidemiology of some STIs because improved sensitivity is suitable for screening programs allowing for the diagnosis of both symptomatic and asymptomatic individuals. Diagnosis of asymptomatic infections is critical to the control of disease (13). Molecular tests detect organisms whether they are alive or not, making them more robust for places where specialized specimen transfer may not be possible. However, that means that treated individuals may continue to test positive on a molecular test after treatment and beyond the period of infectivity.

The purpose of this chapter is to provide guidance on the role of testing both symptomatic and asymptomatic patients for genitourinary tract infections. We have organized the chapter sections into syndromes that are associated with certain genitourinary tract infections.

VAGINAL DISCHARGE

Three conditions are most frequently associated with vaginal discharge: vaginitis, vaginosis, and cervicitis.

Vaginitis

The vagina normally contains more than a billion colony-forming units of bacteria per gram of vaginal fluid, pre-

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dominantly lactobacillus species that help to maintain the normal vaginal pH (14). Vaginitis can be characterized by vaginal discharge, vulvar itching, vulvar irritation, vaginal odor, dyspareunia, and dysuria (14). Vaginitis can be caused by infections including *T. vaginalis* (trichomoniasis) and *Candida* species (15). Globally, there are over 10 billion cases of vaginitis each year, making it the most common women's health issue.

Vaginitis testing has traditionally included a series of manual laboratory tests such as wet mount microscopy with and without KOH examination and culture. Each test has its challenges in terms of low sensitivity and specificity, dependency on operator technique, and technician subjectivity in reporting results. New molecular tests are being used to determine the etiologic agents of vaginitis.

Trichomoniasis

Infection of *T. vaginalis*, a motile protozoan, is the most common nonviral STI, with an estimated 174 million new cases globally every year (16). *T. vaginalis* is often asymptomatic (17–19). However, even asymptomatic infections are a public health concern. *T. vaginalis* has been associated with a more than 2.7-fold increase in the risk of HIV acquisition (20–22), a 1.3-fold increase in preterm labor, and a 4.7-fold increase in pelvic inflammatory disease (23, 24). Currently, the U.S. CDC sexually transmitted disease treatment guidelines recommend *T. vaginalis* screening for all HIV-infected women when care is initiated and then at least annually, as well as diagnostic testing in symptomatic women presenting with vaginal discharge (15).

T. vaginalis is traditionally diagnosed when motile, pear-shaped trichomonads are viewed microscopically; this is 100% specific (25). However, microscopy is an insensitive method of detection for *T. vaginalis* because of the low parasitic burden and short viability of the organism. Some studies have compared wet mount (a preparation technique conducted by mixing vaginal secretions with saline on a slide) microscopy with highly sensitive molecular detection tests and have shown the poor sensitivity of microscopy, which ranges from 44 to 68%, even under ideal conditions (25–27). In addition, the limited viability of the organism likely leads to underdiagnosis in laboratories that rely on microscopy because motility aids in the detection of the organism. Culture has been used for diagnosis, but culture requires incubation at 37°C up to 7 days. The sensitivity of culture has been estimated to range from 38 to 82% when compared with molecular methods due to problems visualizing low numbers of motile organisms (25–28).

Some nucleic acid tests exist for the detection of *T. vaginalis* including both amplified and nonamplified molecular tests. Nucleic acid tests do not require the organism to be viable at the time of detection, which increases the sensitivity and reduces the need for special transport and temperature conditions. The BD Affirm VPIII microbial identification test (Becton, Dickinson, Sparks, MD) is a nonamplified multianalyte, nucleic acid probe-based system designed to enable the identification and differentiation of organisms associated with vaginitis (*Gardnerella vaginalis*, *Candida* spp., and *T. vaginalis*) (29). The Affirm VPIII test detects nucleic acids using specific oligonucleotide probes. It requires a heating system and processor instrument and takes about 1 hour to perform.

Nucleic acid amplification testing for *T. vaginalis* has provided highly sensitive and specific diagnoses. The transcription-mediated amplification-based Aptima *T. vaginalis* assay (Hologic, San Diego, CA) is an FDA-cleared, qualitative NAAT for the detection of *T. vaginalis* rRNA. The assay requires a high-complexity instrumentation system and trained laboratory personnel. Specimens are typically obtained and placed in Aptima transport medium and sent to central laboratories for testing (Table 1). The Aptima *T. vaginalis* assay is cleared for use in female specimens including clinician-collected endocervical swabs, clinician-collected vaginal swabs, urine specimens, and specimens collected in PreservCyt solution from symptomatic or asymptomatic women. After collection, specimens are transferred into specimen transport tubes. Within the tubes the transport solution releases the rRNA target and protects it from degradation during storage and transport. Once in the laboratory, the Aptima *T. vaginalis* assay performs target capture and amplification. Target capture is a method to isolate the rRNA molecule from the specimen by use of a specific capture oligomer and magnetic microparticles.

A new real-time PCR assay from Cepheid (Sunnyvale, CA) is in clinical trials to obtain FDA clearance. The assay, Xpert TV, might be the first nucleic acid amplification test for *T. vaginalis* cleared for use on male urine specimens.

The high sensitivity of NAATs for *T. vaginalis* (26, 30–32) makes them suitable as screening tests, particularly because noninvasive specimens such as urine and self-collected vaginal swabs can be used (33–36). Additionally, liquid-based specimens collected for Pap cytology can be used for NAATs. NAATs do not require viable organisms, and they provide convenient specimen processing, allowing use in diverse settings.

TABLE 1 Reported performance of nucleic acid amplification tests for the detection of *Trichomonas vaginalis*

Assay	Specimen type	Symptomatic	TMA: Aptima (rRNA target)	
			Sensitivity	Specificity
TV assay	PreservCyt solution liquid Pap	Symptomatic	100 (94.8, 100)	99.2 (97.8, 99.7)
		Asymptomatic	100 (85.7, 100)	100 (98.8, 100)
	Endocervical swab	Symptomatic	100 (95.8, 100)	99.1 (97.8, 99.7)
		Asymptomatic	100 (87.1, 100)	99.7 (98.4, 99.9)
	Female urine	Symptomatic	95.2 (86.7, 98.3)	98.9 (97.1, 99.6)
		Asymptomatic	95.5 (78.2, 99.2)	99.0 (97.1, 99.7)
	Clinical-collected vaginal swab	Symptomatic	100 (95.8, 100)	99.1 (97.7, 99.6)
		Asymptomatic	100 (86.2, 100)	98.8 (96.8, 99.5)

Candida spp

Vulvovaginal candidiasis is a common fungal infection of the vaginal epithelium that affects up to 75% of women over their life time (37). Between 85 and 95% of yeast strains isolated from the vagina belong to the species *Candida albicans* (37–39). Use of antibiotics, oral contraceptives, hormone replacement therapy, pregnancy, and uncontrolled diabetes mellitus increase the risk of vulvovaginal candidiasis (37, 40–42). Common symptoms include vulvar itching, soreness, irritation, and erythema, but vulvovaginal candidiasis can also be asymptomatic (42, 43). Vaginal discharge can be present and is often white and "cheese-like" with a normal vaginal pH (≤4.5). Vulvovaginal candidiasis can also cause pain or discomfort during intercourse or urination, and chronic or severe infection may cause vulvar fissuring, edema, and excoriation (43). Most patients with symptomatic vaginitis can be readily diagnosed by microscopic examination of vaginal secretions. Additionally, diagnosis can be made by ruling out other potential causative organisms if microscopy is negative (37).

The Affirm VPIII, a semiautomated test that uses DNA probe technology, can be used to detect and differentiate *Candida* ssp., G. *vaginalis*, and *T. vaginalis*. The test takes only 1 hour but is conducted by a physician and requires specialized equipment (43, 44). The Affirm VPIII has very good sensitivity and specificity for the diagnosis of *Candida* spp. in both symptomatic and asymptomatic women (44). PCR-based technology is not widely commercially available for the detection of *Candida* spp.; however, reference laboratories use their own laboratory-developed assays (29, 45, 46).

BV

Bacterial vaginosis (BV) is often associated with vaginal discharge, cervicitis (47–52), and vaginitis (14). BV is a disturbance in the normal lactobacilli-predominant vaginal microbiota (53, 54). It is the most common cause of vaginal discharge but is often asymptomatic (15, 54). BV is associated with several adverse outcomes including preterm delivery and the acquisition and transmission of other STIs and HIV (3–6, 53, 55, 56). Screening and subsequent treatment for BV might prevent adverse pregnancy outcomes including preterm birth, recurrent abortions, postabortal sepsis, early miscarriages, and stillbirths (57–60). Symptoms of BV include vaginal discharge and/or odor. The odor is usually described as fishy and may become more noticeable after condomless sex or during menses (54).

A single etiologic agent has not been identified for BV. A study using broad-range bacterial PCR analysis of 16S rDNA from the vaginal fluid of subjects with BV showed a high level of species diversity, with 35 bacterial phylotypes detected in 16 samples (61). Because BV has no specific causative organism, it is diagnosed by the use of clinical criteria called Amsel's Diagnostic Criteria or by Gram stain using the Nugent score (62). A swab of vaginal wall secretions is obtained to test for BV. For a confirmed diagnosis of BV using the Amsel criteria at least three of the following should be present: (i) thin, white, gray, homogeneous discharge; (ii) clue cells on microscopy; (iii) pH of vaginal fluid less than 4.5; and (iv) release of a fishy (amine) odor after addition of a 10% KOH solution (63). To examine for clue cells, the vaginal fluid is mixed on a glass slide with a drop of normal saline and covered by a coverslip, and 10 fields are examined under a power of 400× (1). Clue cells are squamous vaginal epithelial cells that are covered with a bacterial biofilm of either small curved rods or cocci, which gives them a stippled or granular appearance (1).

In settings with limited laboratory capabilities, objective point-of-care tests have been developed for the diagnosis of BV, but their performance is variable (64–67).

An alternative to using the Amsel criteria is to use a Gram-stained vaginal smear (68, 69). The advantages of using a Gram stain for diagnosis of BV are that it includes interpretation by standardized criteria by a microbiologist and specimens can be stored and diagnosis can be confirmed later (1). According to the package insert, the BD Affirm VPIII has 95.2% sensitivity and 100% specificity for detecting G. *vaginalis* using Gram stain as the reference test. Another study found that the Affirm VPIII test is a more sensitive diagnostic test than conventional clinical examination and wet mount testing for identification of symptomatic vaginosis (70).

Research using molecular detection techniques for BV-associated bacteria has revealed species not previously reported or isolated by culture, often called BV-associated bacteria (BVAB-1, BVAB-2, BVAB-3) (61, 71–74). Laboratory-developed tests for BV offered by reference laboratories often use different targets for BV-associated bacteria and for other species commonly seen in the bacterial overgrowth associated with BV such as *Gardnerella* species. However, well-performed clinical studies validating those tests are lacking.

Cervicitis

Cervicitis is an inflammatory condition of the cervix and is often asymptomatic (75, 76). Cervicitis is usually caused by the STIs *C. trachomatis*, *N. gonorrhoeae*, and occasionally *T. vaginalis* or HSV. Other organisms that have been implicated as causing cervicitis include *Mycoplasma genitalium*, *Ureaplasma urealyticum*, cytomegalovirus, and adenoviruses (49, 52, 75, 77, 78). Signs and symptoms that can be used to identify cervicitis include mucopurulent discharge or friability (easily induced bleeding) at the endocervical os (75, 79). More subtle signs include edematous ectopy (edema of the cervical ectopion). Leukorrhea (>10 white blood cells per high-power microscopic field of vaginal fluid) can occur and has been associated with chlamydial and gonococcal infection of the cervix (15). Where inflammatory vaginitis is not present, leukorrhea may be used as a sensitive indicator of cervicitis.

Complications of cervicitis include ascending infections such as endometritis and pelvic inflammatory disease and adverse outcomes of pregnancy and infections in the newborn. Chronic cervical inflammation has also been linked to pathogenesis of cervical cancer (75). Cervicitis may also be associated with increased sexual and mother-to-child transmission of HIV infection through the increased susceptibility to HIV infection and increased HIV viral shedding (20, 22, 80–83). Women with signs of cervicitis should be assessed for pelvic inflammatory diseases and tested for *C. trachomatis*, *N. gonorrhoeae*, *T. vaginalis*, and if available, *M. genitalium*. *C. trachomatis* and *N. gonorrhoeae* account for less than half of cases of cervicitis (75, 84, 85).

Microscopic definitions of cervicitis utilize Gram stain analysis of mucus from the endocervical canal. The presence of more than 10 polymorphoneutrophils (PMNs) per high-power field is associated with cervicitis (79, 85, 86). However, microscopy is subject to intra-observer variability

and error due to PMNs from outside the cervical canal. Identification of the etiologic agents is necessary for appropriate treatment.

Gonococcal and Chlamydial Infections

Sexually transmitted infections of *C. trachomatis*, causing chlamydia, and *N. gonorrhoeae*, causing gonorrhea, continue to place an immense health burden on men and women worldwide. *C. trachomatis* and *N. gonorrhoeae* infections are the most common STIs and accounted for 211.8 million cases globally in 2008 (87). In the United States alone, over 1.4 million chlamydial infections, a rate of 456.7 per 100,000 population, were reported to the CDC in 2012, making it the most common notifiable disease in the United States (88). Gonorrhea is the second most commonly reported notifiable disease in the United States, with over 330,000 cases reported in 2012, a rate of 107.5 per 100,000 population (88).

C. trachomatis can cause urethritis, epididymitis, proctitis, cervicitis, acute salpingitis, and pelvic inflammatory disease. *C. trachomatis* infections are often asymptomatic in both males and females. Children born to *C. trachomatis*-infected mothers are at higher risk for conjunctivitis and chlamydial pneumonia (89). Gonococcal infections are more likely to be symptomatic and may cause lower and upper genital tract infections and occasionally disseminated infections with gonococcemia, with a characteristic rash, tenosynovitis, and arthritis (90). The immunologic response triggered by lower genital tract infections with *C. trachomatis* and *N. gonorrhoeae* leads to significant inflammation of the cervico-endometrial tissue (91). Due to infection and the associated chronic inflammatory response, several important sequelae may result from these infections, including pelvic inflammatory disease, ectopic pregnancy, and infertility (1, 15, 91, 92).

For decades, bacterial culture was the standard diagnostic modality for *C. trachomatis* and *N. gonorrhoeae* infections. Culture is specific, but studies have demonstrated that the molecular-based enzyme immunoassay or nucleic acid amplification has a higher clinical sensitivity than culture (93, 94). Due to its lower clinical sensitivity and variable performance between laboratories, culture has been largely replaced in laboratories by NAATs (10, 95–108). NAATs have shown high performance in determining infection from symptomatic and asymptomatic individuals and in many specimen types (10, 95–99, 101–107, 109–113). Urine specimens, however, tend to have lower sensitivities. Self-collected vaginal specimens have been used for diagnosis, and the sensitivity observed has been comparable to or better than other specimen types.

Chlamydiae are nonmotile, Gram-negative bacteria. They are obligate intracellular bacteria, which makes them difficult to grow in culture. The *C. trachomatis* species comprises 15 serovars (A, B, Ba, C, D, E, F, G, H, I, J, K, L1, L2, and L3) that can cause disease in humans; serovars D through K are the major cause of genital chlamydial infections in men and women (114). *C. trachomatis* culture technique requires specific methods of specimen collection, transport, and storage and the use of a sensitive cell line. Importantly, because of the equipment and technique required, chlamydia culture is also relatively expensive. A further disadvantage to culture is that because chlamydiae are intracellular bacteria, their specific culture requirements are not available in all laboratories, and the culture system has modest sensitivity. Historically, clinical laboratory methods for *C. trachomatis* detection have also included direct fluorescent antibody testing and enzyme immunoassay.

N. gonorrhoeae is a Gram-negative diplococcus bacterium. Historically, the diagnosis of *N. gonorrhoeae* infection required isolation of the organism on selective media or the observation of diplococci in Gram-stained smears of clinical specimens. *N. gonorrhoeae* does not survive long outside the host and is highly susceptible to adverse environmental conditions such as drying and alternations in temperature, which makes culture of *N. gonorrhoeae* difficult. Culture of *N. gonorrhoeae* requires plating on selective media and incubation at 36°C in a CO_2-enriched, low oxygen atmosphere (112). That process is followed by colony identification by morphology, oxidase positivity, and confirmation using various carbohydrate utilization chemical tests, direct fluorescent antibody, or Gonostat (a DNA transformation assay for the detection of *N. gonorrhoeae* in clinical specimens). Gonococcal cultures can be falsely negative in the setting of a low bacterial load (112). However, false-positive gonococcal cultures may occur when cultures are taken from sites such as the pharynx, which is commonly colonized with other nongonococcal *Neisseria* species (e.g., *Neisseria meningitis*, *Neisseria flavus*, *Neisseria cinerea*, etc.). Culture methods can have good clinical sensitivity but are dependent on proper specimen handling. Improper specimen storage and transport can result in the loss of organism viability and yield false-negative results. In addition, poor sampling technique, toxic sampling materials, and the inhibition of growth by the components of body secretions can also result in false-negative results.

Commercially available, FDA-cleared, real-time PCR nucleic acid amplification assays for the detection of *C. trachomatis* and *N. gonorrhoeae* include Xpert CT/NG (Cepheid, Sunnyvale, CA), Cobas Amplicor CT/NG (Roche Molecular Systems Inc., Basel, Switzerland) and RealTime CT/NG (Abbott Molecular, Abbott Park, IL). Those assays can be used with a variety of genitourinary specimens including female endocervical swabs, patient-collected vaginal swabs, and male and female urine specimens. The nucleic acid targets for those tests are described in Table 2.

The Xpert CT/NG assay can be used with genital and urine specimens collected in urine and endocervical/vaginal specimen collection kits that preserve patient specimens to allow transport to the laboratory for analysis. Test results are obtained in approximately 90 minutes and are displayed in tabular and graphic formats on a computer system. The GeneXpert system is an example of a system with internal quality control mechanisms to ensure ideal test functioning and conditions. Reagents for quality control mechanisms include the detection of a sample processing control, a sample adequacy control, and a probe check control, included in the cartridge. The sample processing control is present to control for adequate recovery and amplification of the target bacteria using DNA from the nonpathogen *Bacillus globigii*. The sample processing control verifies that binding and elution of target DNA have occurred if the target organisms are not found to be present in the sample. The sample adequacy control reagents detect the presence of the gene encoding hydroxymethylbilane synthase, a single-copy human cellular housekeeping gene, to monitor whether the sample contains human DNA. A negative sample adequacy control indicates that inadequate numbers of human cells were present in the sample due to sample degradation, insufficient mixing, or because of an inadequately collected specimen. The probe check control

TABLE 2 Nucleic acid amplification test assays and targets for the detection of *C. trachomatis* and *N. gonorrheae*

Technology target amplification method/detection method	PCR	Strand displacement amplification (SDA)	Transcription-mediated amplification (TMA)
Commercially available test platforms	GeneXpert (Cepheid, Sunnyvale, CA) Cobas Amplicor (Roche Molecular Systems Inc., Basel, Switzerland) m2000 RealTime (Abbott Molecular, Abbott Park, IL)	ProbeTec SDA (Becton Dickinson, Sparks, MD)	Aptima TMA (Hologic, San Diego, CA)
Targets	Xpert CT/NG: DNA (one unique chromosomal gene sequence for *C. trachomatis* and two unique chromosomal gene sequences for *N. gonorrhoeae*) Amplicor CT/NG: DNA (207 nucleotides within the cryptic plasmid DNA of *C. trachomatis* and a sequence of approximately 201 nucleotides within the M·Ngo PII gene of *N. gonorrhoeae* Abbott RealTime CT/NG: DNA (plasmid DNA of *C. trachomatis* and genomic DNA of *N. gonorrhoeae*)	Genomic DNA	rRNA

verifies reagent rehydration, PCR tube filling in the cartridge, probe integrity, and dye stability.

The Abbott RealTime CT/NG assay uses fluorescent-labeled oligonucleotide probes to amplify and detect the *C. trachomatis* and *N. gonorrhoeae* target sequences. The process occurs in a closed system without the need to open the sealed plate. The probes only generate signal when specifically bound to the amplified product. The targets for the assay are *C. trachomatis* cryptic plasmid (occurring at about 7 to 10 copies per chlamydial organism) and the multicopy opacity gene of *N. gonorrhoeae* (repeated up to 11 times per organism). The limit of detection for that assay is about 320 copies of each target, which is equivalent to approximately 30 to 40 organisms.

The BD ProbeTec ET *Chlamydia trachomatis* and *Neisseria gonorrhoeae* Amplified DNA Assays, when used with the BD ProbeTec ET system, use homogeneous strand displacement amplification technology for primer amplification and fluorescent energy transfer (ET) as the detection method to test for the presence of *C. trachomatis* and *N. gonorrhoeae* DNA in clinical specimens (115, 116). The ProbeTec ET can be used with endocervical swabs, male urethral swabs, and in female and male urine specimens as evidence of infection with *C. trachomatis*, *N. gonorrhoeae*, or of co-infection with both *C. trachomatis* and *N. gonorrhoeae*. Specimens may be from symptomatic or asymptomatic females and males.

The Aptima Combo 2 Assay is a second-generation NAAT that uses hybrid capture and transcription-mediated amplification to streamline specimen processing and amplify the target rRNA and uses a dual kinetic assay to detect the amplicon (97, 111). The Aptima Combo 2 Assay detects a specific region of the 23S rRNA from *C. trachomatis* and a specific region of the 16S rRNA from *N. gonorrhoeae*.

Several studies have evaluated the utility and performance of self-collected specimens for *C. trachomatis* and *N. gonorrhoeae* testing (10–12). NAAT performance for the detection of *C. trachomatis* and *N. gonorrhoeae* infection can be seen in Table 3.

Over the past 75 years, *N. gonorrhoeae* infections have become increasingly resistant to antimicrobial therapy: first to sulfa-based antibiotics, then penicillins, tetracyclines, fluoroquinolones, and now to extended-spectrum cephalosporins (117). Modern molecular techniques of PCR or genetic sequencing demonstrate that *N. gonorrhoeae* DNA antimicrobial-resistance-associated mutations can be easily and reproducibly identified for surveillance (118–120). NAAT is more sensitive than culture but does not readily allow for bacterial isolation and phenotypic drug susceptibility testing. The validation of newer methods of susceptibility testing based on molecular markers of resistance is urgently needed.

M. genitalium

M. genitalium is an important sexually transmitted bacterium (121). The role of *M. genitalium* in the etiology of cervicitis has been well supported (77, 122–128). *M. genitalium* has also been shown to be associated with endometritis and pelvic inflammatory disease (124, 129–132), urethritis in males and females (133–136), balanoposthitis (137), prostatitis (138), genital tract disease in men (124, 139), and perhaps male and female infertility (140, 141). *M. genitalium* may also be associated with increased rates of preterm delivery (141, 142). It cannot be diagnosed by symptoms alone, because symptoms vary and many infected patients are asymptomatic. The prevalence in symptomatic patients varies from 11 to 25%, indicating a need for routine testing in those with genitourinary symptoms (77, 78, 125, 132, 143).

The diagnosis of *M. genitalium* has improved with the use of PCR, but laboratory screening and diagnostic algorithms vary between sites and regions. Many large clinical laboratories in the United States have developed

TABLE 3 Reported performance of nucleic acid amplification assays for the detection of *C. trachomatis* and *N. gonorrhoeae*[a]

Organism	Specimen	Symptom status	PCR						SDA: BD ProbeTec SDA		TMA: Aptima Combo 2	
			Abbott Real-Time PCR		Cobas Amplicor System (vs. culture/DFA)		GeneXpert					
			Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
C. trachomatis	Patient-collected vaginal specimen	Symptomatic	98.4 (91.5, 100)	98.9 (97.5, 99.6)			100 (95.4, 100)	98.4 (97.5, 99.0)				
		Asymptomatic	97.3 (85.8, 99.9)	99.1 (97.9, 99.7)			99.2 (95.5, 100)	99.5 (99.2, 99.8)			98.4 (91.2–100)	96.8 (95.0–98.1)
	Endocervical swab	Symptomatic	93.8 (84.8, 98.3)	99.8 (99.0, 100)	98.9 (97.6–100)	98.6 (97.8–99.3)	96.2 (89.3, 99.2)	99.6 (99.0, 99.9)			92.4 (86.7–96.1)	96.7 (95.1–97.9)
		Asymptomatic	NR	NR	95.0 (93.7–96.3)	98.6 (97.9–99.3)	95.9 (90.7, 98.7)	99.5 (99.2, 99.8)			98.4 (91.3–100)	98.8 (97.4–99.6)
	Female urine	Symptomatic	91.3 (82.8, 96.4)	99.7 (98.9, 100)	92.1 (86.5–97.7)	97.7 (96.8–98.6)	98.8 (93.6–100)	99.7 (99.2–99.9)	77.0 (64.5–86.8)	98.2 (97.0–99.3)	93.8 (88.5–97.1)	98.8 (97.7–99.5)
		Asymptomatic	93.5 (82.1, 98.6)	99.7 (98.9, 100)	89.5 (82.6–96.4)	98.6 (97.9–99.3)	97.6 (93.2–99.5)	99.9 (99.7–100)	83.9 (72.3–92.0)	98.3% (97.0–99.1)	96.8 (88.8–99.6)	99.0 (97.7–99.7)
	Clinical-collected vaginal swab	Symptomatic	98.4 (91.6, 100)	100 (99.3, 100)			NR	NR	88.7 (78.1–95.3)	98.5 (97.1–99.4)	96.5 (91.3–99.0)	96.4 (94.7–97.7)
		Asymptomatic	97.2 (85.5, 99.9)	99.3 (98.2, 99.8)			NR	NR	96.8 (89.0–99.6)	97.9 (96.6–98.8)	96.7 (88.7–99.6)	97.2 (95.5–98.4)
	Male urine	Symptomatic	95.5 (91.4, 98.1)	99.1 (98.0, 99.7)	87.6 (82.8–92.3)	91.9 (90.2–93.5)	97.6 (93.0–99.5)	99.7 (98.8–100)	95.4 (89.6–98.5)	89.4 (85.9–92.4)	98.5 (95.7–99.7)	98.4 (96.8–99.3)
		Asymptomatic	96.6 (90.3, 99.3)	99.3 (98.2, 98.8)	92.0 (85.9–98.1)	95.7 (94.1–97.3)	100 (95.1–100)	99.8 (99.6–99.9)	89.5 (66.9–98.7)	95.8 (91.6–98.3)	96.3 (89.4–99.2)	98.8 (96.8–99.7)
	Urethral swab	Symptomatic	93.4 (87.9, 97.0)	98.3 (96.8, 99.2)	96.3 (93.6–99.0)	95.2 (93.9–96.5)	NR	NR	95.5 (89.7–98.5)	92.9 (89.9–95.3)	96.4 (92.8–98.6)	96.9 (94.9–98.2)
		Asymptomatic			98.7 (97.1–100)	97.7 (96.5–98.9)			89.5 (66.9–98.7)	97.0 (93.2–99.0)	94.6 (86.7–98.5)	98.4 (96.3–99.5)

(*Continued on next page*)

TABLE 3 Reported performance of nucleic acid amplification assays for the detection of *C. trachomatis* and *N. gonorrhoeae*[a] (*Continued*)

Organism	Specimen	Symptom status	PCR						SDA: BD ProbeTec SDA		TMA: Aptima Combo 2	
			Abbott Real-Time PCR		Cobas Amplicor System (vs. culture/DFA)		GeneXpert					
			Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity	Sensitivity	Specificity
N. gonorrhoeae	Patient-collected vaginal specimen	Symptomatic	96.2 (80.4, 99.9)	99.6 (98.7, 100)			100 (87.2–100)	99.8 (99.4–100)				
		Asymptomatic	100 (80.5, 100)	100.0 (99.4, 100)			100 (86.3–100)	>99.9 (99.8–100)			100 (83.9–100)	99.5 (98.6–99.9)
	Endocervical swab	Symptomatic	88.0 (68.8, 97.5)	99.8 (99.1, 100)	94.5 (86.6–98.5)	98.7 (98.0–99.4)	100 (87.2–100)	99.9 (99.6–100)			100 (96.2–100)	98.1 (96.9–98.9)
		Asymptomatic	NR	NR	98.0 (89.3–99.9)	98.7 (98.1–99.4)	100 (86.3–100)	100 (99.8–100)			96.9 (83.8–99.9)	99.6 (98.7–100)
	Female urine	Symptomatic	93.8 (79.2, 99.2)	99.6 (98.8, 99.9)			96.6 (82.2–99.9)	100 (99.7–100)	83.7 (70.3–92.7)	99.6 (98.6–100)	92.6 (85.3–97.0)	99.1 (98.2–99.6)
		Asymptomatic	82.6 (61.2, 95.0)	99.5 (98.7, 99.9)			92.0 (74.0–99.0)	>99.9 (99.8–100)	86.5 (71.2–95.5)	99.3 (98.4–99.8)	87.5 (71.0–96.5)	99.5 (98.5–99.9)
	Clinical collected vaginal swab	Symptomatic	96.3 (81.0, 99.9)	100 (94.4, 100)			NR	NR	96.1 (86.5–99.5)	99.3 (98.1–99.8)	96.2 (87.0–99.5)	99.1 (98.1–99.6)
		Asymptomatic	100 (80.5, 100)	100 (94.4, 100)			NR	NR	97.4 (86.2–99.9)	99.6 (98.9–99.8)	95.5 (77.2–99.9)	99.3 (98.3–99.8)
	Male urine	Symptomatic	98.7 (96.3, 99.7)	99.2 (98.0, 99.7)	96.0 (93.9–98.1)	97.6 (96.6–98.6)	99.1 (94.9–100)	100 (99.4–100)			98.4 (96.3–99.5)	99.8 (98.7–100)
		Asymptomatic	100 (71.5, 100)	100 (99.4, 100)	75.0 (42.8–94.5)	99.6 (98.8–99.9)	92.3 (64.0–99.8)	99.9 (99.7–100)			100 (75.3–100)	99.5 (98.1–99.9)
	Urethral swab	Symptomatic	99.5 (97.1, 100)	99.0 (97.6, 99.7)	99.4 (97.9–99.9)	96.2 (95.0–97.5)	NR	NR	98.4 (95.5–99.7)	94.8 (91.6–97.0)	99.0 (97.2–99.8)	98.8 (97.2–99.6)
		Asymptomatic							95.5 (72.7–99.9)	99.3 (98.3–98.7)	100 (78.2–100)	96.7 (94.3–98.3)

[a]Abbreviations: NR, not reported; SDA, strand displacement amplification; TMA, transcription-mediated amplification; DFA, direct fluorescent antibody

Mycoplasma species-specific NAATs using quantitative PCR. Isolation of the fastidious and slow-growing *M. genitalium* from clinical specimens has proven difficult. It has the smallest known genome of a self-replicating organism (144) and contains 521 genes, 482 of which are able to encode proteins (135, 145, 146). Additionally, *M. genitalium* genotypic macrolide resistance has been documented (147).

GENITAL ULCER DISEASE

More than 20 million cases of genital ulcer disease are estimated to occur globally each year (148). Genital ulcer disease is characterized by ulcerating lesions on the vulva, vagina, perineum, perianal skin, penis, or scrotum. It is commonly caused by sexually transmitted infections including syphilis and HSV and less commonly by *Haemophilis ducreyi* (chancroid). However, genital ulcer disease is commonly caused by other infectious and noninfectious etiologies. Genital ulcer diseases may facilitate HIV acquisition and transmission (2, 149, 150). In HIV co-infected patients, genital ulcer diseases can increase the transmission of HIV by increasing local viral shedding (151, 152), and syphilis infection can increase plasma HIV viral load (153, 154).

The clinical evaluation of genital ulcers is insufficient for the diagnosis of disease because of frequent atypical presentation, the possibility for co-infections, and because the treatment differs by etiologic agent. Conventional laboratory methods for the diagnosis of genital ulcer diseases include serology, dark-field microscopy, culture, and histopathology (biopsy). Genital ulcer diseases can be diagnosed using PCR; multiplex PCR assays can help to speed the time to diagnosis by testing for multiple infections (155–158). Several large laboratories offer multiplex PCR assays for the diagnosis of genital ulcer disease, which include targets for HSV-1, HSV-2, *Treponema pallidum*, and *H. ducreyi*.

Syphilis

Syphilis is a curable disease that is easily diagnosed with a simple blood test and treated with penicillin, yet there are 10 million new syphilis infections each year (87). In the United States there were 49,903 cases of syphilis reported in 2012, a rate of 16.0 per 100,000 population (88). Syphilis is an infection caused by the spirochete *T. pallidum*, which is able to persist for decades in the mammalian host but is extremely fragile *ex vivo* (159). The manifestations of the disease vary depending on in which of the four stages it presents: primary, secondary, latent, and tertiary. The primary stage classically presents with a single chancre (a firm, painless, nonitchy skin ulceration), secondary syphilis with a diffuse rash which frequently involves the palms of the hands and soles of the feet, latent syphilis with few to no symptoms, and tertiary syphilis with gummas, neurological, or cardiac symptoms. However, syphilis frequently has atypical presentations that may be difficult to differentiate from other sexually transmitted infections, making effective diagnostics essential to the identification and control of infection (160).

The primary route for transmission of syphilis is through sexual contact. However, it can also be transmitted from mother to fetus during pregnancy or at birth, resulting in congenital syphilis. If syphilis remains untreated during pregnancy, it can lead to fetal loss or stillbirth or, in a live-born infant, neonatal death, prematurity, low birth weight, or syphilis infection in the infant. In syphilis-infected pregnant women, adverse birth outcomes are common and have been shown to be 4.5 times higher in those with untreated syphilis than those without syphilis (161). Congenital syphilis can be prevented by screening early in pregnancy, treating seropositive pregnant women, and preventing reinfection (161, 162).

Syphilis infection, like other genital ulcer diseases, may facilitate HIV acquisition and transmission. Anogenital chancres interfere with the natural mucosal and epithelial barriers and cause inflammation, providing a portal of entry for HIV (2, 149). Additionally, people with HIV infection are at increased risk for neurosyphilis (160, 163). Furthermore, syphilis infection during pregnancy is associated with over a 2.7-fold increased risk of mother-to-child HIV transmission (163, 164).

In patients with a current lesion who seek health care, early diagnosis can be made using dark-field microscopy to visualize the motile spirochetes, using the exudate collected from the lesion, because the chancre may precede syphilis seroconversion. However, most patients have no signs or symptoms, so in clinical settings serological tests are typically used to make diagnoses of syphilis.

There are two classifications of serological tests for syphilis: nontreponemal and treponemal. Nontreponemal tests are based on the reactivity of cardiolipin-cholesterol-lecithin antigen with serum from patients with syphilis. Nontreponemal tests have the benefit of being rapid, relatively low cost, and easy to perform; however they are nonspecific and less sensitive but often used for initial syphilis screening. In contrast, treponemal tests detect antibodies particular to the members of the genus *Treponema* and thus tend to be more specific than nontreponemal tests.

Molecular testing is not commonly used to diagnose syphilis infections. However, a recent meta-analysis was conducted of 46 studies showing the performance of PCR for the detection of *T. pallidum* infection. The study showed that sensitivity using whole blood specimens was 36.1% (95% CI: 24.2%, 50.0%) in primary syphilis cases and 54.2% (95% CI: 41.0%, 66.8%) in secondary syphilis. Lesion swab specimens had higher sensitivity than whole blood at 75.9% (95% CI: 68.5%, 82.0%) (165). The specificity was very good, with a range from 83.3 to 96.6% for primary and secondary syphilis infections in all specimen types (165).

To identify networks of transmission, researchers have conducted *T. pallidum* strain typing. The CDC developed a *T. pallidum* typing method using 60-bp repeats in the acidic repeat protein (*arp*) gene and using sequence differences in the *Tpr* subfamily II genes (*tprE* [tp0313], *tprG* [tp0317], and *tprJ* [*tp0621*]) (166). That strain typing method has been applied to patient samples and used in epidemiological studies (167–172). Studies have shown diverse strain types worldwide. However, studies using strain typing in San Francisco and Seattle showed that most infections were of the same subtype, making interpretation of results difficult and necessitating the development of a strain typing system with better discrimination (168, 169).

HSV

Over 500 million people are infected with HSV-2 (173). HSV-1 also affects a large proportion of people, with a seroprevalence of around 80% of adults in the United States (174, 175). The association of HSV with the increased risk of newborn disease and infant mortality as well as the increased risk of HIV transmission is well recognized (81, 176).

A clinical examination of potential herpes lesions should always be confirmed by an antigen-specific diagnostic test.

HSV can be detected with viral culture, antigen, nucleic acid, and antibody tests. To use viral culture to detect clinical disease, culture may have to be performed more than once since the virus is not always present at the site of the lesion (177). Serological tests, such as enzyme immunoassay and Western blot, can detect specific antibodies to HSV-1 and HSV-2; however, antibodies may not develop until several weeks or months after initial infection (178). Molecular tests for HSV can be performed on lesions and body fluids including cerebrospinal fluid and have high sensitivity compared to HSV culture (177). PCR tests can distinguish between HSV-1 and HSV-2 types through several mechanisms. One mechanism is through PCR with melting point analysis and fluorescence resonance energy transfer probes (179). Another method of type differentiation is through melt analysis of a gene encoding glycoprotein D (180). A third mechanism is through amplification of glycoprotein B, a viral envelope protein, encoded by a highly conserved gene. Highly specific probes then detect polymorphisms in that gene for HSV-1 and HSV-2 typing (181).

Currently, there are three FDA-cleared molecular tests for the detection of HSV infections. Information on those tests can be found in Table 4. HSV encephalitis or meningitis must be diagnosed using cerebrospinal fluid, and other noncommercial assays can be used for these cases but will not be discussed here.

Chancroid

In 2012, only 15 cases of chancroid were reported in the United States (88), but chancroid remains a cause of genital ulcer disease in other countries. The etiologic agent of chancroid is *H. ducreyi*, a fastidious Gram-negative coccobacillus. Chancroid is characterized by one or more deep, painful genital ulcers with regional lymphadenopathy. Often diagnosis is made after the exclusion of HSV and *T. pallidum* infection at least 7 days after ulcer onset. However, co-infections of *H. ducreyi* and those other infections are common. Chancroid has traditionally been more prevalent in lower socioeconomic populations and in certain high-risk groups (182). Additionally, males have a higher incidence than females, and incidence is particularly high among uncircumcised men (183, 184). Chancroid may enhance the transmission of HIV (185, 186).

Laboratory diagnosis of *H. ducreyi* can be difficult because the organism does not grow well *in vitro*. Gram stain of exudate is insensitive but can be suggestive of *H. ducreyi* (Gram-negative, slender rods or coccobacilli in a "school of fish" pattern). Definitive diagnosis of *H. ducreyi* is made if identified on culture. Culture has been shown to be at best 75% sensitive in comparison with molecular assays, so molecular tests have replaced culture as the diagnostic test of choice (156, 187).

Nucleic acid-based tests have been developed for *H. ducreyi* using amplification of the 16s rRNA gene, the *rrs* (16S rRNA)/*rrl* (23S rRNA) intergenic spacer region, the gene encoding a 27-kDA *H. ducreyi*-specific protein, and the *groEL* gene and the *recD* gene (188). Because *H. ducreyi* is not the only cause of genital ulcer disease, and it is uncommon, the most useful PCR formats are those that can distinguish between other genital ulcer diseases. Seeplex multiplex-PCR panels (Seegene, Seoul, South Korea) are available, which include the STD4 ACE Detection test, which can detect and distinguish between *H. ducreyi*, *T. pallidum*, HSV-1/2, and *C. albicans*. However, that test has not been evaluated for the performance of *H. ducreyi* detection in settings where chancroid is still endemic (188).

MALE URETHRAL DISCHARGE

Male urethral discharge is characterized by abnormal purulent or mucoid secretions from the urethral meatus. Urethral discharge usually indicates urethritis and can be caused by infection. The etiologic agents associated with male urethral discharge include *C. trachomatis*, *N. gonorrhoeae*, *M. genitalium*, *T. vaginalis* (189, 190), and HSV. *U. urealyticum* and adenovirus may also cause male urethral discharge.

Urethritis is associated with PMNs in a urethral smear, urinary sediment, or uncentrifuged urine. Generally, to diagnose urethritis, counts of at least five PMNs per high-power field (×1,000) in the urethral smear or at least five white blood cells per high-power field (×400) in the urinary sediment are used (144, 191, 192). Highly sensitive and specific NAATs, as mentioned previously for the diagnosis of causes of cervicitis, can be used for diagnostic testing to determine the etiology of urethral discharge. Those same tests that are used for cervicitis in women can be used with male urethral or urine specimens. Urine specimens are preferred because they are noninvasive and easy to collect.

ASYMPTOMATIC INFECTIONS

STIs may be asymptomatic, and therefore knowledge of the local STI epidemiology and systematic screening is important to reduce the burden of these infections. STI screening creates the opportunity to treat asymptomatic disease, prevent adverse sequelae, prevent further transmission to others, identify likely infected partners for testing and treatment, and reduce the burden of disease. Sensitive NAATs should be used for screening. Up to a third of infections of *T. vaginalis* in females are asymptomatic, and most infections in men are asymptomatic (16, 190). The observed high prevalence of asymptomatic *T. vaginalis* in

TABLE 4 FDA-cleared tests for the detection of HSV by using oral or anogenital specimens

Assay name	Method	Specimen types	Sensitivity	Specificity
MultiCode-RTx kit (Luminex, Austin, TX)	PCR	Vaginal swabs	92.4 (HSV-1) 95.2 (HSV-2)	98.3 (HSV-1) 93.6 (HSV-2)
ProbeTec HSV Qx test (Becton Dickinson, Sparks, MD)	SDA	Anogenital lesions from men and women	96.8 (HSV-1) 98.4 (HSV-2)	97.6 (HSV-1) 83.7 (HSV-2)
IsoAmp HSV Assay (Biohelix, Beverly, MA)[a]	Isothermal helicase-dependent amplification	Male and female genital or oral lesions	97.1 (HSV-1 and HSV-2)	93.4 (HSV-1 and HSV-2)

[a]The Biohelix HSV Assay does not distinguish between HSV-1 and HSV-2.

male partners highlights the use of PCR testing in this population and the need for improved partner management and new disease control strategies (190).

EXTRAGENITAL INFECTIONS

NAATs are the recommended test method for rectal and oropharyngeal specimens for testing of *C. trachomatis* and *N. gonorrhoeae*, but currently, NAATs are not FDA cleared for use with extragenital specimens. Therefore, clinical laboratories must be in compliance with federal regulations (Clinical Laboratory Improvement Act) to test extragenital specimens for infection (112, 193).

FUTURE DIRECTIONS

Patient self-collected specimens for the diagnosis and screening of urogenital infections are increasingly being used and evaluated. Those specimens allow for home-based specimen collection, expedited testing in the clinic, and specimen collection outside of the home at nonclinical venues. The continued evaluation of patient self-collected specimens should be conducted to understand the accuracy and benefit of those specimen types and to improve the frequency of regular screening for STIs.

Multiplex diagnostics (tests that use one specimen to test for multiple infections) have the capacity to streamline diagnosis and the differentiation of infections. An additional advantage of multiplex tests is that they can detect co-infections. An example of a multiplex testing platform is the BD Max System Vaginitis, which will be a semiquantitative molecular diagnostic system that detects the full range of organisms associated with vaginitis/vaginosis including *Candida* spp., *Lactobacillus* spp., *G. vaginalis*, *Atopobium vaginae*, BVAB-2, *Megasphaera*, and *T. vaginalis*. That test is currently under development. Further research should be conducted to systematically evaluate the performance and benefits of multiplex testing.

Finally, combining patient self-collection of specimens with rapid, point-of-care, multiplex systems with information technology enabling the timely delivery of test results directly to the patient may allow people to get screened or tested for STIs outside of the physician's office and obtain their results in less than an hour. Such increased access to screening and decrease in time from testing to treatment could profoundly change medical and public health practice regarding the control and prevention of STIs (194). With a shortened time to diagnosis, patients may be treated and rendered noninfectious the same day, resulting in reduced complications from untreated infection and decreased spread of infection to others.

The use of NAATs has revolutionized the management of STIs, enabled the use of various specimen types, and improved the detection of known and previously unrecognized infections. The future is promising, with expected improvements in sexual and reproductive health.

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Syndromic Diagnostic Approaches to Bone and Joint infections

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29

INTRODUCTION: THE SIGNIFICANCE OF OSTEOARTICULAR INFECTIONS

Bone and joint infections are among the more common infections of normally sterile body sites. These infections can lead to loss or compromise of function, long-term damage, and mortality. Prompt diagnosis, including microbiological diagnosis, is needed for appropriate management to reduce these adverse outcomes.

Prosthetic joint infections are the highest-incidence osteoarticular infections (1). Kurtz et al. (2) studied the frequency and cost of these. In the United States, the incidence of joint infection is estimated to be 2.18% among patients with prosthetic hip and knee joints. There are an estimated 14,802 knee infections and approximately half that many hip infections, and the rate of these infections has increased over time. The length of hospital admission for prosthetic joint infections is 9.5 days and 7.2 days for hip and knee infections, respectively. Treatment of these infections is expensive, costing an average of $30,300 per case for hip infections and $93,600 for hip revisions. In 2009, the total national cost of treating prosthetic joint infections was an estimated $566 million. An earlier estimate of the annual cost of hospital care for prosthetic joint infections in 2004 was $283 million (3). The incidence of native joint infection is not well studied, but it is lower than that of prosthetic joint infection (4, 5).

The epidemiology of vertebral osteomyelitis, a disease that occurs primarily in adults, has been well studied (6). The incidence of vertebral osteomyelitis is estimated to be 2.4 cases per 100,000 people. The incidence rises markedly with age; among those 70 years old and older, the incidences are approximately 5 and 9 cases per 100,000 persons in women and men, respectively. The length of hospital admission for osteomyelitis can be quite long, with a mean of 25 days and median of 14 days (6). Outcomes from osteomyelitis can be severe. In a study of patients with vertebral osteomyelitis in the Cleveland area, 11% died, 14% relapsed, and one-third had residual disability (7).

Osteoarticular infections are fairly common in children. Acute, hematogenous osteoarticular infections are estimated to occur in 1 in every 10,000 children younger than 12 years of age each year in Norway and from 1.1 to 1.4 in every 10,000 children 1 to 16 years of age in Lithuania (8, 9). The highest incidence is in children under 3 years of age, and approximately twice as many cases occur in boys than girls (10, 11). These numbers reflect the incidence in developed nations, and the incidence in children in the developing world is thought to be higher (10). Hospital admissions for osteomyelitis in children can be lengthy, ranging from medians of 15.7 to 50 days (9, 12). The duration of hospital admission for septic arthritis in children is somewhat shorter, at a median of 9.6 days (12).

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CLINICAL CONTEXT: OSTEOMYELITIS

Osteomyelitis, or infection of the bone, is classified primarily by the route of entry of pathogens into the bone. Pathogens enter the bone either by contiguous spread following a break in the protective layers over the normally sterile bone or through hematogenous spread. Within this primary classification, secondary groupings of age, site of infection, duration of infection, and source of the infection are also clinically relevant.

Osteomyelitis Following Contiguous Spread

Pathogens can enter the bone following a variety of different breaks in the overlying structures. Surgery, accidental wounds (including bites from humans or animals), and open fractures are common opportunities for organisms to gain access to bone. The presence of a foreign object within the bone increases the chance of infection and may require surgical intervention for recovery. Patients with diabetes are at risk for osteomyelitis of the foot bones due to vascular compromise, which reduces the host's ability to clear infection, as well as peripheral sensory neuropathy, which leads to frequent injury and inadequate wound care due to absence of pain (13). These lead to chronic, often untreated ulcers of the feet which can progress to deeper infections, including those of the bone. Chronic decubitus ulcers in patients confined to bed or wheelchairs can also progress to sacral osteomyelitis (14).

The manifestations of osteomyelitis due to contiguous spread of pathogens are pain and fever, often accompanied by erythema and swelling due to an overlying soft-tissue infection (10, 15, 16). These symptoms may be ascribed to the overlying tissue injury or trauma, so the osteomyelitis may go untreated until severe manifestations such as sinus tract drainage occur and lead to thorough medical

evaluation. Chronic osteomyelitis is more likely to occur when there is a foreign object at the site.

Microbiology of Osteomyelitis Following Contiguous Spread

Osteomyelitis following contiguous spread of pathogens is often polymicrobial, with the mixed infection being caused by organisms from the normal flora of the skin or, less often, the gastrointestinal tract. *Staphylococcus* species, mainly *Staphylococcus aureus*, are the most commonly isolated pathogens. Other Gram-positive organisms, in particular streptococci and enterococci, are also commonly found. Gram-negative bacilli, usually enterobacteriaceae, are also common. Some examples will illustrate this.

About half of the cases of osteomyelitis associated with overlying chronic ulcers such as diabetic foot ulcers or sacral decubitus ulcers are polymicrobial (14, 17). *S. aureus* is the most common pathogen found in these infections, occurring in about half the patients. Coagulase-negative staphylococci are only slightly less common than *S. aureus*, and streptococci are frequently found as well. Gram-negative bacilli are often present, mainly enterobacteriaceae (*Escherichia coli*, *Klebsiella pneumoniae*, and others) and occasionally *Pseudomonas aeruginosa*. The pathogens that cause sternal osteomyelitis following surgical sternotomy are similar, with a predominance of *S. aureus* and, less often, coagulase-negative staphylococci and various Gram-negative bacilli (18). Osteomyelitis following bites from dogs and cats is also often polymicrobial and frequently includes infection with *Pasteurella* species, particularly *Pasteurella multocida* (19). Human bites can lead to osteomyelitis with a variety of oral and skin flora, again including *S. aureus*, but also oral organisms including *Capnocytophaga* and anaerobes such as *Bacteroides*, *Prevotella*, *Porphyromonas*, *Fusobacterium*, and anaerobic Gram-positive cocci (20, 21).

Osteomyelitis Following Hematogenous Spread

Osteomyelitis caused by hematogenous spread of microorganisms falls into two main groups, based on the site of infection. Vertebral osteomyelitis tends to occur in older adults, while osteomyelitis in bones other than the vertebrae tends to occur in children and intravenous drug users.

Hematogenous spread from the urinary tract, mouth, or other colonized body sites is the most common route of entry of pathogens into the vertebrae, but entry of organisms into the vertebrae can also occur by spinal surgery or direct extension from infected soft tissues. Subacute back pain that progresses over a period of weeks is the most common presenting symptom; other symptoms, including fever and tenderness at the site of infection, are not always present (16).

The long bones are common sites of acute osteomyelitis in children, the femur or tibia being the most common, making up 23 to 29% and 19 to 26% of cases, respectively, with other sites each accounting for less than 10% of cases (10, 11). Acute vertebral osteomyelitis does occur in children, but makes up less than 5% of cases (22). Both the relatively high rates of osteomyelitis and the anatomic distribution of osteomyelitis in the long bones in children are thought to be due to the slow blood flow through fenestrated capillaries in the growth plates of long bones, which allows bacteria to escape the circulation and enter the bone. Although many children have a history of minor trauma that precedes onset of osteomyelitis, it is not clear that this is truly a risk for development of the infection because minor trauma is very common in children (23). Finally, it is common for both the bone and adjacent joint to be involved in pediatric infections (24). The most common manifestations of acute osteomyelitis in children are limping or refusal to walk, with fever and tenderness at the site of infection (10).

Microbiology of Osteomyelitis Following Hematogenous Spread

Osteomyelitis following hematogenous spread of microorganisms is nearly always caused by a single species of bacteria. The organisms detected in samples from patients with osteomyelitis due to hematogenous spread are summarized in Table 1. About half of cases of vertebral osteomyelitis are caused by *S. aureus* in the developed world (7, 25). Coagulase-negative staphylococci cause 6.6 to 12% of cases (7, 25). Streptococci are found in 9 to 19.7% of cases, with *Streptococcus pneumoniae* and viridans-group streptococci being most common (7, 25). Gram-negative bacilli are also common causes of vertebral osteomyelitis, making up 12 to 23% of cases (7, 25), most of which are caused by *E. coli*, *P. aeruginosa*, and *K. pneumoniae* (7, 25). Fungi are uncommon causes of osteomyelitis of any type, with *Candida* species and *Aspergillus* species causing a small number of cases, mainly following surgery or in the presence of immunocompromise (6, 25–27).

TABLE 1 Causes of acute osteomyelitis due to hematogenous spread of pathogens in adult vertebral ostomyelitis and children[a]

Pathogen	Frequency in adults (vertebral osteomyelitis)	Frequency in children	Comments
Staphylococcus aureus	+++	++++	Most common cause
Coagulase-negative staphylococci	+ to ++		
Streptococci	+ to ++	+	*Streptococcus pneumoniae* in adults and children
			Viridans-group streptococci in adults
			Streptococcus pyogenes in children
Enterobacteriaceae	++	+	*Escherichia coli*, *Klebsiella pneumoniae* most common
			Salmonella in children with sickle cell disease
Haemophilus influenzae		++	Occurs in unvaccinated children; otherwise rare

[a]Approximate frequency with which pathogens cause infections is indicated by the number of plus signs: ++++, >60%; +++, 40 to 60%; ++, 10 to 39%; +, less than 10%. The absence of a symbol indicates that the pathogen is uncommon or that the literature is limited. See text for references and details.

S. aureus is the cause of 79 to 89% of cases of acute hematogenous osteomyelitis in children (10, 24, 28, 29). Streptococci make up most of the remaining cases, with *S. pneumoniae* and *Streptococcus pyogenes* being the most common species (28–30). Before the use of the *Haemophilus influenzae* type B vaccine, this organism was the second most common cause of this infection, causing 17% of cases in a large case series in Finland before vaccine use but no cases after 1990, when the vaccine was in widespread use (29). *Salmonella enterica* is the most common cause of osteomyelitis in children with sickle cell disease (31). It is important to note that anaerobes are also a cause of osteomyelitis, but they enter the bone through contiguous spread rather than by hematogenous spread (32). *K. kingae* can also cause osteomyelitis in children, but this organism more often causes septic arthritis, so it is discussed in that section.

CLINICAL CONTEXT: INFECTIOUS ARTHRITIS

Clinical Manifestations of Infectious Arthritis

Infectious arthritis is inflammation of the synovial fluid and synovial membrane caused by a pathogen within the joint. Hematogenous spread to the joint is the most common route of entry of pathogens, although direct inoculation can also occur and is particularly important with infections of prosthetic joints at the time of surgery (1). The infection usually affects a single joint, and onset is rapid (acute), with swelling, erythema, warmth, and pain in the affected joint (33). Chronic infectious arthritis or the involvement of multiple joints suggests the involvement of specific pathogens, discussed below (33). Underlying joint disease, such as rheumatoid arthritis, is a significant risk factor for infectious arthritis (4, 33).

The hips and knees are the most common sites of prosthetic joint infections (1, 34). The manifestations are those already mentioned (swelling, erythema, warmth, and pain). Sinus tracts and local abscesses often occur, usually when the pathogen is introduced during surgery (1).

Lyme disease causes recurrent prolonged (weeks to months) or persistent arthritis, typically of the large joints, although small joints can also be affected (35). Lyme arthritis is a late manifestation of Lyme disease, occurring weeks to months after the initial infection (36). Because most patients are treated early in the course of infection, Lyme arthritis occurs in less than 10% of patients (36).

Microbiology of Infectious Arthritis

The age of the patient is a major factor in determining the specific pathogens that cause infectious arthritis. The organisms detected in samples from patients with infectious arthritis are summarized in Table 2. *S. aureus* is the most common organism isolated in most studies of children (e.g., from 42% of cases in a single hospital in Australia [12] and from 38% of cases in Dallas [28]). Detection of *K. kingae* requires use of special methods which are discussed below. In studies which have included these techniques, most find *K. kingae* to be the second most common pathogen in this population (37–39). However, studies in France and Israel found *K. kingae* to be the most common organism detected in osteoarticular infections of young children, being present in 45% and 48% of cases, respectively (40, 41). In areas where brucellosis occurs, such as Israel, *Brucella melitensis* has been found in 8 to 17.5% of cases of infectious arthritis in children (39, 41). Streptococci, including *S. pneumoniae*, *S. pyogenes*, and *S. agalactiae*, and Gram-negative bacilli are found in a small number of cases in children (12, 28, 40, 42). *H. influenzae* type B is also a cause of infectious arthritis in children in the absence of vaccination (43).

In adolescents and adults, *S. aureus* is the most commonly isolated bacterium in infectious arthritis, being present in about half of cases (33, 43–45). Coagulase-negative staphylococci are found in 19% of cases of prosthetic joint infection but in only 6% of native joint infection (45). Streptococci are found in approximately a quarter of cases of infectious arthritis in adults, most often *S. pneumoniae* and *S. pyogenes* (43, 45). *Neisseria gonorrhoeae* is found in a small number of cases among sexually active patients, but it appears that this organism has become less common as a cause of infectious arthritis (44). Mycobacteria, usually *Mycobacterium tuberculosis*, are found in small numbers of cases, causing 1% and 7.7% of cases in two studies which included culture for these organisms (43, 44). In addition to coagulase-negative staphylococci, prosthetic joints

TABLE 2 Causes of acute infectious arthritis in adults and children[a]

Pathogen	Frequency in adults	Frequency in children	Comments
Staphylococcus aureus	+++	+++	
Coagulase-negative staphylococci	+ to ++		More common with prosthetic joint infection
Streptococci	++	+	*Streptococcus pneumoniae* and *Streptococcus pyogenes* in adults and children
			Streptococcus agalactiae in newborns
Enterobacteriaceae	+	+	More common with prosthetic joint infection
Haemophilus influenzae		+	Occurs in unvaccinated children; otherwise rare
Kingella kingae		+++	
Neisseria species	+		
Mycobacteria	+		*Mycobacterium tuberculosis* most common
Propionibacterium acnes	+		More common with prosthetic joint infection
Brucella melitensis		+ to ++	In endemic areas

[a]Approximate frequency with which pathogens cause infections is indicated by the number of plus signs: +++, 40 to 60%; ++, 10 to 39%; +, less than 10%. The absence of a symbol indicates that the pathogen is uncommon or that literature is limited. See text for references and details.

become infected with *Enterobacteriaceae* and, albeit less often, with anaerobes such as *Propionibacterium acnes* that are inoculated into the joint during surgery (1, 34).

Lyme arthritis, caused by *Borrelia burgdorferi*, is a late manifestation of Lyme disease. It occurs in children and adults with exposure to *Ixodes* ticks in areas where the disease is endemic. Antibody testing is the usual method of diagnosis (35), but molecular testing of synovial fluid can also be performed, as discussed below.

MOLECULAR TESTS FOR OSTEOARTICULAR INFECTIONS

Molecular tests for pathogens associated with osteoarticular infections will be discussed in detail in this section. Those molecular assays that have been evaluated in comparison to some "gold" (reference) standard in systematic studies will be included, and the emphasis will be on the diagnostic yield of the molecular test in comparison to the gold standard. In general, case reports will not be discussed unless they add unique information. First, the detection of single agents by molecular methods will be considered. Next, the use of molecular assays for simultaneous detection of multiple pathogens will be discussed. Multiplex assays and molecular panels will be discussed separately from broad-range PCR assays because these assays differ significantly in design.

Molecular Assays for Detection of Individual Pathogens in Bone and Joint Infections

Staphylococcus Species

Staphylococcus species can be readily detected by either culture or PCR. Methicillin-resistant *S. aureus* (MRSA) and methicillin-susceptible *S. aureus* are often considered separately in the studies discussed below, and some of the PCR tests distinguish between these by detection of the *mecA* gene, which mediates methicillin resistance. Several studies in which multiplex PCRs were used to detect *Staphylococcus* species are discussed in the section on multiplex assays, below.

In most studies, the sensitivities of PCR and culture for *S. aureus* are similar. PCR can have the advantage of reduced turnaround time compared to culture if the PCR is run frequently enough. This is significant, because Gram stain, while rapid, is insensitive for staphylococci in osteoarticular infections, detecting only about a third of these (46).

A commercial PCR test for staphylococci was assessed in a single institutional study in France, in which specimens from 72 patients with osteoarticular infections were studied, along with specimens from 33 control patients undergoing revision of bone and joint prostheses (46). The patients with osteoarticular infections had a variety of infections, but most had prosthetic joint infections or septic arthritis. The assay studied was the Xpert MRSA/SA SSTI PCR test run on the GeneXpert platform, produced by Cepheid. This assay is intended for detection of *S. aureus* and the methicillin-resistance trait in swab samples collected from patients with skin and soft tissue infections. The authors used swabs dipped into synovial fluid or into saline containing crushed bone biopsies for PCR and culture with both solid (agar) media and blood culture bottles. Compared to culture, the Xpert MRSA/SA SSTI test had sensitivity of 100% (16/16 detected) and specificity of 98.3% for methicillin-susceptible *S. aureus* (119 out of 121 negative results). Only two patients had MRSA, and both were detected by PCR. All patients without MRSA were correctly identified, for a specificity of 100%. While there were no statistically significant differences in the sensitivity or specificity of the Xpert MRSA/SA SSTI PCR test and culture, hands-on times for culture and Xpert MRSA/SA SSTI PCR were markedly different (25 and 2 minutes, respectively), as were the turnaround times until notification of results (79 hours and 72 minutes, respectively). Commercial assays such as this are expensive, and the cost of the test must be weighed against the potential benefit of reduced turnaround time.

Variable results have been obtained with laboratory-developed tests for *S. aureus* in bone and synovial fluid samples. A study using a laboratory-developed PCR test for *S. aureus* found a modest increase in the detection of *S. aureus* using PCR for the *Nuc* gene in addition to culture that included both solid (agar) media and culture of synovial fluid and blood in separate blood culture bottles (47). The study was conducted at a single hospital in France and included patients under 15 years of age. Out of 24 patients with *S. aureus* infection detected, 54% were detected by culture of osteoarticular samples, 25% were detected only by blood culture, and 12.5% were detected only by PCR. A significant caveat to this study, which was primarily a clinical description of the manifestations of infection, is that it is not clear that all samples were tested by all the methods, so these data should be interpreted with caution. Two small studies also found that laboratory-developed PCR tests were less sensitive than culture for detection of *S. aureus* in osteoarticular samples (37, 48).

K. kingae

K. kingae can be detected by culture or by PCR. As already discussed, this organism is a cause of infectious arthritis in children, most often those 6 to 36 months of age (49). Culture for this organism using solid (agar) media is insensitive, but inoculation of synovial fluid into blood culture broth increases its detection. One study compared culture of 100 specimens from children using several agarose media (including lysed sheep's blood agar) and thioglycollate broth to culture in a BACTEC 460 aerobic 6B blood culture bottle (39). *K. kingae* was detected in a total of 11 samples; 1 was detected by conventional culture methods with agarose media or thioglycollate broth, while all 11 were detected by culture in the blood culture bottle. In a smaller study of 18 cases of *K. kingae* osteoarticular infections in children, culture of synovial fluid in blood culture bottles had a sensitivity of 65%, while culture with solid (agar) media had a sensitivity of only 13.5% (38). Although PCR is significantly more sensitive for *K. kingae* than culture by any method (discussed below), culture in blood culture bottles is an accessible method for detection of this organism in laboratories that do not perform PCR for it. Furthermore, some studies have found that use of blood culture media increases detection of organisms other than *K. kingae* in synovial fluid (50, 51), although others have not (39).

There are a number of studies on the utility of PCR for detection of *K. kingae*, and these vary somewhat in design. Several of these studies include both PCRs specific for *K. kingae* and broad-range PCRs for bacteria using amplification and sequencing of 16S rRNA genes. The results obtained using broad bacterial PCRs are discussed in a separate section below, but it is worth noting that PCRs specific for *K. kingae* were consistently more sensitive than

broad bacterial PCRs for detection of *K. kingae* in these studies (40, 52).

Among the first studies of the use of PCR to detect *K. kingae* was one in which a PCR for the *cpn60* gene (which encodes a chaparonin in *K. kingae*) was performed on synovial fluid samples from children with clinical signs of infectious arthritis (40). Only those samples that had been tested in culture without detection of an organism were tested for *K. kingae* by PCR. The culture included incubation of synovial fluid using a blood culture broth. Of 131 samples, 59 were positive by culture (of these, 25 grew *S. aureus* and 17 grew *K. kingae*). Only 61 culture-negative specimens were available for PCR; 22 of these contained *K. kingae* as determined by PCR. Because of the study design, it is not possible to determine the clinical sensitivity of the PCR for *K. kingae*, but these results suggest that PCR was significantly more sensitive for *K. kingae* than was culture.

To accurately compare the sensitivities of PCR and culture for *K. kingae*, studies must use a validated PCR test for *K. kingae* and apply the appropriate tests (PCR and culture for *K. kingae*) to all specimens in the study. At least three studies fulfill these criteria. In these studies, a positive PCR or culture result for *K. kingae* is considered to be correct, creating a reasonable composite gold standard for sensitivity of the PCR, although the specificity of the PCR cannot be determined. First, a study conducted in France tested synovial fluid from all children with clinically suspected infectious arthritis using a PCR for *cpn60* and culture that included use of a blood culture bottle and solid media (53). In the analytical validation, the PCR detected several clinical isolates of *K. kingae*, with an analytical sensitivity of 200 copies of DNA per reaction, and it did not cross-react with several species of bacteria related to *K. kingae* or unrelated species that commonly cause infectious arthritis. Out of 31 total cases of *K. kingae* infectious arthritis, culture of synovial fluid detected 3 (10% sensitivity) using solid agar, 7 using blood culture bottles (22% sensitivity), and 31 using PCR (100% sensitivity). Neither culture nor PCR of peripheral blood was positive in any patient with infectious arthritis due to *K. kingae* (sensitivities of 0%). Of note, PCR for *K. kingae* remained positive in a majority of samples after 2 to 6 days of treatment with antibiotics.

In a second study, conducted in Switzerland, PCR using two primer and probe sets for two genes (*rtxA* and *rtxB*) within a toxin locus of *K. kingae* was compared to bacterial culture using solid agar and brain-heart infusion broth but no blood culture media (52, 54). Synovial fluid samples from children under 4 years of age with clinical signs of infectious arthritis were tested. Twenty three cases of *K. kingae* infectious arthritis were detected by the PCR test, and none were detected by culture, for sensitivities of 100% and 0%, respectively. Finally, a study conducted at two institutions in the United States used primers for both *cpn60* and *rtxA* and found that of the 12 children 18 years of age or younger, 4 had PCR positive for *K. kingae* (37). Only one of these specimens was cultured in blood culture media, and that culture was positive for *K. kingae*.

K. kingae is found in the oropharyngeal flora of young children, and this has led to the suggestion that oropharyngeal testing might serve as a surrogate for testing of joint fluid in patients with signs and symptoms of infectious arthritis. One group used PCR on oropharyngeal swabs to detect the organism in patients with osteoarticular infections (54). They studied a group of patients with clinical presentations suggestive of osteoarticular infection: 30 patients with *K. kingae* infection diagnosed by culture or PCR of blood, joint fluid, or bone aspirate and 84 without *K. kingae* infection by this definition. They found that 100% of 30 patients with *K. kingae* osteoarticular infections also had the organism detectable by PCR in the oropharynx, but the organism was also found as a commensal in 9.5% of children who did not have *K. kingae* osteoarticular infections, so the specificity of this method is only 90.5%. Another study used culture of oropharyngeal specimens (rather than PCR) as a method for testing children known to have *K. kingae* osteoarticular infections by PCR or culture of synovial fluid or blood, and found that 66.7% of children had oropharyngeal colonization as detected by culture (54).

N. gonorrhoeae

The literature on use of specific molecular assays for detection of *N. gonorrhoeae* species in joint fluids is limited to case reports and small case series of patients with infectious arthritis due to *N. gonorrhoeae*. The declining incidence of this manifestation of *N. gonorrhoeae* infection explains the lack of larger studies. Because of the lack of large, high-quality studies, it is not possible to draw conclusions about the utility of PCR for *N. gonorrhoeae* in infectious arthritis.

A PCR using primers that amplify a region of the 16S rRNA gene specific to pathogenic *Neisseria* species, including *N. gonorrhoeae* and *Neisseria meningitidis*, was used to test samples from eight adult patients who met clinical criteria for gonococcal arthritis and 38 samples from patients with arthritis from other causes (55, 56). Six of the nine patients with gonococcal arthritis had at least one positive PCR, including seven that had cultures positive for *N. gonorrhoeae*. Four of the positive cultures were of synovial fluid, while three were cultures of genital samples from patients with negative synovial fluid cultures. Of the samples from patients without a clinical diagnosis of gonococcal arthritis, 37 of 38 were negative by PCR and one was positive, which was attributed to contamination.

A nested PCR that amplifies the gene for outer membrane III was used to test 14 synovial fluid samples from 10 adult patients who met clinical criteria for gonococcal arthritis and 27 synovial fluid samples from patients with arthritis of other causes (57). All the samples from patients with clinically diagnosed gonococcal arthritis had negative cultures on chocolate agar. Of the 14 samples from patients with gonococcal arthritis, 11 had positive results in the PCR, while none of the samples from patients with arthritis of other causes had positive results. The PCR had an analytical sensitivity of fewer than 10 viable *N. gonorrhoeae* organisms per ml, and the analytical specificity was demonstrated using a range of 23 organisms, including *N. meningitidis* and *Neisseria sicca*.

B. burgdorferi

The mainstay of diagnosis of Lyme disease is the use of two-tier immunodiagnostic testing, with a screening immunoassay followed by a confirmatory Western blot test for positive samples (58). This test can take weeks to become positive after initial infection (35). Lyme arthritis is a late manifestation of sustained infection, so the immunoassay should be positive in patients with this disease. In many patients, a typical clinical presentation of Lyme arthritis with an appropriate exposure history and positive serology is adequate to make the diagnosis (35). In areas that have

endemic Lyme disease, a significant proportion of the population can be seropositive. For example, in Long Island, NY, 5% of the population had serological evidence for previous exposure to *B. burgdorferi* (59). With a high background rate of seropositivity, it might be difficult to interpret the results of serological testing in a patient with joint disease, and molecular testing for synovial fluid can be useful for confirmation of Lyme arthritis. It is important that PCR tests for *B. burgdorferi* be performed only in patients with serological evidence of infection, because positive PCR results from some laboratories that performed tests on patients with negative serological testing led to unfortunate outcomes (60, 61). The organism is only rarely detectable by culture of synovial fluid (62, 63).

PCR for *B. burgdorferi* in synovial fluid of patients with Lyme arthritis is reasonably sensitive and very specific. One of the largest studies included samples from 88 patients with Lyme arthritis defined by clinical criteria, antibody testing, and exclusion of other causes of arthritis, as well as 69 control patients with arthritis from other causes (64). Four primer and probe combinations were tested, three of which amplified regions in the plasmid-encoded *ospA* gene and one of which amplified a region of the chromosomal 16S rRNA gene. The sensitivity of amplification of the *ospA* gene was 64 to 76%, but the sensitivity of PCR for the 16S rRNA gene was only 48%. None of the samples from patients who did not have Lyme arthritis were positive for *B. burgdorferi* PCR (100% specificity). The rate of positivity of PCR for Lyme arthritis declines with the duration of antibiotic treatment. In patients who had received no antibiotics and those who received less than a month of oral therapy, 94 to 100% had at least one positive PCR for *B. burgdorferi*. However, in patients who had received either parenteral antibiotics or oral antibiotics for a month or more, only 37% had at least one positive PCR result.

Smaller studies have also demonstrated good sensitivity and high specificity for *B. burgdorferi* PCR used on synovial fluid in patients with Lyme arthritis. For example, a chromosomal target of amplification was positive in six of seven synovial fluid samples from patients with Lyme arthritis (85% sensitivity), including patients who had been symptomatic for several months (62, 65). A PCR for the *recA* gene was positive in 10 of 14 (71% sensitivity) synovial fluid samples from patients with untreated Lyme arthritis (66). Quantitative PCR tests for *B. burgdorferi* have been analytically validated and used in samples from experimentally infected mice and in experimental studies in humans, but the utility of these tests in patient care has not been demonstrated (66, 67).

Lyme borreliosis in Europe is caused by *B. burgdorferi sensu stricto* (as in the United States), as well as additional species, *Borrelia afzelii*, *Borrelia garinii*, and *Borrelia spielmanii*. Diagnostic testing for these agents can be found in a recent review (68).

Tropheryma whipplei

Recurrent arthritis is the early manifestation of classic Whipple's disease, followed by weight loss and diarrhea (69). The presence of *T. whipplei* in affected native and prosthetic joints has been demonstrated in several case reports about patients with arthralgia (70–72) and a case series of patients with various manifestations of *T. whipplei* infection (73). The case series included a total of 137 patients with definite *T. whipplei* infections. In the patients with classic Whipple's disease, defined by pathological demonstration of the presence of the organism in the small intestine, four of five synovial fluid and two of two synovial tissue samples tested for *T. whipplei* by PCR were positive (73). Several primer pairs specific for *T. whipplei* have been described (70–72), and two of these have been characterized for quantitative use (74). The clinical specificity of one primer pair was demonstrated by negative results from 40 control patient specimens (72). Publications describing systematic validation of analytical sensitivity and specificity testing are lacking for all primers designed for detection of *T. whipplei*.

Mycobacterium Species

There are few high-quality studies of the utility of PCR for detection of *Mycobacterium* species in bone or synovial fluid samples. Two studies of multiplex PCR for *M. tuberculosis* and *Brucella* species are discussed in the section on multiplex assays in bone and joint infections, below. These are the largest studies of the use of PCR for detection of *M. tuberculosis* in bone, and the reader can refer to that section for more information.

Mycobacterium ulcerans has been recently studied as a cause of osteomyelitis using PCR analysis of patient specimens (75). This environmental organism is the cause of "Buruli ulcer," a superficial infection that can invade more deeply to cause scarring and deformities that can require surgical intervention in addition to antibiotic treatment (76). Pommelet et al. studied a subset of patients from a large cohort who were diagnosed with *M. ulcerans* infections in Benin, using a PCR test for IS*2404* from this organism (75). The PCR test was, unfortunately, not described in detail, and results for microscopic analysis of acid-fast stains and for mycobacterial culture were not available or not described in detail, so little can be said about the utility of the PCR test other than that it was used to make the diagnosis. The assay was performed on a variety of specimen types, including bone and skin biopsies, pus (drainage), fine-needle aspirates, and swabs of overlying skin lesions. In 81 patients with osteomyelitis caused by *M. ulcerans*, most (60.5%) had osteomyelitis that was contiguous with an overlying skin infection, but a significant minority of patients (14.8%) had bone infections at anatomically remote sites from skin infection (metastatic infection), and 24.7% had no known history of superficial infection.

The other publications about diagnosis of mycobacterial infections in bone and joints are case reports that will be described only briefly. An isolate of *Mycobacterium bovis* BCG was identified using PCR for regions of difference (RD) RD1, RD9, and RD10 as markers within the *M. tuberculosis* complex to identify this organism from culture of a vertebral specimen from a patient who received intravesicular therapy with this organism 1.8 years earlier (77). *Mycobacterium massiliense* (*Mycobacterium abscessus* subsp. *bolletii*) was identified from culture grown from a patient with otomastoiditis using PCR for *erm* (41) and sequence analysis of the 16S rRNA and *rpoB* genes (78). A case of *Mycobacterium microti* osteomyelitis of the hip was detected by a commercial assay, the GenoType MTBC reverse hybridization assay (Hain Lifescience GmbH), performed on pus from the site when the organism did not grow in culture (79).

Two case reports of *M. tuberculosis* osteomyelitis are particularly interesting. First, *M. tuberculosis* was detected in a case of sternal osteomyelitis using PCR for an unspecified target when culture and AFB stain had not detected the organism (80). Second, spinal tuberculosis was detected in

the remains of a man from the 12th or 13th century by using PCR for IS*6110* and IS*1081* (81).

MOLECULAR ASSAYS FOR SIMULTANEOUS DETECTION OF MULTIPLE PATHOGENS IN BONE AND JOINT INFECTIONS

Multiplex and Panel PCR Assays for Specific Pathogens

Because a large number of pathogens cause osteoarticular infections, a large number of primer and probe combinations are needed to detect them. It is challenging to combine these into multiplex reactions. The laboratory-developed tests discussed below either keep the PCRs separate rather than multiplexed (panel assays) or detect only a few pathogens by combined PCRs run in multiplex as a supplement to culture. Some commercial panels do detect large numbers of pathogens in multiplex assays, and these are also discussed below.

Laboratory-Developed Tests

The performance of a PCR panel including 10 primer pairs and multiple probes for detection of 14 pathogens was tested using disrupted biofilm samples from 434 patients with explanted knee and hip prostheses (82). The prostheses were processed by vortexing and sonication to disrupt biofilms and release organisms for detection by culture or PCR. This method of disrupting biofilm material and testing the "sonicate" specimen has been demonstrated to increase the sensitivity of culture for diagnosis of prosthetic joint infection (83). In this study of the PCR panel, joint infection was defined using criteria that excluded culture results but included the presence of a sinus tract or evidence of acute inflammation within the joint (82). By this definition, 144 of the patients had joint infection, and the remaining 290 did not. The overall sensitivity of PCR was 77.1%, which was greater than that of culturing tissue (70.1%) or culturing sonicate material (72.9%). The specificities of all three methods were high (>97%). In a separate study, the same PCR panel was used to test periprosthetic tissue samples from patients undergoing arthoplasty with tissue culture, but the method had a sensitivity of only 16% for prosthetic joint infection using this sample type (84).

The diagnostic utility of a laboratory-developed multiplex PCR test for *M. tuberculosis* and *Brucella abortus* in vertebral or paravertebral samples was investigated in a study performed at a single hospital in Spain (85). The assay included primers for an intergenic region of *M. tuberculosis* (SenX3-RegX3) and a gene for an immunogenic protein of *B. abortus* (bcsp31). Patients with vertebral osteomyelitis caused by these organisms were identified by clinical manifestations, culture results, anatomic pathology, or serology (the last only for *B. abortus* infection). Nine control samples from patients with other pathologies of bone were included as negative controls. The multiplex PCR detected all 5 infections with *B. abortus* and 9 out of 10 infections with *M. tuberculosis*. There was one false-positive result: a sample from a patient with *M. tuberculosis* infection was falsely positive for *B. abortus* by PCR. In a subsequent study, several of the same authors found that the combination of the SenX3-RegX3 primers for *M. tuberculosis* and an alternative primer pair for *B. abortus* (IS*711*) provided optimum detection of these organisms in multiplex PCR for extrapulmonary samples from patients infected with these organisms (86). This study included samples of many types, including vertebral and paravertebral tissues, and the PCR assays performed well using the samples from patients with vertebral osteomyelitis.

Commercial Tests

Some groups have studied the use of multiplex PCR kits intended for detection of bacteria and fungi in blood for detection of osteoarticular infections. These kits are intended to be used either directly with blood or with broth from blood culture bottles that are positive for growth. The kits that have been studied are not available for clinical use in the United States. These kits have generally performed well at detecting the pathogens that they are intended to detect, but of course, they will not detect pathogens that are omitted from the panel.

The Septifast kit, produced by Roche Diagnostics, can detect 25 of the common bacteria and yeast found in bloodstream infections using both signal detection to detect amplification and melting curve analysis for further differentiation of organisms (87, 88). The assay is run with the LightCycler 2.0 system, also produced by Roche Diagnostics. Many of the pathogens detected in this assay cause osteoarticular infections. Significant pathogens that cause osteoarticular infections that are missing from the kit include *K. kingae*, *P. acnes*, and mycobacteria. Achermann et al. tested the utility of Septifast for detecting pathogens in disrupted biofilm (sonicate) material from explanted prosthetic joints in patients with suspected prosthetic joint infection (89). Results of PCR were compared to culture results from periprosthetic tissue and sonicate. A total of 39 cases were positive for bacteria or yeast. Culture of periprosthetic tissue culture, culture of sonicate, and PCR testing of sonicate detected 24 (65%), 23 (62%), and 29 (78%) cases, respectively. The Septifast kit detected more cases of staphylococcal infection than the culture methods. Five cases of *P. acnes* infection were detected in culture but were not detected by the PCR test. This kit appears to have good potential for detection of pathogens that cause osteoarticular infections in adults, perhaps in combination with culture or for use on specimens found to be negative by culture.

The Seeplex Sepsis assay, produced by Seegene Inc., consists of screening PCR tests that include limited identification of either Gram-positive organisms or fungi with Gram-negative organisms. Subsequent multiplex PCR tests are done to specifically identify pathogens detected in the screening reactions. The kit can detect 64 organisms, including many common causes of osteoarticular infections. Significant pathogens that cause osteoarticular infections that are missing from the Seeplex Sepsis kit include *K. kingae*, *P. acnes*, and mycobacteria. The PCR products are analyzed by electrophoresis and optical (or visual) analysis. The use of this kit was evaluated with 80 synovial fluid samples from patients with infectious arthritis. The results were compared to culture and 16S rRNA analysis. Twenty samples were positive by culture, and 19 of these were positive by the Seeplex Sepsis test. Six specimens were positive in the Seeplex Sepsis test and negative by culture; five of these were also positive by 16S rRNA analysis (three streptococci, one *S. aureus*, and one *Enterobacter aerogenes*). One specimen containing *M. tuberculosis* was detected by both mycobacterial culture and 16S rRNA analysis but not by the Seeplex Sepsis test. Although the total number of positive specimens was small, these data do suggest that the Seeplex Sepsis test is sensitive for

detection of bacterial causes of infectious arthritis. The detection of PCR products by electrophoresis raises concern about higher rates of PCR contamination than detection using closed (e.g., real-time) detection methods, and this is a potential concern with the Seeplex Sepsis test.

Broad-Range PCR Amplification with Product Analysis for Identification

Broad-range molecular assays are those that detect conserved genetic sequences found in a wide range of bacteria, usually by PCR amplification using primers complementary to the conserved sequences. If there are genus- or species-specific variable sequences in the PCR product, then sequencing of the PCR product and analysis of the sequence can be used to identify the species of bacteria. The 16S rRNA gene has become the most popular target for broad-range molecular assays for bacteria, because it has appropriate conserved regions flanking sequences that vary between species or genera of bacteria (90, 91). The main advantage of 16S rRNA analysis over culture is that is allows detection of bacteria that grow poorly or not at all in conventional culture conditions. As has already been noted, PCRs that are specific for a species of bacterium are, at least in some cases, more sensitive than broad-range PCRs (40, 52). Other genes have also been used for broad-range PCR with product sequencing for bacterial identification, including topoisomerase subunits, *gyrB*, and *parE* (92). An alternative technique for broad-range PCR with product analysis for identification is PCR with electrospray ionization mass spectrometry (PCR-ESI/MS) (93).

16S rRNA gene analysis has been widely used for detection of bacterial causes of septic arthritis and osteomyelitis. Many case reports describing the use of 16S rRNA analysis to detect unusual organisms that are difficult to grow or that do not grow under usual culture conditions have been published, and some examples are shown in Table 3. These illustrate the utility of 16S rRNA analysis when performed on specimens that have already been tested by culture and found to be negative. Because the literature about 16S rRNA is extensive, it will be discussed below by specific patient groups: children with osteoarticular infections and adults with infection of prosthetic joints or vertebral osteomyelitis.

Pediatric Osteoarticular Infections

Several studies demonstrate increased detection of bacteria using 16S rRNA analysis over culture for osteoarticular infections in children. In all of these studies, the organism that is most commonly detected only by PCR (and not by culture) is *K. kingae*. Since this organism has already been discussed in detail, only specific findings using 16S rRNA gene analysis will be included here. In a study performed at a single institution in France, 171 synovial fluid and bone biopsies were tested by culture (including using blood culture bottles when specimen volume permitted), and those that were negative by culture were then tested by 16S RNA gene analysis. Of these, 64 specimens were positive by culture, most of which grew *S. aureus* (30), β-hemolytic streptococci (10), *K. kingae* (9), or coagulase-negative staphylococci (7, 94). 16S rRNA analysis of the 107 culture-negative specimens detected *K. kingae* in 15 specimens and no other organisms. In a subsequent study by the same group, similar results were obtained using separate specimens, with a large increase in detection of *K. kingae* by 16S rRNA analysis and a small number of other organisms also detected only by 16S rRNA analysis (one each of *S. pyogenes*, *N. meningitidis*, *Streptococcus constellatus*, *Streptococcus* species, and an *Enterobacteriaceae* (40). As in the previous study, a large number of *S. aureus* organisms were detected by culture, and 16S rRNA gene analysis did not increase detection of this organism. Other studies have also found significant increases in the detection of *K. kingae* by use of 16S rRNA gene analysis, with small increases in the detection of other species of bacteria using synovial fluid samples from children with suspected septic arthritis (95, 96). In conclusion, since PCR specific for *K. kingae* is more sensitive than 16S rRNA analysis and since this is the main organism detected by 16S rRNA analysis, specific PCR for *K. kingae* should generally be preferred over 16S rRNA analysis of osteoarticular samples from children.

Prosthetic Joint Infections

The diagnosis of prosthetic joint infection is complex. There are several guidelines available for this, and each of them incorporates criteria that are more or less heavily weighted in making a diagnosis. This is very well described in a recent review of prosthetic joint infection by Tande and Patel (1). For purposes of this discussion, a few important points from that review will be summarized. First, culture is not considered the sole, definitive method of diagnosis of prosthetic joint infections. Culture can be performed on a variety of specimen types including drainage from sinus tracts, synovial fluid, and periprosthetic tissue samples. Each of these has imperfect sensitivity and specificity, the latter due to frequent contamination of the samples. Culture of multiple periprosthetic biopsies is usually performed, and growth of the same organism from multiple biopsies is an important criterion in determining that infection is present.

The criteria defined by the Musculoskeletal Infections Society (MSIS) are often used to define prosthetic joint infections in the studies discussed below (97). Briefly, these define definite prosthetic joint infection as present

TABLE 3 Use of PCR for 16S rRNA gene PCR and sequencing to detect bacterial causes of septic arthritis and osteomyelitis that were not detected by conventional bacterial culture[a]

Organism	Specimen	Patient history	Reference
Fusobacterium nucleatum	SF	10-year-old boy with SA and OM	106
Helicobacter sp.	Periosteal lesion	5-year-old immunocompetent girl with OM	107
Legionella bozemanii	SF	71-year-old woman with amyopathic dermatomyositis and SA	108
Legionella dumoffii	SF	58-year-old woman with systemic lupus erythematosus-like disease and SA	109
Legionella micdadei	SF	83-year-old woman with SA of prosthetic joint	110
Mycoplasma pneumoniae	Pus	25-year-old man with common variable hypogammaglobulinemia and OM	111

[a]Abbreviations: SA, septic arthritis; SF, synovial fluid; OM, osteomyelitis

when any of the following are met: the presence of a sinus tract communicating with the prosthesis or detection of a pathogen from two separate tissue or fluid cultures from the affected joint or the presence of any four of six other criteria. These six criteria are (i) elevated markers of inflammation (erythrocyte sedimentation rate or C-reactive protein), (ii) elevated synovial leukocytes, (iii) elevated synovial neutrophil percentage, (iv) purulence in the joint, (v) isolation of a microorganism from one culture of tissue or fluid, and (vi) elevated neutrophils detected by histological analysis.

As discussed above, dislodged biofilm material (sonicate) from explanted prosthetic joints can be tested by different methods for organisms causing prosthetic joint infection (82, 83). 16S rRNA analysis was performed on sonicate material and compared to culture results using synovial fluid, tissue, and sonicate material using samples from 366 patients (98). Joint infection was defined using criteria that excluded culture results but included the presence of a sinus tract or evidence of acute inflammation within the joint. With this definition, 135 patients had prosthetic joint infection and 231 did not. Culture and 16S rRNA analysis results were concordant for 90% of samples at the genus level. The sensitivity and specificity of 16S rRNA analysis depended on the crossing point value used to determine whether PCR for the 16S rRNA gene was positive. If a lower value was used (crossing point <26 cycles), the sensitivity and specificity were 70.4% and 97.8%, respectively, which were essentially equivalent to the values for culture of the biofilm material. If a higher crossing point value was used as the cutoff (<27.59, indicating a weaker signal in the PCR), then the sensitivity increased to 80.0% and specificity fell to 90.9%.

16S rRNA analysis was found to be much more specific but slightly less sensitive than culture for detection of prosthetic joint infection in two studies (99, 100). The larger of these will be discussed in more detail (100). This study included 40 patients with prosthetic joint infections and 82 patients without prosthetic joint infections. The authors used a gold standard for diagnosis of infection that included purulence in the joint, acute inflammation in histological analysis of periprosthetic tissue, or a sinus tract draining the prosthesis. Since this definition of disease did not include culture results, the performance of culture and 16S rRNA analysis could be independently evaluated and compared. Multiple samples of periprosthetic tissue were collected, and the analyses included evaluation of the sensitivity and specificity of individual samples, as well as of the total number of positive samples. The sensitivities of independent (single) culture and PCR results were 75.9% and 67.1%, respectively, while the specificities were 81.2% and 97.8%, respectively. When the total number of positive samples was considered, the two tests had similar sensitivities, but the specificity of 16S rRNA analysis was much higher than that of culture. The sensitivity of one or more positive samples by culture was 87.8%, while the sensitivity of one or more positive 16S rRNA results was 80.5%. The specificity of one or more positive samples by culture was 67.1%, while the specificity of one or more positive 16S rRNA results was 96.3%. If two or more positive samples were taken to indicate the presence of infection, then culture and 16S rRNA analysis were 93.9% and 98.8% specific, respectively, but the sensitivity of each was only about 60%.

In a separate, somewhat smaller study including 16 patients with prosthetic joint infection and 51 without infection, the results of culture and 16S rRNA analysis were similar (101). Prosthetic joint infections were defined using criteria similar to those in the previously discussed study. Of 13 patients determined to have prosthetic joint infections with positive cultures, 12 were positive and 1 was negative by 16S rRNA analysis. Of the patients without prosthetic joint infection, three had positive cultures, and all of these also had positive 16S rRNA results. There were a small number of positive specimens for which the number of organisms detected was different for the two methods.

Alternative genes for broad-range PCR are used in the Prove-it Bone and Joint test produced by Mobidiag in Finland. The genes for topoisomerase subunits, *gyrB* and *parE*, are amplified using broad-range primers, and then the PCR products are analyzed using an oligonucleotide microarray (92). Primers and oligonucleotides to detect the *mecA* gene, for methicillin resistance, are included in the assay. The assay is designed to detect over 60 organisms, including the common bacterial causes of osteoarticular infections. It was evaluated in a study using joint tissue and fluid samples from 61 patients with suspected prosthetic joint infections (102). True infection was defined using the MSIS criteria (97), and the Prove-It Bone and Joint test results were compared to those of culture. A total of 38 patients met the MSIS definition of prosthetic joint infection, and 31 and 28 of these had an organism detected by the Prove-it Bone and Joint test or by culture, respectively. The results of culture and the Prove-it Bone and Joint test matched in 25 samples. Three samples that were positive for MRSA by culture were positive only for *mecA* (not for other genes of *S. aureus*) in the Prove-it Bone and Joint test. There was a trend toward a higher positivity rate using Prove-it Bone and Joint compared to culture in the small number of patients who had received antibiotics prior to sample collection.

Broad-range PCR as well as specific PCRs for detection of a wide range of bacteria, *Candida* species, and limited antibiotic resistance genes (*blaKPC*, *mecA*, *vanA*, and *vanB*) were all included in the BAC detection assay, produced by Ibis Biosciences (Abbot) (103). Analysis of the PCR products was performed using PCR-ESI/MS, which determined the base composition of the PCR product(s) calculated from the mass-to-charge ratio determined by mass spectrometry (93). A curated, commercial database was used to interpret these data and determine the identity of the organism and antibiotic resistance genes present. Melendez et al. used this kit to test synovial fluid samples from patients with prosthetic joint infections defined using the MSIS criteria (93, 97, 103). Samples from 21 patients with prosthetic joint infection and 82 patients with aseptic prosthetic joint failure were included in the study. Culture and PCR-ESI/MS detected bacteria in 18 (sensitivity 86%) and 17 (sensitivity 81%) samples, respectively, using samples from patients with prosthetic joint infections. Culture and PCR-ESI/MS were negative for 82 samples (specificity 100%) and 78 samples (specificity 95%) from patients with aseptic prosthetic joint failure. Eleven staphylococci were detected by both culture and PCR-ESI/MS, allowing comparison of susceptibility results to PCR testing for oxacillin resistance by *mecA* detection. For 10 isolates, the results of susceptibility testing and PCR testing for *mecA* matched. Results were discordant for one isolate of coagulase-negative *Staphylococcus*; the organism was resistant to oxacillin by testing *in vitro*, but *mecA* was not detected by PCR-ESI/MS.

Vertebral Osteomyelitis

Two single-institution studies demonstrated that 16S rRNA analysis is more sensitive than culture for detection of bacterial causes of vertebral osteomyelitis. In the first of these, two pairs of primers were used to amplify the 16S rRNA gene sequence, and the results were combined (104). From samples from 45 patients with a clinical diagnosis of vertebral osteomyelitis, culture detected an organism in 13 (28.9%) and 16S rRNA detected an organism in 24 (53.3%). Multiple cases of staphylococcal infection (five *S. aureus*, four coagulase-negative staphylococci) and single cases of five other organisms were detected only by 16S rRNA analysis. Two positive results by 16S rRNA analysis were judged to be false-positive results based on clinical implausibility. Importantly, five patients (11.1% of the total) had positive culture results with negative 16S rRNA analysis. Two of these had positive cultures for *M. tuberculosis*, and one each had positive cultures for *S. aureus*, *Staphylococcus epidermidis*, and *Enterococcus faecium*. Therefore, the authors suggest that 16S rRNA analysis and culture be used in combination for detection of bacterial causes of vertebral osteomyelitis. The study did not include any negative control biopsies from patients lacking infectious osteomyelitis.

Another study also found that 16S rRNA analysis was more sensitive than culture, although the difference between the two was not as large as that discussed above (105). The study included 18 patients with clinically diagnosed infectious osteomyelitis and 20 with other diseases of bone (mainly cancer). Nine (50%) patients with infectious osteomyelitis had positive culture results that were judged to be true positives, while 11 (61%) had positive results by 16S rRNA analysis. Organisms that were detected only by 16S rRNA analysis, and not by culture, included one each of *K. kingae*, *Clostridium histolyticum*, viridans-group *Streptococcus*, and *K. pneumoniae*. Two infections with *S. aureus* were detected by culture but not by 16S rRNA analysis, again demonstrating the importance of including culture in such testing. Discrepant results were obtained in one sample by culture (*S. aureus*) and 16S rRNA analysis (*Streptococcus mutans*, detected three times by repeat testing). There was one false-positive culture result and no false-positive 16S rRNA analysis results using the biopsy samples from the patients with noninfectious bone disease. Prolonged antibiotic treatment was associated with negative results in both culture and 16S rRNA analysis.

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section VI

VIROLOGY

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Molecular Detection and Characterization of Human Immunodeficiency Virus Type 1

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30

AIDS was first described in clinical terms in the United States in 1981, and in 1983, the cause was determined to be infection with a transmittable human retrovirus, eventually named human immunodeficiency virus type 1 (HIV-1). Globally, an estimated 35.3 (32.2 to 38.8) million people were living with HIV in 2012, with 2.3 (1.9 to 2.7) million new HIV infections globally, and 1.6 (1.4 to 1.9) million AIDS deaths in 2012 (http://www.unaids.org/sites/default/files/en/media/unaids/contentassets/documents/epidemiology/2013/gr2013/UNAIDS_Global_Report_2013_en.pdf). This pandemic has highlighted the utility of laboratory diagnostics and its relevance to patient care and treatment monitoring. The concern for transmission via blood products motivated the development of an antibody test in 1985 (1). The current versions of these enzyme-linked immunoabsorbent assays detect IgG seroconversion within a median of 3 to 4 weeks after infection (2). Antiretroviral therapy (ART) came into use, starting with AZT monotherapy, in 1985, and eventually the available number and classes of drugs expanded, which led to a better understanding of viral dynamics. Currently, the standard for managing HIV-1 infected individuals is combination therapy with three or more drugs (http://aidsinfo.nih.gov/guidelines/html/1/adult-and-adolescent-arv-guidelines/0). During this phase of active drug development, viral load testing became the standard of care for managing response to therapy. With advances in therapy, long-term (years) suppression of viral load became possible, but selective drug pressure eventually led to drug resistance. The use of genotypic and occasionally phenotypic resistance testing to accurately direct changes in nucleotides that imply resistance to drug therapy is routine in HIV clinical practice. This chapter will focus on the available molecular tests for diagnosis, monitoring, and management of HIV-1 infected individuals.

DESCRIPTION OF THE VIRUSES

HIV types 1 (HIV-1) and 2 (HIV-2) are enveloped RNA viruses that are members of the genus *Lentivirus* and the family *Retroviridae*. They are genetically related to the simian immunodeficiency viruses found in mostly nonhuman African primates. HIV-1 is thought to be the result of transmissions from chimpanzees (*Pan troglodytes*) to humans (3), and HIV-2 is likely derived from sooty mangabeys (*Cercocebus atys*) (4). The majority of the AIDS pandemic is due to HIV-1 infection, in contrast to HIV-2 which follows a less pathogenic course and is limited to a few countries in West Africa (5). This chapter will primarily focus on molecular methods as they apply to the diagnosis and management of HIV-1.

HIV-1 is further divided into groups described as M (major), O (outlier), and N (nonmajor and nonoutlier) that phylogenetically correlate with three separate transmission events (6). Recently, a new group P has been identified that is closely related to a gorilla SIV (7). Viruses in the M group involve subtypes (formerly clades) A-G, H-K, and N-P, and at least 55 circulating recombinant forms (CRFs) as described by the Los Alamos National Laboratory (LANL 2013 Sequence Compendium). These are classified based on sequence diversity in the *gag* and *env* genes; differences in the *env* gene between subtypes may be as much as 25% (8). Subtype B predominates in Europe and North America, subtype C predominates in Africa and India, and subtype E occurs in much of Southeast Asia. Genetic diversity plays an important role in the design and interpretation of viral load and resistance assays, and provides clues to epidemiology and transmission of HIV-1.

The life cycle of HIV-1 begins with conversion of the virion single-stranded RNA into double-stranded DNA by reverse transcription after entry into the cell. The double-stranded DNA is then integrated into the host genome; this integrated retroviral DNA is referred to as the provirus, which persists and produces progeny throughout the lifespan of the cell (9). After the functional proteins are synthesized, the viral capsids are assembled in the plasma membrane; budding particles are coated in the cellular membrane that contains specific viral proteins. HIV-1 uses CD4 as a receptor in addition to a coreceptor, either CXCR4 or CCR5 for entry into the cell. CXCR4 coreceptors are usually found on T-cells, while CCR5 coreceptors are found on macrophages. HIV-1 may be tropic (i.e., have a predilection) for either of these coreceptors or both. The identification of the cellular tropism has become more important as the coreceptor CCR5 is the target for a new class of antiretroviral agents (10). Prior to initiating treatment with a CCR5 inhibitor, the patient's virus must be analyzed to determine whether it is tropic for

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CCR5, CXCR4, or both. This assay will be described in detail later in the chapter.

CLINICAL APPLICATIONS

HIV-1 Viral Load Assays

The first HIV-1 viral load test was FDA approved in 1996 and rapidly became the standard of care for monitoring response to ART. Early studies found that patients who had higher viral loads progressed more rapidly to AIDS and death than those with low viral loads (11–13). A study done in the mid-1990s in homosexual males showed that an initial viral load of greater than 100,000 genome equivalents/ml predicted a 10-fold increase in the risk of developing AIDS over the next 5 years (14). Moreover, patients with a viral load less than 1,000 genome equivalents/ml did not progress to AIDS during this same time period (14). It is now widely accepted that viral load testing is a marker of response to ART. The guidelines for initiation of therapy based on viral load have changed as our understanding of disease progression at higher CD4 cell counts has improved. With the availability of newer more potent and less toxic drugs, ART is recommended for all HIV-infected individuals regardless of their CD4 cell count or viral load (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov). Viral load testing is not used routinely to determine when to initiate ART, although viral load results may influence the timing of initiating therapy. For patients with viral load values greater than 100,000 copies/ml, there is a more urgent need to initiate therapy. After treatment is initiated, viral load testing is crucial for monitoring response to therapy and should be measured in all HIV-infected individuals at the time of entry into care, when therapy is initiated, and at a regular intervals (usually 3 to 4 months) while on therapy. The standard of care is to treat with a combination of antiretroviral drugs, which are classified based on their viral targets: nucleoside reverse transcriptase inhibitors (NRTIs), non-nucleoside reverse transcriptase inhibitors (NNRTIs), protease inhibitors (PIs), fusion inhibitors, integrase inhibitors (also known as integrase strand transfer inhibitors), and CCR5 entry inhibitors. A complete description of the clinically available antiretroviral drugs is beyond the scope of this chapter; the currently FDA-approved drugs are listed in Table 1. Current guidelines (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov) recommend an initial regimen of two NRTIs and either an NNRTI, a PI, or an integrase strand transfer inhibitor. The pretreatment viral load values influence the treatment regimen, as some regimens are less effective in patients with high viral load levels. After initiation of appropriate therapy, there is typically a 2 $\log_{10}$ or greater decrease in viral load within 2 to 3 months. The goal for a patient is to achieve a viral load level below the limit of detection of the most sensitive assays (20 to 50 copies/ml). Data have shown that the lower the absolute viral load, the better the clinical and virologic outcomes (15, 16). Guidelines recommend measuring plasma HIV-1 RNA levels immediately before initiating therapy and 2 to 8 weeks later, with the goal of achieving an undetectable viral load level within 16 to 24 weeks of initiating therapy (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov). It is important to determine early in the treatment course if there is suboptimal viral load suppression, so that factors affecting adherence can be assessed and, if needed, the regimen altered. After the initial response has been characterized, viral load levels should be monitored every 3 to 4 months to ensure the response to therapy is sustained (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov). Of note, viral blips (viral load becomes detectable at low levels, usually <400 copies/ml) occur in successfully treated individuals and are not predictive of virologic failure (17). For this

TABLE 1 Current FDA-approved antiretroviral drugs[a]

Class	Target	Abbreviation/generic name/trade name
Nucleoside/nucleotide reverse transcriptase inhibitors	Reverse transcriptase enzyme	AZT/zidovudine/Retrovir
		ddI/didanosine/Videx
		ddC/zalcitabine/Hivid
		d4T/stavudine/Zerit
		3TC/lamivudine/Epivir
		ABC/abacavir/Ziagen
		FTC/emtricitabine/Emtriva
		TDF/tenofovir/Viread
Non-nucleoside reverse transcriptase inhibitors	Reverse transcriptase enzyme	DLV/delavirdine/Rescriptor
		NVP/nevirapine/Viramune
		EFV/efavirenz/Sustiva
		ETV/etravirine/Intelence
		RPV/rilpivirine/Edurant
Protease inhibitors	Protease enzyme	SQV/saquinavir/Invirase, Fortovase
		RTV/ritonavir/Norvir
		IDV/indinavir/Crixivan
		NFV/nelfinavir/Viracept
		LPV/RTV/lopinavir/ritonavir/Kaletra
		ATV/atazanavir/Reyataz
		FPV/fosamprenavir/Lexiva
		TPV/tipranavir/Aptivus
		DRV/darunavir/Prezista
Fusion inhibitor	Prevents conformational changes required for the fusion of viral and cellular membranes	T20/enfuvirtide/Fuzeon
Integrase inhibitor	Integrase enzyme	RAL/raltegravir/Isentress
		DTG/dolutegravir/Tivicay
		EVG/elvitegravir/Vitekta
CCR5 inhibitor	Small molecule inhibitor of CCR5 coreceptor for entry	MVC/maraviroc/Selzentry
Pharmacokinetic enhancer	Used to decrease enzymatic metabolism of other drugs	cobi/cobicstat/Tybost

[a]This table does not include combination drugs.

reason, DHHS and the AIDS Clinical Trials Group now define virologic failure as a confirmed viral load of >200 copies/ml.

Viral load testing has also been used to aid in the diagnosis of acute HIV-1 infection (the window period after infection that occurs prior to antibody production), although the currently available viral load assays are not FDA-approved for diagnostic purposes. The clinical presentation of this acute retroviral syndrome has signs and symptoms resembling mononucleosis including fever, fatigue, rash, lymphadenopathy, and oral ulcers. During this period of early infection, patients typically have very high viral load levels ranging from 10^5 to 10^7 copies/ml (18). It is possible to see lower levels of the virus if testing is done within a week of infection. When using viral load testing to diagnose acute HIV-1 infection, vigilance for false-positive viral load results must remain high and testing should include patient counseling and consent, and communication with the laboratory. It is also recommended that antibody testing accompany viral load testing, and all positive viral load results be confirmed by documentation of eventual seroconversion. This is especially critical if the viral loads are in a low range, i.e., of <10,000 copies/ml.

Qualitative Proviral DNA and RNA Assays

Serologic assays for the detection of HIV-1 antibodies cannot be used to diagnose primary HIV-1 infection in neonates as maternal IgG can cross the placenta and cause positive antibody results into the second year of life. Therefore the diagnosis of HIV-1 infection in neonates in the United States is dependent on the use of HIV-1 RNA or proviral DNA tests (Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection, http://AIDSinfo.nih.gov). Infants under the age of 18 months should be tested at the following intervals: 14 to 21 days, 1 to 2 months, and 4 to 6 months after birth, with some experts also recommending testing at birth. HIV-1 infection is diagnosed by two positive RNA or DNA tests performed on separate blood samples regardless of age. Infection is confirmed by a positive antibody test at age ≥18 months of age (Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection, http://AIDSinfo.nih.gov). RNA and DNA tests have comparable sensitivities and specificities, although specimens with a low viral load (<10,000 copies/ml) should be repeated. An advantage of proviral DNA tests is that they remain positive even in individuals receiving ART (19). The impact of highly active ART on the sensitivity of RNA tests is unknown. The diagnosis of HIV can presumptively be ruled out with two or more negative tests, with one at 14 days or older and the second at 1 month or older. To definitively exclude HIV infection in non-breast-fed infants, two negative tests are needed at 1 month or older and at 4 months or older. Many experts recommend HIV antibody testing at 12 to 18 months to confirm the infant is HIV negative (Guidelines for the Use of Antiretroviral Agents in Pediatric HIV Infection, http://AIDSinfo.nih.gov). The Aptima HIV-1 RNA Qualitative Assay (GenProbe, San Diego, CA) is the first nucleic acid test FDA approved for the diagnosis of HIV-1 infection, and can be used to diagnose neonatal HIV-1 infection and acute HIV-1 infection, confirm a repeatedly positive antibody screen, or to resolve indeterminate Western blots. Currently, there is not an FDA-approved assay for the detection of proviral DNA; however, there are real-time PCR assays commercially available as research use only (RUO) tests for the detection of HIV-1 RNA and proviral DNA that will be discussed below (see "Available Assays"). Given the availability of HIV-1 RNA tests, they are commonly used for the diagnostic indications discussed above, with proviral DNA testing reserved for clarifying results from RNA testing. However, an important role for the proviral DNA test is in the diagnosis of HIV-1 infection in neonates born to HIV-1-infected mothers; DNA may be more reliably detected than RNA in this situation since the neonate will be taking ART.

With the recent changes to the HIV diagnostic algorithm, qualitative RNA assays now have a role in confirming HIV-1 infection. The new guidelines recommend initial testing with a combination immunoassay that detects both HIV-1 and HIV-2, preferably a fourth-generation assay that includes p24 antigen detection. All reactive specimens undergo supplemental testing with an immunoassay that distinguishes HIV-1 from HIV-2 antibodies. Discrepant results between the two antibody tests are resolved with HIV-1 RNA testing (http://stacks.cdc.gov/view/cdc/23447). It is anticipated that the number of samples requiring RNA testing will be low, and given the lack of availability of a rapid, random access, fully automated HIV RNA test, laboratories may choose to send testing to a reference laboratory. Laboratories have the option of using viral load testing for confirmatory testing, but the assay will need to be validated by the laboratory for this indication.

Resistance Testing

Mutations are common in the HIV-1 genome due to the lack of proofreading activity of the reverse transcriptase enzyme. It has an error rate as high as 10^{-4} mutations per site and 2 to 3 recombination events per genome per replication cycle (20). As a result, mutations accumulate at a rate of about one error per replicative cycle; on average there are 1 billion viral particles (21, 22) produced each day, so that a random mutation at each position occurs with each replicative cycle. This error rate during replication yields a "quasi-species," or a cluster of variant viruses that arise from mutations over time within a viral isolate. This high rate of mutation accounts for the ability of HIV-1 to evade the host immune system and to develop resistance against antiretroviral medications. The goal therefore is viral suppression, which requires the use of preferably three active drugs from two or more drug classes. Combination therapy, using drugs from multiple classes, has been the most effective approach to controlling viral replication (23). As described earlier, HIV-1 viral load testing has become the method by which the efficacy of these combinations can be monitored, with complete suppression of viral replication to undetectable levels as the goal for therapy. With the use of combination therapy, viral replication can be suppressed below a detectable level in plasma for years.

HIV-1 resistance testing can be performed using either genotypic or phenotypic methods. Genotypic methods examine the gene sequence directly to identify mutations, while phenotypic assays are performed by the creation of a pseudoviral vector, measuring its replicative capacity in varying concentrations of drug, and comparing this to replication of the wild-type virus. Both genotypic and phenotypic methods are used clinically for assessing resistance in patients, with phenotypic testing usually reserved for drug-experienced patients with complicated resistance profiles.

The guidelines recommend initial genotypic resistance testing for drug-resistance testing for persons with HIV infection at entry into care, regardless of whether ART will be initiated immediately. These drug resistance mutations can be in a minority population as compared to the wild-type virus, and can lead to decreased treatment efficacy after initiation of treatment with ART (24). Even though in the absence of therapy resistant viruses may decline over time, when therapy is eventually initiated, resistant viruses, even at a low level, may still increase the risk of treatment failure (25). In patients with transmitted drug resistance, the virological response to a regimen approaches the responses observed in patients with wild-type viruses, but these patients are at a higher risk of harboring minority drug-resistant variants (26). In a 2003 study, paired genotypic and phenotypic susceptibility data were retrospectively analyzed for 317 ART-naive, HIV-infected subjects from 40 cities in the United States. The study found that 8% had reduced susceptibility to at least one drug (27). By class, <1% had reduced susceptibility to PIs, 1% had reduced susceptibility to NRTIs, and 7% had reduced susceptibility to NNRTIs. Reduced susceptibility to a drug was significantly higher for white subjects (27).

Current guidelines recommend that resistance testing be performed in the following scenarios: (i) prior to initiation of ART in treatment-naive patients, (ii) the selection of active drugs when changing antiretroviral regimens, (iii) management of suboptimal viral load reduction, and (iv) in all pregnant women prior to the initiation of therapy (DHHS Panel on Antiretroviral Guidelines, http://AIDSinfo.nih.gov).

Several clinical trials have evaluated the utility of genotypic resistance testing. The earliest trials studied patients who had failed a treatment regimen(s) and compared the use of a genotypic resistance assay to clinicians making decisions based on the past regimen the patient had received to determine the most appropriate next regimen for the patient. These studies showed an improved response to therapy as compared to the control group, with the outcome measured being suppression of the viral load (28–30). Genotypic resistance assays have become the guidebook in terms of which ART patients should be switched to if they are failing their regimen. The clinical utility of phenotypic assays has also been evaluated in clinical trials enrolling patients that have failed a treatment regimen which included protease inhibitors. The VIRA3001 study showed that patients had improved virologic outcomes when their providers had phenotypic testing results (31) as compared to using the patient's treatment history and published guidelines. The NARVAL trial compared phenotypic or genotypic testing to the standard of care when no resistance testing was available, and neither type of resistance testing was shown to improve outcomes, except in a subset of patients who had limited PI use (32). The California Collaborative Treatment Group Study 575 randomized patients with treatment failure to receive either phenotypic testing or the standard of care. At time intervals of 6 and 12 months, there was not a significant difference in reduction of viral load (33) in the phenotypic group compared to the standard of care group. The Evaluation of Resistance trial randomized trial compared genotypic testing alone to genotypic and phenotypic testing in 300 patients who had virologic failure. There was no difference at 12 months between the groups in terms of their viral load suppression achieved with guidance of the clinicians by each of these methods (34). Although these trials have differing results in terms of the utility of genotypic and phenotypic testing, and their impact on virologic outcomes, it is clear that these methods are helpful in managing patients in many situations and they have been implemented with frequent use in clinical practice. Both genotypic and phenotypic testing are used in the management of patients; however, given the higher cost and longer turnaround time for results using phenotypic testing, most clinicians use genotypic testing for initial evaluation of resistance. Phenotypic testing can however be very useful for patients with complex drug resistance mutations, and may provide insights in determining salvage regimens that may have appeared as unlikely candidates based on genotypic testing.

The advent of next-generation sequencing HIV-1 assays has demonstrated benefit for individuals with drug-resistant HIV infection. Detection of mutants can be to the level of 1%, whereas typical Sanger sequencing can detect mutations at a level of 25 to 20% of the population in the quasi-species. A retrospective, multicenter, cohort study in ART-experienced, HIV-1-infected adults who initiated salvage ART, had Sanger and 454 sequencing of plasma HIV-1 used to generate separate genotypic sensitivity scores using various algorithms (35). The predictions of virological outcomes were improved with ultrasensitive genotyping in these patients, but prospective trials are needed to confirm these findings.

Tropism Assay

In conjunction with the development of the CCR5 inhibitor, maraviroc, a tropism assay called the Trofile (Monogram Biosciences, Inc, South San Francisco, CA) was developed to detect whether the patient's virus uses predominantly CCR5 (R5-tropic) or CXCR4 (X4-tropic) or both (dual/mixed or D/M-tropic) as a coreceptor for entry. This has been improved over time and has enhanced sensitivity. There is also a genotypic assay that is commercially available, but according to guidelines, should be used as an alternative test to predict HIV-1 coreceptor usage. Most viruses are solely CCR5-tropic and the tropism has not been found to be predictive in terms of response to standard ART. It has, however, been shown that patients who have X4-tropic virus have a faster rate of decline in CD4 count, and increased clinical complications, with an increased relative risk of having a clinical event (36) as compared to patients with an R5-tropic virus. The tropism assay must be performed on a patient's virus prior to initiating maraviroc, or any other CCR5 inhibitor, in order to determine whether the virus is R5-tropic. This drug would not be indicated for use if the patient's virus was X4- or D/M-tropic. There is also a recommendation that coreceptor tropism testing might also be considered for patients exhibiting virologic failure on a CCR5 inhibitor, since one possibility for their failure would be that their viral population was no longer predominantly R5-tropic. Phenotypic assays characterize the coreceptor usage of plasma-derived virus by the generation of laboratory viruses that express patient-derived envelope proteins, and are then used to infect target cell lines that express either CCR5 or CXCR4 (37). After the patient-derived virus is generated, the coreceptor tropism is confirmed by testing the susceptibility of the virus to specific CCR5 or CXCR4 inhibitors *in vitro*. This assay takes about 2 weeks to perform and requires a plasma HIV RNA level ≥1,000 copies/ml (http://aidsinfo.nih.gov/guidelines/html/1/adult-and-adolescent-arv-guidelines/8/coreceptor-tropism-assays).

AVAILABLE ASSAYS

There are several FDA-approved assays for the detection, quantification, and characterization of HIV-1; the conventional viral load tests are no longer available and have been replaced with real-time reverse transcriptase-PCR (RT-PCR) viral load tests. This section will cover real-time viral load tests as well as RNA and proviral DNA tests for the detection of HIV-1, resistance testing, and the tropism assay. There are a number of different tests available, so an understanding of the most appropriate test to use in any given clinical situation is important. Tests available for the detection of HIV-2 will also be briefly discussed.

Viral Load Assays

Currently, there are two FDA-approved tests available for the quantification of HIV-1 RNA from plasma specimens. These tests use real-time PCR technology (Cobas AmpliPrep/Cobas TaqMan HIV-1, version 2.0 [Cobas TaqMan], and the Abbott Molecular m2000 RealTime System [Abbott RealTime]). These assays differ slightly in their limit of detection, linear range, gene target, and input plasma volume (Table 2). Real-time RT-PCR assays offer several advantages over the previously used conventional viral load assays, including more extensive automation, broader linear range, and decreased risk of carryover contamination. The lower limit of quantification is an important characteristic of all viral load assays; the real-time tests have a slightly lower limit of detection (20 to 40 copies/ml) compared to the conventional tests, but they require a larger input volume of plasma to achieve this level of sensitivity (Table 2). The lower limit of quantification coupled with the 6 $\log_{10}$ to 7 $\log_{10}$ linear range of the real-time tests has led to their widespread use in clinical laboratories.

There is genetic diversity of HIV-1 isolates from individuals in the United States, primarily from those who have emigrated from Africa and Asia (38, 39) or in military personal infected overseas (40), so detection of all non-B subtypes is critical for these patients. The ability to detect all subtypes and CRFs differs among the various viral load tests. The Abbott RealTime test, which targets the integrase gene, quantifies all group M and N viruses, CRFs, and group O viruses (41, 42). The Cobas TaqMan v2 assay is a dual target assay targeting both the long terminal repeat and the *gag* gene, which substantially improved the quantification of non-B subtypes, circulating recombinant forms, and group O virus compared to the Cobas TaqMan v1 test. A recent study found excellent agreement and correlation in viral load values between the Cobas TaqMan and Abbott RealTime tests when testing samples from B and non-B subtypes; the mean difference in viral load values was 0.04 $\log_{10}$ (43). The Abbott RealTime assay gave slightly lower viral load values for subtype A1 samples (mean difference 0.24 $\log_{10}$) compared to the Cobas TaqMan test. When comparing viral load values at a cut-off of 50 copies/ml the percent agreement between the two tests remained very high (90.9%; mean difference 0.01 $\log_{10}$) confirming the excellent agreement between the two tests. At a cutoff of 20 copies/ml, the agreement between the two assays dropped to 67% (43). A subsequent study found a 97% correlation between the Cobas TaqMan and Abbott Realtime tests when evaluating samples covering the full linear range of the tests, the correlation dropped to 78% when assessing samples with viral load values less than 1,000 copies/ml (44). Overall, there is very good correlation of viral load values between the real-time tests, and both of these assays accurately quantify group M, group O, and CRFs and the Abbott RealTime test can also quantify group N viruses. There are ongoing international surveillance projects of HIV-1 isolates to ensure that these assays maintain their ability to detect evolving viral genetic diversity, which occurs because of the high recombination activity within the HIV-1 subtypes and CRFs (45). One concern with the Abbott RealTime assay is that the gene target (integrase gene) is also a drug target (integrase inhibitors). Also, with the increased use of integrase inhibitors, it will be very important to monitor the performance of the test in patients with drug induced mutations in the integrase gene, to ensure that mutations that occur in the integrase gene do not affect the binding of primers or probes leading to reduced viral load values.

Given the relatively low homology between HIV-1 and HIV-2, it is easy to understand how HIV-2 infection can be missed by quantitative viral load testing. If patients are determined to be HIV-2 positive, they cannot be monitored for response to therapy, or a decision made to initiate therapy with the use of HIV-1 quantitative viral load testing. Viral load testing for HIV-2 is not widely available, but can be found through a few referral laboratories. One laboratory which offers this testing is the University of Washington (http://menu.labmed.washington.edu/oltg/display?mnemonic=HIV2VL). The New York State Department of Health (46) and the U.S. Military HIV Research Program at the Walter Reed Army Institute of Research (47) have also published their validation for HIV-2 viral load testing.

Viral load testing has also been assessed in resource-poor settings (48) given its ease of use and reproducibility; however, it requires a constant power supply and high capital investment, so in these settings it will remain a tool for research and for use in reference laboratories. The Cavidi

TABLE 2 FDA-approved molecular tests for quantification of HIV-1 RNA

Test	Method	Target	Specimen vol[a]	Range
Cobas AmpliPrep / Cobas TaqMan HIV-1, version 2.0 (Roche Diagnostics, Indianapolis, IN)	Real-Time RT-PCR	HIV-1 *gag* gene and LTR[b]	1 ml	20–10,000,000 copies/ml
Abbott Molecular m2000 RealTime System (Abbott Molecular, Des Plaines, IL)	Real-Time RT-PCR	HIV-1 integrase gene	1 ml	40–10,000,000 copies/ml

[a]For tests that use an automated extraction instrument (AmpliPrep and RealTime), the specimen volume listed refers to the volume of sample that is loaded on the instrument, which is greater than the actual volume of specimen taken through the extraction.
[b]LTR, long terminal repeat.

ExaVir viral load assay (Cavidi, Uppsala, Sweden), which measures reverse transcriptase activity, and the ultrasensitive p24 antigen assay (Up24) have been evaluated as alternative approaches to monitoring viral load levels in resource-limited settings, as these tests are less expensive and simpler to perform than PCR-based viral load tests. In a comparison with the Roche Amplicor HIV Monitor test, the Cavidi assay was superior to the Up24 test in accuracy, precision, sensitivity, specificity, and negative and positive predictive values, and is an alternative for monitoring viral load in resource-limited settings (49). Another approach to viral load testing is the Samba HIV semi-quantitative test, which is designed to distinguish plasma specimens with a viral load above and below 1,000 copies/ml. RNA is extracted from plasma using the Samba sample preparation instrument, followed by isothermal amplification and dip stick detection within the Sambaamp instrument. When compared to the Roche TaqMan test, the Samba assay had a very high correlation (~95%) for distinguishing plasma samples with less than or greater than 1,000 copies/ml. With this reliable 1,000 copies/ml cutoff, the Samba assay can identify individuals not responding to therapy and could be used for monitoring response to ART in resource-limited settings (50). The Cepheid Xpert HIV-1 viral load test has received a CE-IVD (*In Vitro* Medical Device) status for use in resource-limited countries.

In order to ensure the accuracy of test results, samples must be collected, processed, and stored in a manner that ensures that there is minimal RNA degradation. Plasma is the preferred specimen type for viral load testing, and the different tests have slightly different recommendations for collection and storage of samples, so it is important to follow the recommendations in the package inserts. For the Roche TaqMan test, EDTA is the preferred anticoagulant; plasma anticoagulated in either EDTA or acid citrate dextrose can be used in the Abbott RealTime test. In order to minimize RNA degradation, it is recommended that plasma be separated within 4 to 6 hours of collection. Plasma specimens can be stored at 4°C for a few days, and can undergo three to five cycles of freezing and thawing. For long-term storage, plasma samples should be frozen at or below −70°C. Plasma preparation tubes (PPTs), which contain a gel barrier that physically separates plasma from the cellular components, can be used for collection of blood specimens. These tubes are convenient as they provide a closed sample collection system, which is safe, and are a practical approach to shipping specimens collected at sites remote from the laboratory. However, use of this tube must be validated by the laboratory and may not be compatible with all viral load assays. PPTs have an advantage in that whole blood can be collected in these tubes and held at room temperature for up to 6 hours prior to centrifugation and shipped in the original tube at ambient temperature without affecting the viral load values (40). Plasma samples should not be stored frozen in PPTs since this can falsely elevate viral load values (51–53); therefore, after centrifugation PPTs should either be stored refrigerated, poured off prior to freezing, or spun again prior to running. Although Fernandes et al. showed that it could be done when using the Abbott platform, this is likely due to the additional centrifugation prior to testing (54). If PPTs are centrifuged and then shipped, they should be re-centrifuged prior to testing (53).

Clinically, plasma is the standard specimen for quantification of HIV-1 RNA, but some of the tests have been adapted for use with other specimen types, such as serum, dried blood spots, cerebrospinal fluid (CSF), seminal fluid/semen, cervical secretions, or breast milk. Both whole blood and plasma dried spots can be used for viral load testing; in fact, viral load levels from dried plasma spots are equivalent to those obtained from fresh frozen plasma specimens (55, 56). When serum is used, the viral load is decreased by 50% (57). RNA from dried blood spots has been shown to be stable for up to 1 year at room temperature or cooler (58). Viral load testing can also be done using CSF specimens; detection of HIV-1 in CSF can occur in patients with neurologic involvement from HIV-1, an opportunistic infection, or with a low CD4 cell count (59).

HIV-1 Qualitative Proviral DNA and RNA Assays

The Aptima HIV-1 RNA Qualitative Assay (Gen-Probe Inc., San Diego, CA) is the first nucleic acid test licensed for the diagnosis of HIV-1. The test requires a 500-µl plasma specimen that can be collected in EDTA, acid citrate dextrose, sodium citrate, or in PPTs. The test is manual, and it amplifies and detects the 5′ long terminal repeat and *pol* gene of the HIV-1 genome (Fig. 1). The specimen first undergoes target specific capture to isolate HIV-1 RNA, followed by transcription-mediated amplification. The amplicons are detected using a hybridization protection assay and a dual kinetic assay that allows detection of the HIV-1 target and internal control in the same reaction. This assay has been shown to detect all HIV-1 group M, N, and O viruses and has a limit of detection of 30 copies/ml with a specificity of 99.8%. An advantage of the Aptima test is that it can detect RNA 12 days earlier than EIA detection of antibody and 6 days earlier than testing for p24 antigen (package insert).

The Roche Amplicor Qualitative DNA assay (Roche DNA), which was available as RUO for the diagnosis of neonatal HIV-1 infection, is being phased out, so there is a need for alternative tests for the diagnosis of neonatal HIV-1 infection. A recent study assessed the Aptima test and a qualitative version of the Abbott RealTime test (not FDA-approved) as alternatives to the Roche DNA test. Dried blood spot samples from both exposed uninfected infants and HIV-1 infected infants were tested. The Aptima test had a sensitivity (96.5%) that was comparable to the Roche DNA test (98.8%) for samples with a viral load of >400 copies/ml. The Abbott RealTime test was the least sensitive of the three assays with a sensitivity of 95% for dried blood spot samples with a viral load of >1,000 copies/ml. All three tests had excellent specificity (60). The Aptima test appears to be a reliable for diagnosing neonatal HIV infection. There are RUO tests available for the detection of HIV-1 RNA and proviral DNA. The Roche TaqMan HIV-1 Qualitative RUO test detects HIV-1 RNA from plasma specimens and proviral DNA in either whole blood specimens or dried blood spots. The Abbott

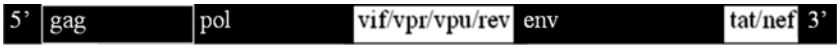

FIGURE 1 Simplified HIV-1 genome with its nine genes and the long terminal repeat regions. The enzymes reverse transcriptase, protease, and integrase are all encoded within the *pol* gene.

RealTime RUO is designed to detect HIV-1 RNA in plasma and proviral DNA in whole blood samples. Currently there is no FDA-approved proviral DNA test available to aid in the diagnosis of neonatal HIV infection.

For the detection of HIV-1 DNA in infants, dried blood spots have been used as an alternative to venous blood and studies have found the specimens to be stable in various storage conditions, up to 6 months at room temperature, and longer with refrigeration (58, 61). The use of real-time PCR on these dried blood spots has increased the sensitivity and specificity of this method to 98.1% and 100% (62) as compared to whole blood samples. Another study done in Uganda showed that the volume of whole blood could be reduced from 500 µl to 100 µl without a loss in sensitivity for detection of neonatal infection (63). This is very important as collecting large volumes of blood from neonates is difficult.

Resistance Testing

Within the last several years, resistance testing for HIV-1 has expanded from evaluation of sequence mutations (genotypic testing) to actual *in vitro* testing of virus replication in the presence of various drugs (phenotypic testing) (Table 3). There are two FDA-cleared HIV-1 genotypic tests, the TruGene HIV-1 Genotyping Kit (Siemens Healthcare Diagnostics, Tarrytown, NY) and the ViroSeq HIV-1 Genotyping System (Abbott Molecular, Des Plaines, IL). However, the TruGene platform in its current form is no longer available. These tests detect mutations in the reverse transcriptase and protease genes that are found in the *pol* gene (Fig. 1) but do not include mutations associated with resistance to fusion inhibitors, CCR5 inhibitors, or integrase inhibitors. Tests assessing these genes are available separately by commercial laboratories. Genotypic resistance testing is complex, requiring sequence alignment and editing, mutation detection by comparison to a standard wild-type sequence, and interpretation of the significance of the mutations identified. The commercially available genotypic resistance tests provide reagents for testing, as well as software programs for the alignment, editing, and interpretation of the sequences. The databases provided by the manufacturer require regular updating as new drugs and resistance mutations are identified. Some clinicians prefer to cross reference mutations to the Stanford database because it is updated more frequently than the commercial databases (http://hivdb.stanford.edu/index.html, or IAS-USA). It has been shown that both the TruGene and Viroseq tests identify similar mutations after accounting for the differences in their reference databases and comparison wild-type viral strains (64). These assays have been compared in the evaluation of a panel of 34 well-characterized HIV-1 viral stocks of subtypes A through H and produced equivalent nucleotide data (65). The Viroseq system also has a RUO product for detection of integrase mutations. The advent of next generation sequencing technology has clear implications for use in HIV-1 genotyping. An international multicenter study showed high consistency of drug resistance mutation detection, well below the detection of routine genotyping (66). A commercial assay that uses next-generation sequencing of proviral DNA (GenoSure Archive, Monogram Biosciences) allows evaluation of resistance mutations in patients with undetectable viral load. Genotypic resistance testing is available for all classes of drugs as well as HIV-1 coreceptor tropism testing.

HIV-1 phenotypic testing involves insertion of the RT and protease genes into a pseudoviral vector that is then grown in permissive tissue culture cells. These cells are then placed in increasing concentrations of antiretroviral drugs and the concentration that inhibits 50% of viral replication (IC_{50}) is calculated; the ratio of the IC_{50} of patient and wild-type virus is reported as the fold change in susceptibility. The PhenoSense HIV test (Monogram Biosciences, South San Francisco, CA) uses a modified HIV-1 vector containing a luciferase reporter gene into which the RT-PCR products of the protease and RT genes are inserted. Replication in the presence of the drug is then quantified by

TABLE 3 Selected assays for resistance testing

Assay	Method	Comments
Viroseq HIV-1 Genotyping System (Abbott Molecular, Des Plaines, IL)	Genotypic	FDA-cleared; detects only protease and reverse transcriptase mutations
GenoSure PRIme (Monogram Biosciences, South San Francisco, CA)	Genotypic	Detects protease, reverse transcriptase, integrase
GenoSure PRIme MG (Monogram Biosciences, South San Francisco, CA)	Genotypic	Detects protease, reverse transcriptase mutations at low levels (500 copies/ml)
GenoSure Archive (Monogram Biosciences, South San Francisco, CA)	Genotypic	Detects mutations in proviral DNA; detects reverse transcriptase, protease, and integrase using next-generation sequencing
PhenoSense HIV (for Reverse Transcriptase and Protease Inhibitors) (Monogram Biosciences, South San Francisco, CA)	Phenotypic	
PhenoSense for Entry Inhibitor Susceptibility (Monogram Biosciences, South San Francisco, CA)	Phenotypic	Measures susceptibility to entry inhibitors (Fuzeon)
PhenoSense Integrase (Monogram Biosciences, South San Francisco, CA)	Phenotypic	Measures susceptibility to integrase inhibitors
Trofile Coreceptor Tropism (Monogram Biosciences, South San Francisco, CA)	Tropism	Used prior to initiating therapy with CCR5 inhibitors
Trofile Coreceptor Tropism DNA (Monogram Biosciences, South San Francisco, CA)	Tropism	Used prior to initiating therapy with CCR5 inhibitors, uses proviral DNA

measuring luciferase activity (68). This technology is also used for the PhenoSense Entry and PhenoSense Integrase tests, which measure susceptibility to the entry inhibitor and integrase inhibitor, respectively. The currently available range of resistance testing (genotype, phenotype) provides clinicians with tools to better assess how to tailor the antiretroviral regimen, as well as the ability to determine which drugs are considered "resistant" by genotype which may be usable in a salvage regimen after analysis by a phenotypic testing or virtual phenotype.

The current recommendations for specimen collection and processing for HIV-1 resistance testing are the same as for viral load testing. The assays are quite sensitive to RNA degradation as a large portion of the genome is amplified (~1,300 to 1,800 nucleotides) for testing. A viral load level of at least 500 to 1,000 copies/ml is recommended for genotypic resistance testing, as results are less reproducible at very low viral load levels. For viral load values less than 5,000 copies/ml, some protocols recommend high-speed centrifugation (23,000 × *g* or higher) prior to RNA extraction in order to concentrate virions. However, concentration of specimens by centrifugation can lead to concentration of other plasma components, which may interfere with the quality of sequencing. Although clinical resistance testing is done on plasma specimens, dried blood spots have been used successfully for genotypic resistance testing, and this is an important component of global HIV strategies. One study found that dried blood spots stored at −20°C for 5 to 6 years could be used to successfully sequence a large portion (~1,000 nucleotides) of the polymerase gene, while those stored at room temperature could not. There was also good agreement of sequencing data obtained from the stored dried blood spots compared to the stored plasma samples (69). Others have shown that successful genotyping from dried blood spots was significantly influenced by the size of the amplicon. For dried blood spots stored at 4°C for 1 year, only 58% could be amplified using the Viroseq test, which generates a 1,800-base-pair amplicon, compared to a 95% success rate using a laboratory-developed assay that amplifies a 1,000-base-pair fragment (70). Dried blood spots may be a good alternative for resistance testing, although proviral DNA can contribute significantly to the viral sequences (69), and storage conditions are critical (71).

Tropism Assay

Prior to initiating therapy with maraviroc (CCR5 inhibitor), it is necessary to determine whether the patient's virus uses CCR5 as a coreceptor, as viruses that use CXCR4 as a coreceptor will not be susceptible to this drug. There is one commercially available tropism assay (Trofile, Monogram Biosciences, South San Francisco, CA). In the cell-based Trofile assay, pseudo-viruses are generated using full-length *env* genes amplified from the patient's virus. Coreceptor tropism is then determined by measuring the ability of the pseudoviral population to infect CD4+/U87 cells that express either CXCR4 or CCR5. Depending on which cells they infect, the viruses are then designated X4-tropic, R5-tropic, or D/M-tropic (37). Patients are candidates for a CCR5 inhibitor if their virus is solely CCR5 tropic. For patients with CCR5-tropic virus that are treated with maraviroc, there are reports of development of resistance to the drug due to either (i) mutations that allow the virus to adapt and use CXCR4 coreceptors or (ii) structural changes in the envelope of a R5-tropic virus that prevent the drug from being effective (72, 73).

The Trofile assay has been enhanced over time, but there is still a sensitivity issue in that there may be R4-tropic virus that is below the limit of detection and may allow individuals to fail the use of maraviroc (74). The Trofile assay is the only phenotypic coreceptor tropism assay that is commercially available. This assay has been modified to use cell-associated viral DNA or proviral DNA, which allows for testing when plasma RNA is undetectable. In recent years, there has been a genotypic method that involves sequencing the V3-coding region of the *env* gene coupled with a predictive algorithm. The genotypic methods can be less sensitive (~50 to 70%) to detect an X4-tropic virus, and in one study, would have excluded 8% of individuals who had R5-tropic virus (75). However, there are studies that argue favorably for genotypic coreceptor testing given the test cost and accessibility (76), which the European guidelines endorse.

INTERPRETATION OF THE RESULTS

Viral Load Assays

In order to be used effectively, both clinicians and laboratorians must understand what change in viral load represents a clinically significant change in viral replication. This requires data on both the biological variation of the virus and the assay performance. In untreated individuals, the amount of virus in the plasma is relatively stable over time; the biological variation is approximately 0.3 $\log_{10}$ copies/ml (57, 77). The RealTime assay is very precise, with intra-assay variation ranging from 0.1 to 0.2 $\log_{10}$ copies/ml when testing multiple replicates in the same run, although this value varies depending on where in the linear range reproducibility is measured. There is greater variability near the limit of quantification for all of the assays and may exceed 0.2 $\log_{10}$ copies/ml. Based on these data, describing biological and intra-assay variation, changes in HIV-1 viral load must exceed 0.5 $\log_{10}$ copies/ml (3-fold) to represent biologically relevant changes in viral replication. For patients with low viral load levels, it is very important not to overinterpret small changes in viral load. For example, 5-fold changes in viral load can be reliably determined only when the standard deviation of the assay is less than 0.15 $\log_{10}$ copies/ml (78) and for some viral load tests, the standard deviation may exceed this value for samples with viral load levels near the limit of detection. Reporting viral load values as $\log_{10}$ copies/ml can be very helpful in preventing clinicians from overinterpreting small changes in viral load. Patients who have viral load levels suppressed to <20 to 40 copies/ml while on ART may have viral blips, in which the viral load becomes detectable, usually at a low level of <400 copies/ml. These blips do not predict failure, so in patients previously suppressed, low level viral load values should be repeated to confirm that there is a persistent increase in viral load.

In general, baseline viral load values are not obtained when patients are acutely ill, or when they have an opportunistic infection, as these can lead to a transient increase in viral load values. The same phenomenon has been observed for patients receiving vaccinations for influenza, tetanus, or pneumococcal infection (79, 81). This is not a contraindication for vaccination, as these rises in viral load are transient and usually return to baseline within several weeks of the acute event or vaccination.

False-positive results are a concern with viral load tests and can be due to carryover contamination of amplicons,

cross contamination of specimens during processing, or assay chemistry. Concern about carryover contamination has been greatly reduced with the introduction of the closed automated real-time PCR assays. False-positive results due to cross contamination during the nucleic acid extraction step can occur with any of the tests. Automation decreases this risk, although there may be contamination between specimens when samples have very high viral load levels (10^8 or greater copies/ml). However, it is very rare for HIV-1-infected individuals to have such high viral load values, even if they are untreated, except during acute HIV-1 infection where viral load levels may exceed 10^7 copies/ml.

The method used to calculate the viral load varies with the different tests. For the Abbott RealTime test, an internal control is added to each specimen prior to extraction to monitor for inhibition of amplification. Two assay calibrators are run in triplicate to generate a calibration curve, and the slope and intercept of this curve are stored on the instrument. The concentration of HIV-1 RNA in the specimen is calculated using the stored calibration curve. The curve can be used for 6 months, or until a new lot of reagents is used. For the Roche TaqMan test, a quantitation standard is added to the specimen at a known concentration and is used to adjust the concentration of HIV-1 RNA in the sample. Lot specific calibration constants are used to calculate the viral load values.

Qualitative RNA and DNA Tests

Since qualitative RNA and DNA tests are used to diagnose HIV-1 infection, a false-positive result can have very serious consequences. Ideally, these tests should be performed in laboratories that are experienced with molecular testing. False-positive results can occur due to carryover of amplicons or cross contamination between specimens. With the new CDC guidelines, HIV-1 RNA testing is used for confirming the diagnosis of HIV-1 when there are discordant results between the screening EIA and HIV-1/2 discriminatory test. There are multiple issues to consider when implementing this algorithm in clinical laboratories, whether to test plasma or serum samples, and using the same sample for serologic testing as molecular testing. Using the one sample for serologic and molecular testing raises concerns for cross contamination that may occur between samples during the serologic testing, as specimens are not handled with the same concern for cross contamination as is done in the molecular laboratory. Prealiquoting for RNA testing could be done prior to serologic testing, but given the very low number of samples that would require RNA testing, this approach would be very labor intensive and not practical for most laboratories. Requesting a second specimen to be collected for RNA testing would address the contamination concerns, but requires a second patient visit and may decrease the likelihood that testing is done. Alternatively, the laboratory could collect two tubes of blood for initial testing, but again, this involves the storing of a large number of samples that would never be tested. Laboratories should consult with clinicians to determine the most effective approach for their center.

Resistance Testing

The genetics of HIV-1 resistance are very complex and certain mutations may cause resistance to several drugs, while others may lead to resistance to an entire class of drugs. Moreover, there may be interactions of mutations, so that resistance to one drug may improve or reduce susceptibility to another drug. As a result of these intricate interactions, interpretation of genotypic resistance testing requires considerable experience. To assist clinicians with the interpretation of genotypic resistance testing, the FDA-cleared tests include reagents for testing, as well as software for interpretation of results. A comprehensive review of all drug resistance mutations is beyond the scope of this chapter. The reader is referred to the International AIDS Society-USA website for a complete list of mutations for all classes of drugs, which is updated regularly (http://www.iasusa.org). Mutations are listed which are associated with reduced susceptibility to the drug (24).

Genotypic resistance testing is one of the most complex tests performed in the molecular microbiology laboratory and involves multiple technical steps to generate the sequence, as well as the interpretation of the sequencing data. An error in either of these processes can lead to reporting of incorrect results to the clinician. Quality control of the technical aspects of sequencing involves methods to minimize carryover contamination; this is particularly important as the currently used sequencing methods require post-amplification manipulation to set up the sequencing reaction. The interpretive software provided by the manufacturers serve multiple functions, including assisting in base calling, sequence alignment, and identification of resistance mutations. Once the sequence is generated, it is compared to that of wild-type HIV-1, and any differences from wild type are then assessed to determine if they are associated with resistance to any drugs. This is done using a "rules-based" system; these rules are established by an independent panel of experts and updated as new information becomes available. In general, the manufacturer's rules are not updated as frequently as online databases (http://hivdb.stanford.edu/index.html; http://www.iasusa.org/content/essential-management-hiv-infection). For this reason, it is helpful for the laboratory to report the list of mutations, in addition to the interpretation provided by the manufacturer, as it provides the clinician with the opportunity to obtain a more up-to-date interpretation from an online database. The databases used for interpretation of HIV-1 resistance mutations take into account cross resistance and interactions of mutations. All of these data are condensed in an easy-to-read report that lists the drugs, and provide an interpretation of either "no evidence of resistance," "possible resistance," or "resistance". These "rules-based" interpretations provide the clinicians with a user-friendly report that can be used to manage patients without the need for extensive knowledge of the genetics of HIV-1 resistance. The different manufacturers may use different wild-type sequences and rules-based interpretations, so the interpretation may not be identical between the different tests.

The presence of mutations in general will predict failure of a drug, while the lack of resistance mutation may not predict success of the drug or regimen. There are multiple reasons for failure of a regimen in which all drugs are reported as "no evidence of resistance." In the absence of drug selection pressure, the mutant population may be present at levels below the limit of detection of sequencing tests. Mutant populations that are present at less than 25% of the total viral quasispecies are unlikely to be reliably detected. Once the drug is used to treat the patient, the resistant variant can quickly predominate again, leading to clinical failure. Other reasons for treatment failure in the absence of resistance mutations include lack of adherence to the treatment regimen, sub-therapeutic levels of drug,

drug-drug interaction, or laboratory error in the testing procedure or interpretation (24).

Mutations in HIV-1 are reported using a specific nomenclature, with the amino acids reported as single letter abbreviations. The wild-type amino acid encoded by the nucleotide triplet is followed by the location of the mutation (codon number) and then the mutant amino acid. For example, M184V indicates the methionine (wild type) at codon 184 is replaced by a valine (mutant).

Interpretation of phenotypic testing is based on the fold change in IC_{50} as compared to wild-type virus. A fold change of 1.0 means the patient's virus has the same degree of susceptibility as the wild-type virus, with a fold change of >1.0, the patient's virus is less susceptible than wild-type virus, and with a fold change of <1.0, the patient's virus is more susceptible than wild-type virus. Initially cutoffs in the fold change were based on the technical performance of the assay, and essentially important changes in IC_{50} were determined based on the reproducibility of the assay. Over time, clinical cutoffs have been determined that correlate fold change in IC_{50} with clinical outcome (67).

As with genotypic resistance testing, phenotypic methods rely on amplification of specific gene sequences from a plasma specimen, so care must be taken avoid contamination. Likewise, issues regarding drug selection pressure and low levels of mutant population also apply to phenotypic testing. So both genotypic and phenotypic resistance testing are best used to predict failure of a regimen rather than ensure success.

As described above ("Available Assays"), there are many resistance tests that are accessible to provide clinicians with data about their patients' HIV-1 resistance patterns (Table 3). The DHHS guidelines (http://AIDSinfo.nih.gov) recommend genotypic testing preferred over phenotypic testing due to lower cost, faster turnaround time, and greater sensitivity for detecting mixtures of wild-type and resistant virus. Typically clinicians will start with a genotype, and if drug resistance patterns are complex, or there is resistance to a large number of drugs, then they will proceed to phenotypic testing. It is important to remember that the results of the different types of assays may not always agree; the presence of a resistance mutation does not imply that it will decrease viral replication in the phenotypic test.

LABORATORY ISSUES

There are many factors that a laboratory director would take into consideration when choosing a viral load assay including volume of testing; turnaround time; equipment and space requirements; technical expertise required; cost; and for the real-time automated systems, the extent of the testing menu. Each laboratory may well have a different solution depending on its testing needs, as the various viral load tests have different strengths and weaknesses. The real-time PCR tests have reduced the amount of hands-on time needed to perform the testing. These instruments are quite large and designed for moderate- to high-throughput testing, and may not be the right fit for all laboratories. Fully automated systems that can perform viral load testing on demand are under development and may be very useful in low- to mid-volume laboratories. Additional advantages of the real-time assays are the decreased risk of carryover contamination, broad linear range, and detection of CRFs and group O viruses.

The College of American Pathologists offers proficiency testing for both HIV-1 viral load and genotyping. To date, the genotypic survey has focused on the identification of mutations and not the interpretation of the mutations. The Quality Control for Molecular Diagnostics (QCMD) program offers qualitative and viral load testing for HIV-1 RNA and DNA. They also offer proficiency challenges for HIV-1 genotypic resistance testing, which tests not only the identification of mutations but also the interpretation of these mutations. The QCMD program offers very comprehensive challenges, but they are more expensive than the College of American Pathologists surveys.

CPT codes have been established for both HIV-1 viral load testing, genotypic, and phenotypic resistance testing. These codes are designed to be comprehensive, and additional codes used for other molecular diagnostic procedures cannot be billed in addition to the HIV-specific codes.

FUTURE DIRECTIONS

The current state of HIV-1 testing is automated real-time PCR viral load tests that eliminate contamination and have high throughput, and availability of viral load tests that detect diverse sequences and recombinants. None of these tests are well designed for relatively low-throughput laboratories; there are testing platforms available that are better suited for low-volume or random access testing, and HIV-1 viral load testing is being developed for these platforms. The real-time PCR tests provide a marked improvement in automation, but the instruments are large, the current testing menus are limited, and the throughput would not allow moderate- to high-volume laboratories to use a single instrument for testing multiple analytes. Ideally, a laboratory would purchase a single automated system that could perform a wide array of testing for the laboratory essentially on demand; however, no such system is currently available.

Another unmet need is an FDA approved qualitative or quantitative proviral DNA test. A qualitative DNA assay would be useful for diagnosis of neonatal or acute HIV-1 infection, although neither of these is a frequent event. The current FDA-approved qualitative RNA test fits these needs, but is labor intensive and not really designed for low-volume testing. Ideally, these tests could be made available on a fully automated random access instrument. The role of a quantitative HIV-1 DNA test is unclear at this point. Currently, DNA viral load testing is used as a research tool and is not incorporated into routine patient care. If a test were available, clinical studies would be needed to determine its utility.

The detection of diverse viral populations is markedly improved with the real-time PCR tests, but with ongoing viral recombination, there is a need for continued monitoring of the evolution of these viral sequences. Several of the manufacturers of the currently available tests have active surveillance programs throughout the world to detect emerging variants and recombinants.

Another critical need is a low cost viral load option for resource-limited settings. Currently, the systems for viral load testing require high capital investment in instrumentation, along with reliable electricity, and pure water. As technologies emerge and become more simplified and less expensive, viral load testing may eventually be moved to these settings, likely into local reference laboratories. A particular challenge for testing in these settings is the need to reliably detect unusual viral subtypes and recombinants.

The next-generation sequencing platforms are likely to be in routine clinical use in the next few years. Currently, physicians must order genotypic resistance testing for the protease and reverse transcriptase, but if they also desire integrase testing, then this must be performed separately. Similarly, if tropism profiles are desired, this is also a separate test that is performed. New drugs, such as maturation inhibitors, do not yet have a platform on which to be tested, and so resistance testing is not available. However, with the advent of whole region or whole genome sequencing for HIV-1, the need for multiple separate genotypic resistance testing assays will not be necessary, and more information will be available to use in algorithmic decision-making based on larger amounts of sequence information.

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Molecular Detection and Characterization of Hepatitis C Virus

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31

Hepatitis C virus (HCV) and molecular methods are inextricably linked, in history and in clinical practice. This virus was the first to be identified with molecular methods (1) and nucleic acid tests have become fundamentally important in the diagnosis of infection and therapeutic management.

HCV is a globally significant pathogen, infecting over 150 million individuals. In the United States, it is the most common blood-borne infection, causing an estimated 2.7 million chronic infections (2). In the blood-screening era, most transmission occurs after exposure to a low viral inoculum through intravenous drug use, multiple sexual partners, sex with a chronically infected partner, iatrogenic exposure, and occupational exposure to blood such as needlestick (3, 4).

Significant progress against HCV infection has been achieved in the form of direct acting antiviral (DAA) therapies that have cure rates greater than 90% in clinical trials. However, the optimism instilled by the availability of these highly effective drugs is tempered by recent incidence data: after a period of decline from 2000–2004 and plateau at low levels from 2004–2010, acute infections have increased since 2011 (5) (see http://www.cdc.gov/hepatitis/Statistics/2011Surveillance and http://www.cdc.gov/mmwr/preview/mmwrhtml/mm6350md.htm?s_cid=mm6350md_w#fig1). Therefore, despite considerable gains, HCV will likely have a significant negative impact on health for the foreseeable future. Hospitalization rates for HCV-associated liver disease have increased, and the burden of chronic liver disease is expected to grow in the coming decades due to the progression of disease in a reservoir of chronically infected individuals (6). Given this horizon, the diagnosis and management of HCV infection with nucleic acid tests (NATs) will continue to be important.

BRIEF DESCRIPTION OF HCV

HCV is classified in its own genus *Hepacivirus* within the family *Flaviviridae*. HCV has an ~9.6 kb positive-sense RNA genome composed of a long open reading frame flanked by terminal 5′ and 3′ untranslated regions (UTRs) (Fig. 1). The 5′ UTR is highly conserved and the 3′ UTR has a short variable sequence, a poly(U) tract, and a highly conserved element. Core, p7, E1, and E2 are structural protein genes that encode nucleocapsid, transmembrane, gp33, and gp72 proteins respectively.

During replication, the HCV open reading frame is translated into a single polyprotein (approximately 3,000 amino acids) that is subsequently cleaved by host and viral proteases encoded by NS2, NS3, and 4A genes. The NS3 gene also encodes a helicase. The p7 region encodes a protein that is essential for the replication of infectious viruses (7); however, its specific function is unknown. The RNA-dependent RNA polymerase NS5B lacks efficient proofreading activity, resulting in extensive genome mutation during replication and quasi-species generation within an infected individual. NS5A is a multifunctional nonstructural phosphoprotein (8). It is a key component of the replication complex and binds other constituents of the complex including RNA-dependent RNA polymerase, RNA, and cyclophilin A, a host protein required for HCV RNA replication. It is also appears to play a role in virion assembly.

Currently, there are seven major genotypes of HCV and more than 50 known subtypes based on genomic sequence heterogeneity (9). Genotypes and subtypes differ by 30 to 35% and 20 to 25% of nucleotides, respectively. Genotypes 1, 2, and 3 (Gt1, Gt2, and Gt3) have a worldwide distribution and account for most HCV infections in Europe and North America. In the United States, the majority of HCV infections in all age groups are Gt1 (75%), followed by Gt2 (13.5%), and Gt3 (5.5%). Gt4 is most prevalent in the Middle East and North and Central Africa; Gt5 is found primarily in South Africa; Gt6 occurs throughout Asia; Gt7 has been detected in an immigrant from The Congo and its region of endemicity is unclear. This chapter focuses on Gt1 through 4 given worldwide prevalence.

CLINICAL UTILITY OF HCV NAT TESTING

HCV Infection and Disease

The incidence of acute hepatitis C peaked in 1992 at 2.4 cases per 100,000, then declined for almost a decade, falling to 0.3 cases per 100,000, where it remained until 2010 (10). Surprisingly, incidence started to climb again in 2011; the latest surveillance data (2012) demonstrate the rate had doubled to 0.6 per 100,000 compared to nadir (http://www.cdc.gov/hepatitis/Statistics/2012Surveillance/Commentary.htm#hepC). Increased rates were reported

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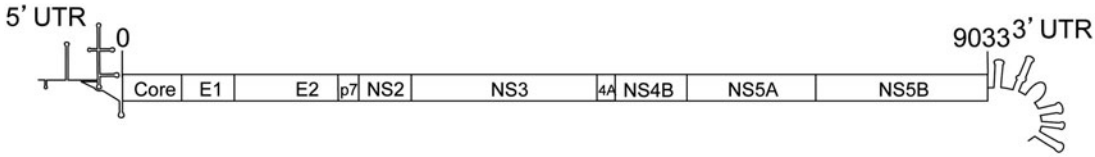

Protein	Role	nuc pos	aa pos	aa length	size
Core	Encapsidation	342	1	191	p21
E1	Receptor binding, entry?	915	192	192	gp31
E2	Receptor binding, entry?	1491	384	363	gp70
p7	viroporin?	2580	747	63	p7
NS2	NS2/3 Zn-dependent protease	2769	810	217	p21
NS3	NS2/3 Zn-dep protease, NS3/4A protease, helicase-NTPase	3420	1027	631	p70
NS4A	cofactor for NS3-4A protease	5313	1658	54	p8
NS4B	membranous web organization	5475	1712	261	p27
NS5A	phosphoprotein	6258	1973	448	p58
NS5B	RNA-dependent RNA polymerase	7602	2421	591	p68

FIGURE 1 HCV genome and protein coding scheme. UTR, untranslated region; NS, nonstructural gene; nuc, nucleotide; pos, position; aa, amino acid; gp, glycoprotein. Numbering according to references 83 and 84.

across all age groups; however, the greatest increases were observed in 0 to 19 and 20 to 29 year olds. Increasing incidence has been noted in young suburban whites who begin by abusing prescription opioids then transition to injection drug use (11), and in HIV-infected men who have sex with men (4).

The clinical features of acute infection are depicted in Fig. 2. The majority of individuals are thought to be asymptomatic. Spontaneous clearance is observed in approximately 25% of acute infections; the remaining individuals become chronically infected. Signs of hepatitis during acute infection are actually positive indicators as they represent early, vigorous antiviral T-cell responses associated with spontaneous virus clearance. These responses are minimal or absent in individuals who progress to chronicity (12).

Why some individuals clear their infection while others progress to chronic HCV infection is unknown. The genetic determinants that govern spontaneous HCV clearance versus persistence have been investigated with genome-wide association studies. This approach has identified a single nucleotide polymorphism (SNP) in *DQA1-DQB2 HLA* class II region (13) found on reference SNP (rs)4273729, and multiple SNPs upstream from the *IL28B* gene (references 14 and 15; reviewed in reference 16), of which the most strongly associated are variants on

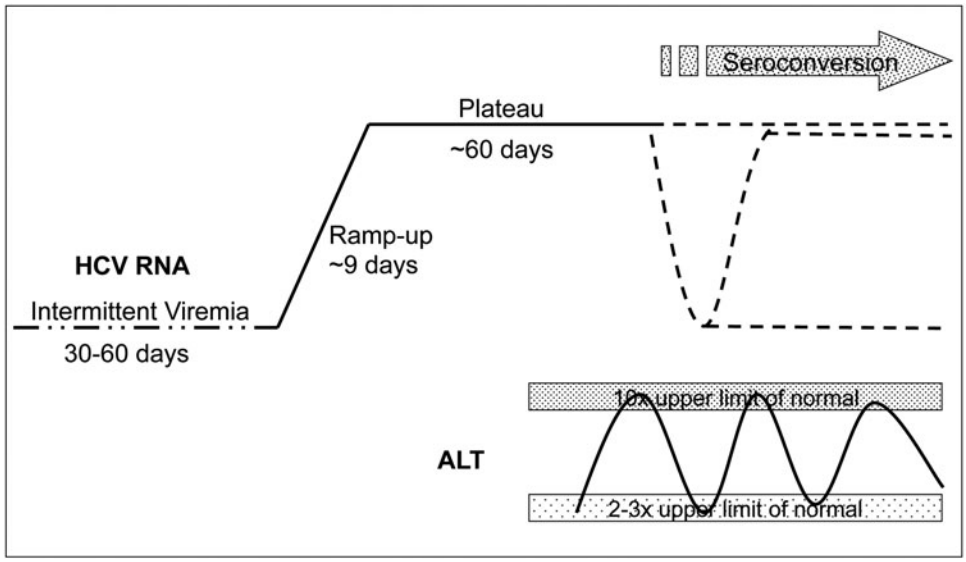

FIGURE 2 Clinical features of acute hepatitis C following exposure to low viral inoculum such as occupational needlestick exposure or community-based exposure (35, 85, 86). Characteristics following higher dose exposure (transfusion with contaminated blood products) may be different. Intermittent viremia phase estimated from needlestick exposure (86). Kinetics of other characteristics derived from seroconversion panels (35). ALT, alanine amino transferase. Dashed lines indicate potential viremia patterns as defined by HCV RNA levels in peripheral blood. Adapted from reference 35.

rs12979860 (13) that are independently associated with clearance and additively effective. The homozygous G allele on *HLA* class II rs4273729 and homozygous C allele on *IL28B* rs12979860 are associated with clearance. The homozygous C allele on *HLA* class II rs4273729 and the homozygous T allele on rs12979860 are associated with chronic infection. Heterozygotes tend more toward chronic infection than clearance. *IL28B* encodes interferon λ3, an interferon that stimulates a signaling cascade similar to interferon α and interferon β, but through a distinct receptor. The worldwide geographic distribution of *IL28B* genotypes appears to explain in part the observed racial differences in acute infection outcome (15, 17). Individuals of East Asian descent have haplotypes associated with resolution, explaining the high rates of spontaneous clearance observed in that region. In contrast, individuals of African descent have haplotypes associated with progression to chronic infection, explaining the high rates of chronic infection observed in Africa. How genetic determinants in *HLA* class II and *IL28B* regulate immune responses that mediate HCV clearance is not yet understood.

Disease progression occurs in a relatively small proportion of patients with chronic hepatitis C infection (Fig. 3). Over decades, progressive liver damage produces cirrhosis in 10 to 20% of chronic infections and liver failure or hepatocellular carcinoma in approximately 5% of chronic infections (18). However, the overall disease burden is significant due to the number of infected individuals. In fact, liver failure due to chronic hepatitis C infection is the leading cause of liver transplantation in the United States (19). Risk factors for disease progression include diseases or behaviors that induce additional hepatic injury (such as concomitant hepatitis B virus infection and alcohol consumption) or impair antiviral immunity (such as HIV infection). Epidemiologic descriptors such as male gender and older age at infection are also associated with higher risk and faster rate of disease progression.

Unlike other chronic viral infections such as HIV and hepatitis B virus, virologic parameters including viral load and genotype do not predict disease progression or indicate disease severity in chronic hepatitis C (20, 21). Viral load remains fairly constant once chronic hepatitis C infection is established (21), and rates of progression have been found to correlate more with disease severity in the liver, as manifested by the extent of fibrosis on initial liver biopsy (22), than on the level of hepatitis C virus replication represented by viremia (23).

"Occult" hepatitis C remains a controversial entity (24, 25). It is defined as the presence of HCV RNA solely in cellular reservoirs of infection such as peripheral blood mononuclear cells and hepatocytes. Its existence has been studied in the contexts of idiopathic hepatitis and HCV clearance (including spontaneous recovery from acute infection and response to treatment of chronic infection). The application of nucleic acid tests to this entity will not be considered further herein since the diagnosis of "occult" hepatitis C remains within the research realm and its biological significance remains unclear.

Treatment of Hepatitis C Infection

Treatment for chronic HCV infection has undergone significant evolution in regard to available agents, duration, and dosing strategy. The utility of interferon alfa in non-A, non-B hepatitis was demonstrated early, prior even to the identification of the specific causative agent (26). The addition of ribavirin resulted in improved overall and genotype-specific responses. The modification of interferon alfa through the addition of a polyethylene glycol (PEG) moiety prolonged drug half-life and increased treatment efficacy while decreasing dosing frequency.

The overall poor efficacy of pegylated interferon/ribavirin spurred the development of direct-acting antiviral drugs (DAAs) for the treatment of chronic HCV infections. The first-generation Gt1-specific protease inhibitors boceprevir and telaprevir were greeted with a good deal of optimism given the findings of clinical trials that demonstrated an improved efficacy of approximately 70% (27, 28). However, high rates of adverse reactions limited tolerability and led to premature treatment discontinuation once these drugs were placed into general clinical use and enthusiasm for them dampened quickly. The next wave of approved DAAs for treatment of Gt1 infections, the NS3 protease inhibitor simeprevir and the NS5b polymerase inhibitor sofosbuvir, had better efficacy, shorter treatment duration, and apparently improved side-effect profiles, but a short lifespan as a first-line treatment of Gt1 infections because they required coadministration with injected pegylated interferon. Current guidelines recommend new drug combinations that can be administered orally for ≤24 weeks and have >90% efficacy for most genotypes (http://www.hcvguidelines.org) (Table 1).

The availability of combination oral DAA regimens has impacted patients infected with less common genotypes.

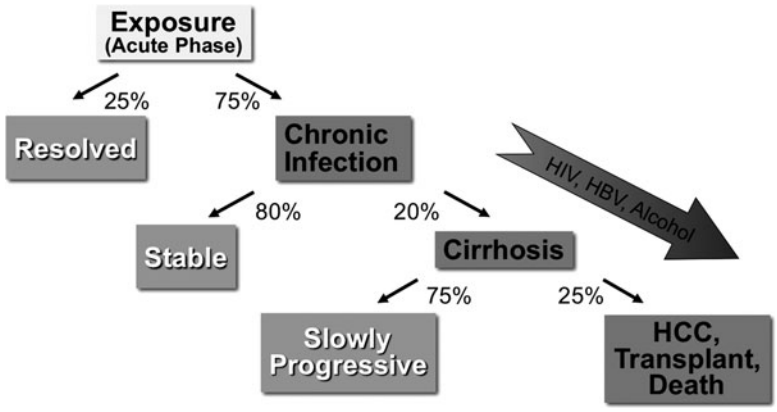

FIGURE 3 Liver disease progression in chronic hepatitis C. Factors listed in notched arrow accelerate histologic progression of disease.

TABLE 1 Combination DAA regimens for chronic hepatitis C treatment

DAA regimen	Genotype(s)	Indication/ treatment duration	Modification for cirrhosis	SVR12
Ledipasvir + sofosbuvir	1a, 1b, 4, 6	12 weeks	None necessary	Gt1, 99% (88, 89)
Paritaprevir/ritonavir + ombitasvir + dasabuvir	1a, 4	12 weeks (and add ribavirin)	Extend treatment to 24 weeks (and add ribavirin)	Gt1a, 95–97% (90, 91)
	1b	12 weeks	Add ribavirin	Gt1b, 98–99% (90, 91)
Simeprevir + sofosbuvir	1a[a], 1b, 4[a]	12 weeks	Extend treatment to 24 weeks	Gt1a, 1b, ≥90% (57)
Sofosbuvir + ribavirin	2, 5	12 weeks	Extend treatment to 16 weeks (Gt2)	Gt2, 93% (56, 92)
	3	24 weeks		91% (92)
Sofosbuvir + ribavirin +pegylated interferon alfa	3, 4, 6	12 weeks	None mentioned in current recommendations	Gt4, 6, 90% (56)

[a]The addition of ribavirin can be considered; initial trials showed no clear benefit but were underpowered. Larger definitive trial results are pending.

Clinical trials for new drug combinations have included patients infected with Gts4-6. At this time, a single regimen for treatment of Gt4 has been approved by the FDA (sofosbuvir/ribavirin), but additional combinations have been recommended based on efficacy data and the desire to avoid ribavirin-induced anemia and neutropenia (Table 1). No treatments have been approved by the FDA for the rare genotypes (Gt5, 6); however, guidelines have issued recommendations based on the high efficacy observed in the small number of enrolled patients (Table 1). The high efficacy of the latest combination DAA regimens has also been beneficial for the treatment of other special patient populations (Table 2).

Numerous DAAs other than those in Table 1 have been developed and are currently in clinical trials. These include N3/4A protease inhibitors, NS5B polymerase

TABLE 2 Treatment of special populations of patients: interferon alfa-based therapies versus combination DAAs

Population	Treatment recommendation		Rationale for change
	Interferon alfa-based therapy	Combination DAA	
HIV-HCV coinfection	Longer treatment required than HCV mono-infected patients because these regimens relied on interferon-induced host antiviral immune responses that were impaired due to HIV infection.	Same treatment duration as HCV mono-infected patients	New DAA combinations are so potent that HIV-HCV coinfected patients are no longer disadvantaged by impaired host antiviral immunity.
Acute infection	Treat early to prevent chronic infection	"Wait and see" management; follow patients monthly with viral loads for 6–12 months to allow for spontaneous clearance. Treat only those who progress to chronic infection.	Chronic infection now readily curable; no need to incur cost of DAA treatment in those who are destined to clear spontaneously.
Compensated cirrhosis	At least 48 weeks of therapy due to refractoriness to treatment; SVR rates 20–30% lower than noncirrhotic patients.	For most combination DAAs, longer treatment duration compared to non-cirrhotic patients, but ≤24 weeks for all regimens (see Table 1)	SVR rates in compensated cirrhosis equivalent to non-cirrhotic patients after extended treatment with combination DAAs.
Decompensated cirrhosis	Guidelines advocated treatment by experienced providers due to potential benefit of preventing allograft infection if HCV RNA negative prior to transplant. However in practice, treatment was generally avoided due to poor response rates and high rates of adverse side effects that included treatment-associated mortality.	Sofosbuvir + ribavirin Sofosbuvir + ledipasvir (+/– ribavirin)	Compared to interferon alfa-based therapies, combination DAAs are safer, better tolerated, and produce higher response rates.

TABLE 3 Utility of HCV RNA tests in different treatment regimens

Drug regimen	Viral genotype/subtype	Viral load	IL28B genotype (rs12979860 haplotype determination)	Resistance mutation detection
Pegylated interferon alfa/ribavirin	Genotype required to determine duration of therapy and probability of response; subtype (Gt1a vs Gt1b) of no utility	All genotypes: Baseline, end of treatment, end of follow-up (to determine SVR) Gt1, 4: On-treatment monitoring for response-guided therapy and futility rules	Gt1: helpful in predicting response to therapy Gt2, 3: Not useful	Not useful
Telaprevir or boceprevir plus pegylated interferon alfa/ribavirin	Genotype required to determine treatment eligibility (Gt1-specific protease inhibitors); subtype (Gt1a vs Gt1b) potentially useful in predicting moderate differences in therapeutic response	Baseline, on-treatment monitoring for response-guided therapy, futility rules, end of treatment, end of follow-up (to determine SVR)	Helpful in predicting response to therapy	Not useful
Simeprevir plus pegylated interferon alfa/ribavirin	Genotype required to determine treatment eligibility (simeprevir approved solely for Gt1); Gt1 subtype necessary (Gt1a should have resistance testing for NS3 Q80K mutation)	Baseline, on-treatment monitoring for futility rules, end of treatment, end of follow-up (to determine SVR)	Helpful in predicting response to therapy	Gt1a: detection of NS3 Q80K mutation at baseline to determine treatment eligibility
Sofosbuvir plus pegylated interferon alfa/ribavirin (Gt1, 4) Sofosbuvir plus ribavirin (Gt2, 3)	Genotype required to determine treatment regimen and treatment duration (Gt3 treated for longer than Gt2)	Prescribing information lacks directives; baseline, end of treatment, and of follow-up helpful in determining response. On-treatment testing likely to be performed to assess compliance	Not useful	Not currently useful
Combination DAAs	Genotype likely necessary to determine regimen and duration; subtype (Gt1a vs Gt1b) potentially useful for protease inhibitors to predict moderate differences in therapeutic response.	Baseline, end of treatment, and of follow-up helpful in determining response. On-treatment testing likely to be performed to assess adherence and response.	Not useful	Not currently useful

inhibitors (nucleotide and non-nucleotide inhibitors), NS5A replication complex inhibitors, and inhibitors of host cell proteins required for HCV genome replication, such as cyclophilin A. Given the efficacy, short treatment period, and ease of dosing of currently approved regimens, it is unclear how many new regimens will be brought to market. A detailed description of the plethora of agents and drug combinations in the pipeline is beyond the scope of this chapter, and instead the reader is referred to several excellent reviews (29, 30) and web-based resources containing the latest recommendations on all aspects of HCV treatment (http://www.hcvguidelines.org, http://www.easl.eu/_clinical-practice-guideline).

Testing during treatment of chronic hepatitis C evolved from a "one size fits all" paradigm when all patients were treated for 48 weeks with pegylated interferon plus ribavirin, to a more complex testing scheme of response-guided therapy which was used primarily during treatment of Gt1 infections with pegylated interferon plus ribavirin and with first-generation DAAs telaprevir and boceprevir. With the currently recommended DAAs, treatment-associated testing is once again fairly simplified and standardized across all genotypes. The types of assays that are useful and their utility during treatment with currently available drugs are summarized in Table 3 and discussed further below.

Diagnostic Applications of HCV NATs

Acute Infection

HCV NATs are useful in establishing the diagnosis of acute HCV infection in seronegative individuals because HCV RNA can be detected as early as 1 week after exposure via needlestick or transfusion (31–33) and at least 4 to 6 weeks prior to seroconversion in a number of transmission settings (34, 35). Before the availability of current combination DAA regimens, it was important to make this diagnosis to institute therapy early, to prevent the development of difficult-to-treat chronic infection (Table 2). Early treatment may still be preferable for those patients with a significant transmission risk; however, for other

patients, "wait and see" management can be considered, reserving treatment with combination DAA regimens for those who do not clear, and avoiding treatment in patients who clear spontaneously. With the "wait and see" approach, the diagnosis remains important to make in order to counsel patients to avoid further hepatotoxic insults such as alcohol, as well as drugs such as acetaminophen, to reduce transmission risk, and for addiction referral for those who likely acquired infection through intravenous drug use.

In the setting of acute infection, the data provided by serology (documentation of potential seroconversion) and NAT (detection of HCV replication) are complementary and confirmatory. They can be used to distinguish previously existing chronic infection versus susceptibility to, or the occurrence of, acute infection. Pre-existing chronic infection should be suspected in seropositive individuals with high viral loads; this can be confirmed if similar results are obtained upon retesting at subsequent time points. Seronegative individuals with detectable HCV RNA are likely in the window period of infection. Additional testing at later times can be used to document resolution of acute infection versus progression to chronicity. Seronegative individuals with no detectable HCV RNA should be retested at multiple subsequent time points regardless of exposure history if acute infection is suspected. It is important to follow antibody and RNA status over time because any single result can be misleading. For example, transient HCV RNA negativity has been documented early postinfection and at the time of seroconversion in individuals who become chronically infected (Fig. 2) (36), while transient positive results have been found in those who spontaneously clear the virus (35, 37). Therefore, serology and RNA assessment every 4 to 8 weeks for 6 to 12 months have been recommended to document the occurrence and outcome of acute HCV infection (http://www.hcvguidelines.org/full-report/management-acute-hcv-infection).

Available guidelines do not make recommendations on the specific NAT type for the diagnosis of acute hepatitis C infection. However, early after a known exposure, when viral loads are known to be low and HCV RNA in peripheral blood is intermittently detected (35), it would be reasonable to use the most sensitive assays, including qualitative (conventional RT-PCR or transcription-mediated amplification [TMA]-based) and sensitive quantitative (RT/real-time PCR) tests. Highly sensitive qualitative assays have been demonstrated to be more effective than a conventional quantitative RT-PCR assay in this setting (38). By inference, branched DNA-based quantitative assessment is also less preferable in the earliest times postexposure since its sensitivity is comparable to conventional quantitative RT-PCR. The utility of latest generation RT/real-time PCR versus TMA-based assays in this setting has not been determined.

Diagnosis of Chronic Infection

The diagnosis of chronic HCV is established with antibody screening tests to document infection, and HCV RNA NAT to document replication. In addition to risk-based testing found in previous hepatitis C screening recommendations (Table 4), new screening recommendations include one-time serology testing for individuals born between 1945 and 1965 (39), the cohort with the projected highest prevalence of chronic hepatitis C. Birth cohort screening has been recommended since previous recommendations were ineffective in identifying what is thought to be the bulk of chronic infections.

TABLE 4 Recommendations for chronic hepatitis C screening (39)

One-time serology should be performed for all individuals born between 1945 and 1965.
Serology should also be performed in individuals with the following risk factors:
Injection drug use (recent or remote; single or multiple episodes)
Conditions associated with hepatitis C prevalence:
HIV
Hemophilia with receipt of clotting factors prior to 1987
Hemodialysis
Unexplained aminotransferase elevations
Blood transfusion or organ transplant prior to 1992
Children born to HCV-infected mothers
Needlestick injury or mucosal exposure to HCV-positive blood
Current sexual partners of HCV-infected persons

Approximately 75% of acutely infected individuals become chronically infected and have stable viremia detectable by NAT (40). NAT testing is particularly advisable in patients with potential impaired humoral immunity before excluding the diagnosis of chronic HCV infection. Roughly 5% of HIV-1 infected individuals have HCV viremia but no HCV antibodies as detected by second- and third-generation serologic assays; low CD4 cell counts (<200 cells/μl) have been found to be a risk factor (41, 42). HCV RNA testing has therefore been recommended for diagnosing the etiology of hepatitis in symptomatic HIV-1 infected patients with no detectable anti-HCV antibodies (http://www.hcvguidelines.org/full-report/hcv-testing-and-linkage-care).

The utility of confirming all positive antibody screening test results prior to HCV NAT is a question that often arises in regard to testing for chronic hepatitis C. Early recommendations advised confirming antibody reactivity by recombinant immunoblot (RIBA) prior to HCV NAT due to the nature of screening tests (emphasis on sensitivity, with the potential for significant numbers of false-positive results) but this practice was never widely adopted by clinicians or laboratorians. Subsequent recommendations included a complex algorithm, advising the confirmation of screening results with low signal/cutoff (S/CO) ratios by RIBA, since false-positives were most likely to occur in these samples (43). RIBA is no longer being manufactured and current testing recommendations for chronic HCV screening (Fig. 4) have largely jettisoned the concept of confirmatory testing unless a spurious positive result is suspected in an individual with no risk factors. In these instances, S/CO ratios are still useful as thresholds for confirmatory testing although they are not formally mentioned.

Unsurprisingly, there is a difference between the type of NAT that should be performed to diagnose chronic hepatitis C and the type of testing that is actually performed. At this time, the only marketed NAT that is approved by the FDA for use in diagnosing chronic infection is APTIMA HCV (Hologic, San Diego, CA). In practice, quantitative tests are used most commonly because chronic infection and baseline viral load can be established with a single test. Considering the biology of infection, this choice is reasonable since chronically infected patients typically

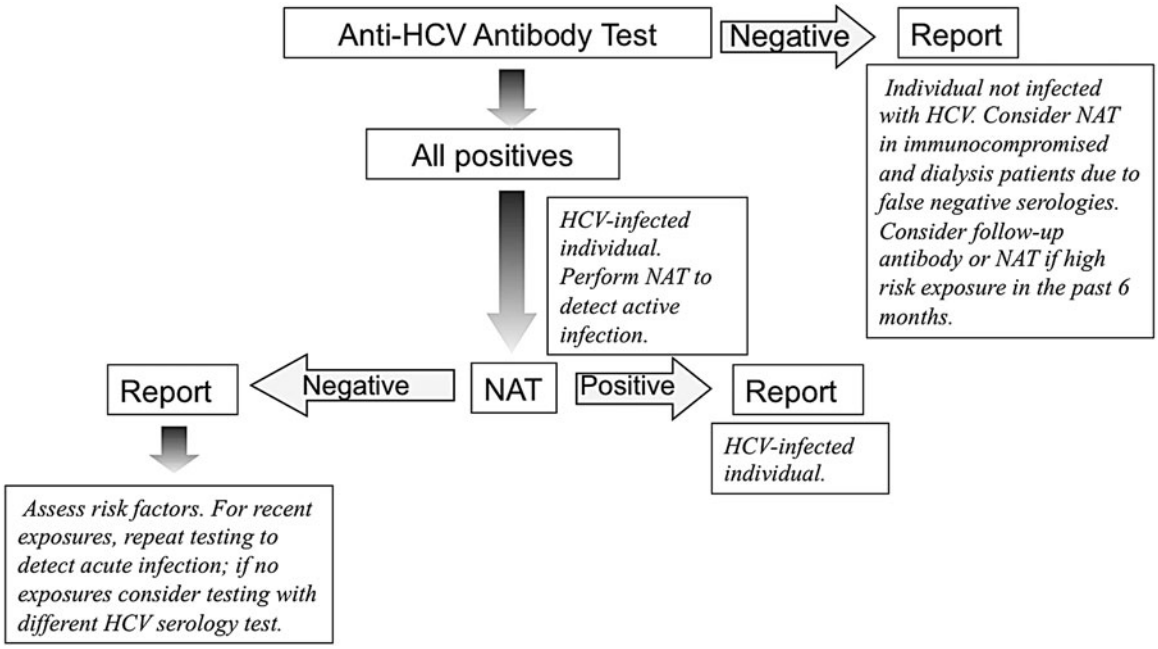

FIGURE 4 Recommended testing algorithm for detection of HCV infection. Derived from reference 87.

have stable viral loads well above the limit of detection of quantitative tests. Real-time PCR-based assays are attractive, clinically practical options for use in initial testing since they provide quantitative data and their low limits of detection (in the range of qualitative assays currently approved for diagnosis) address any theoretical concerns of false-negative NAT results. If off-label use of diagnostic tests is an issue, laboratories may wish to consider offering a panel of tests consisting of a qualitative test for diagnosis followed reflexively by a quantitative test (and genotyping) when a positive qualitative result is obtained. Regulatory requirements such as consulting institutional laboratory advisory committees and offering tests individually should be observed when NAT panels are offered.

NAT Usage in the Management of Therapy for Chronic Hepatitis C

NATs continue to be diagnostically important as new DAAs are introduced; however, their roles are changing (summarized in Table 3). HCV genotype and viral load should be determined at baseline. Genotype is used to determine treatment regimen and duration, depending upon the drug. In the pegylated interferon alfa/ribavirin era, genotype was also useful in predicting response to therapy; however, response rates to DAAs are generally uniformly high across genotypes. Viral load assessments that are common to all therapeutic regimens include baseline, end of treatment, and end of follow-up. Baseline testing is critical as it is the starting point in documenting treatment-induced elimination of viremia. End-of-treatment testing is performed to ascertain whether treatment cleared viremia. End-of-follow-up testing is performed months after end of treatment, to assess durability of clearance in the absence of drug. Individuals who have no detectable viremia by NAT at end of follow-up are defined as having achieved sustained virologic response (SVR) and are considered cured (44). Viral load monitoring for SVR was conventionally performed 24 weeks after end of treatment with pegylated interferon/ribavirin and first-generation DAAs. A follow-up period of 12 weeks has been shown to be comparably predictive of SVR at 24 weeks (45); the attainment of SVR after 12 weeks of follow-up is now incorporated into analysis of all DAA trials and is being used for patient management with all new DAA combinations.

Prescribing information for the newest highly potent DAA combinations contains no recommendations for on-treatment therapeutic monitoring because clinical trials demonstrated that viral load suppression was uniformly rapid and accompanied by high cure rates, therefore on-treatment viral load assessment and response-guided therapy were unnecessary. Guidelines currently recommend viral load assessment after 4 weeks of treatment, at which time viremia in most patients will be undetectable or near the limit of quantification of assays recommended for use in monitoring response to combination DAAs, as described below. Results are used by care providers to counsel patients on the need for compliance, and by insurance companies to provide data that support continued authorization of coverage for extraordinarily expensive drug regimens. At this time, as DAAs transition from clinical trials into general use, it is unclear whether the larger population of chronically infected individuals will have the same rapid durable responses observed in clinical trial subjects. A recommendation to retest at week 6 of treatment if viremia is detectable at week 4 was recently added to the U.S. treatment guidelines, suggesting that responses observed in clinical practice are more heterogeneous than those seen in clinical trials (http://www.hcvguidelines.org/full-report/monitoring-patients-who-are-starting-hepatitis-c-treatment-are-treatment-or-have). Viral load monitoring during combination DAA treatment is mentioned in the current guidelines and is synopsized in Table 5.

TABLE 5 Viral load monitoring recommendations in combination DAA treatment[a]

Timepoint	Utility
Baseline	Establish level of viremia before treatment
Treatment week 4	Determine on-treatment response, assess compliance • Ideally should be <25 IU/ml
Treatment week 6	Recommended if HCV RNA detected at week 4 • Stop therapy if viral load has increased >10-fold[b]
End of treatment	Can be considered to document viral clearance at end of treatment • Helpful in distinguishing between breakthrough (viral load undetectable at week 4, but detectable at end of treatment) versus relapse (viral load undetectable at end of treatment, but detectable 12 weeks later)
After treatment—week 12 (SVR12)	Recommended to document SVR • Should be HCV RNA undetected with a sensitive real-time PCR assay
After treatment—week 24 (SVR24)	Can be considered

[a]http://www.hcvguidelines.org/full-report/monitoring-patients-who-are-starting-hepatitis-c-treatment-are-treatment-or-have
[b]Based on expert opinion; clinical trial data not yet available.

NATs that are acceptable for use in combination DAA treatment regimens are more sensitive and have lower limits of quantification than those that are acceptable for pegylated interferon alfa/ribavirin. Assays that were used to define efficacy in Phase III clinical trials of DAAs had performance characteristics that included a lower limit of quantification of at least 25 HCV RNA IU/ml and a limit of detection of at least 15 HCV RNA IU/ml. Tests that conform to these characteristics should be used to determine response to DAA treatment (Tables 6 and 7).

Host genotype determination became important in the treatment of Gt1-infected individuals late in the era of interferon alfa-containing regimens. Loci upstream of *IL28B* were associated with interferon alfa responsiveness (14, 46–48). The allele on rs12979860 appears to be the most significant; the CC haplotype was found to be the strongest pretreatment predictor of response, was associated with more rapid virus clearance kinetics in the first 48 hours of treatment, and occurred in a high proportion of individuals in whom viremia was no longer detectable after only 4 weeks of treatment (48). The role of *IL28B* SNPs in responsiveness of Gt2 and Gt3 infections to pegylated interferon/ribavirin is controversial; some studies demonstrate supportive data while others have failed to find an association (49). The antiviral activity of the currently available combination DAA regimens is so high that determination of host genotypes associated with interferon responsiveness is no longer clinically useful (Table 3).

HCV QUALITATIVE AND QUANTITATIVE NATS

NAT formats that have been developed for diagnosis and management of hepatitis C infection include qualititative, quantitative, genotyping, and blood screening assays. Available methods and tests for each format are described below. HCV RNA extraction is reviewed first due to its contribution to assay performance characteristics.

HCV RNA Extraction

Nucleic acid extraction is the first step in almost all HCV NATs. Some extraction methodologies are HCV-specific, while others target total RNA, total nucleic acids, or total viral RNA in clinical samples (Tables 6 and 7). Although semiautomated assays that consist of manual extraction with automated downstream processes are still available, most testing is currently performed with automated commercial test systems that include instrumentation for front end nucleic acid extraction, reaction set-up, and back-end qualitative detection or quantification (Tables 6 and 7). There have been no comprehensive comparisons of these

TABLE 6 Commercial HCV RNA qualitative tests[a]

Test (manufacturer)	Test format	Extraction chemistry	Amplification chemistry	Limit of detection (IU/ml)
APTIMA HCV (Hologic)	Manual	Target capture (HCV RNA)	TMA	10
cobas Amplicor v2.0 (Roche)	Semiautomated[b]	Guanidine HCl (total RNA)	Reverse transcription/PCR	50
cobas Ampliprep/cobas Amplicor v2.0 (Roche)	Automated	Silica-based (total nucleic acid)	Reverse transcription/PCR	50
Versant HCV RNA (Siemens)	Manual	Target capture (HCV RNA)	TMA	10

[a]All tests are FDA approved and Conformité Européenne (CE)-marked according to European *In Vitro* Diagnostic Directive 98/79/EC.
[b]Manual extraction, automated reaction set-up.

TABLE 7 Commercial HCV RNA quantification tests[a]

Test (manufacturer)	Extraction chemistry	Amplification method	Measurable range (log_{10} IU/ml)[b]	Limit of detection (log_{10} IU/ml)	Reference(s)
cobasAmpliPrep/cobas TaqMan v1.0 (Roche)	Silica-based (total nucleic acid)	RT-Real-time PCR	1.6–7.8[c]	1.2	(93)
cobas AmpliPrep/cobas TaqMan v2.0 (Roche)	Silica-based (total nucleic acid)	RT-Real-time PCR	1.2–8.0	1.2	(55)
High Pure/cobas TaqMan v2.0 (Roche)	Guanidine HCl/glass fiber column (total viral nucleic acid)	RT-Real-time PCR	1.4–8.5	1.3	(94, 95)
RealTime HCV (Abbott)	Magnetic microparticle/total nucleic acid	RT-Real-time PCR	1.1–8.0	1.1	(72, 73, 95–97)
Versant HCV RNA 3.0 (Siemens)	Lysis without extraction	Branched DNA	2.8–6.9	2.8	(98)

[a]All tests are FDA approved and Conformité Européenne (CE)-marked according to European *In Vitro* Diagnostic Directive 98/79/EC.
[b]Lower limit of quantification—upper limit of quantification of undiluted specimens.
[c]Analytical measuring range. Expanded clinical reportable range (1.6–9.8 log_{10} IU/ml) can be obtained by maximum dilution of 1:100.

automated extraction platforms using a single HCV NAT to determine extraction efficiencies and overall performance.

Qualitative Assays

Qualitative NATs that employ reverse transcription/conventional PCR and TMA are available (Table 6). TMA is more sensitive than conventional RT-PCR. Qualitative NATs are approved for the detection of HCV RNA in seropositive individuals to document chronic infection. None of the available assays are approved for use in assessment of treatment response to DAAs; however, TMA-based tests are adequately sensitive (limit of detection, <15 IU/ml) for use in accurately determining clearance of viremia.

Quantitative Assays

A WHO international calibration standard has been available since 1997; HCV RNA is therefore quantified in International Units per milliliter. The Fourth International Standard is currently in use. It consists of plasmas from three different Gt1a-infected individuals collected prior to seroconversion that have been pooled, then further diluted in HCV-seronegative/HCV RNA-negative pooled human plasma. It is a biological standard whose concentration was derived by testing at multiple sites with currently approved quantitative HCV NATs (Table 7) plus one additional commercial assay not approved for use in the United States (Versant HCV kPCR v1.0; Siemens, Tarrytown, NY). To permit longitudinal consistency in quantification, it has been calibrated in parallel with Second and Third International Standards and has been assigned a value of 5.4 log_{10} IU/ml.

Most quantitative NAT testing is performed with commercially available tests that have obtained regulatory approval (Table 7); some large reference laboratories use their own proprietary laboratory developed NATs. Results are reported as IU per milliliter. The Versant HCV RNA 3.0 is a branched DNA-based microwell plate assay that does not require nucleic acid extraction. HCV virions are lysed and viral genomes are captured with probes. Genomes are quantified by hybridization using a series of probes that contain isoC and isoG oligonucleotides to reduce nonspecific hybridization and increase assay sensitivity. Luminescent signal emission is enhanced through preamplifier and amplifier probe hybridization. Quantification is performed using external calibrators that are included on each plate. The advantages of this assay are its simple genomic isolation procedure, high upper limit of quantification, genotype inclusiveness, and reproducibility. The greatest drawback is its lower limit of quantification/limit of detection which is greater than that of real-time RT-PCR assays. This test is insufficiently sensitive to monitor response to DAA therapy. This product has largely been retired commercially; discussion is included here for historical reference.

HCV RNA quantification can be performed by real-time PCR with commercially available reagents (Table 7) that amplify 5′ UTR sequences but employ slightly different amplicon detection chemistry. The probe in the Abbott Real-Time HCV ASR assay is labeled with a 5′ fluorophor and a 3′ quencher. Unhybridized probe is randomly coiled and fails to fluoresce due to fluorophore and quencher proximity. Major fluorescence occurs upon probe hybridization; a minor amount of fluorescence is produced due to cleavage of the 5′ labeled nucleotide by *Taq* polymerase. Reduced genotype bias is achieved through low-temperature hybridization that permits binding of probes despite probe-target mismatches. Quantification is performed via a calibration curve consisting of two external assay calibrators both tested in triplicate. This assay has a limit of detection and measurable range suitable for use with DAAs (50).

In the cobas AmpliPrep/cobas TaqMan HCV real-time PCR test (Table 7), total nucleic acid extraction and reaction set-up are performed by the AmpliPrep. Amplification occurs on the TaqMan instrument. Two versions are available. The first test version demonstrated genotype bias, with an inability to detect some Gt4 infections and underquantification of others (51–53). A second version has numerous modifications from sample preparation through target amplification to improve genotype inclusivity and increase sensitivity while allowing lower sample input volume (650 µl versus 1 ml for version 1.0 [54, 55]). A third real-time PCR test combines viral RNA extraction with manual spin column (High Pure) with TaqMan 2.0 real-time PCR. These assays are calibrated externally by the manufacturer; lot-specific calibration coefficients are used by system software to calculate HCV RNA concentrations. An internal quantitative standard is added prior to extraction to quantitatively correct for potential inhibitors within individual samples. The 2.0 versions have LODs and measurable ranges suitable for use with DAAs.

GENOTYPING ASSAYS

HCV Genotyping

Genotyping of HCV is useful for determining drug treatment regimen (Tables 1 and 3). In the pegylated interferon alfa/ribavirin era, genotype determination was useful for counseling patients on the likelihood of therapeutic response. For DAAs, the prognostic value of genotyping is likely to be more limited and regimen-dependent. For example, SVR rates to sofosbuvir/ribavirin are at least 30% greater for Gt2 than Gt3; larger differences were observed in cirrhotics and individuals who had failed prior interferon alfa/ribavirin treatment (56).

Subtyping of Gt1 (determination of Gt1a versus 1b) has become relevant in the DAA era due to its impact on treatment efficacy and association with resistance mutations. The importance of subtype for treatment efficacy is regimen-dependent. Ledipasvir + sofosbuvir is equally effective against Gt1a and Gt1b while paretaprevir/ritonavir + ombitasvir + dasabuvir is highly effective against Gt1b but for Gt1a requires the addition of ribavirin to achieve comparable SVR rates. For simeprevir + sofosbuvir, relapse was observed only in Gt1a subjects in clinical trials (57), suggesting subtype may be important in this combination regimen. Additional studies are ongoing to better define the relevance of subtype for this combination regimen. In the meantime, the addition of ribavirin can be considered when treating Gt1a with this regimen.

Subtype has also been important when investigating potential antiviral resistance mutations. For example, individuals with Gt1a infections can harbor viruses with a point mutation in NS3, resulting in glutamine to lysine change at amino acid 80, or Q80K, that confers resistance to simeprevir. Prescribing information for simeprevir + pegylated interferon alfa + ribavirin advised that individuals with Gt1a infections be further tested for Q80K to determine suitability for treatment. This regimen is no longer recommended for treatment of Gt1 infections due to the availability of highly effective interferon-free regimens; however, subtype determination and Q80K mutation detection at baseline may be important for simeprevir + sofosbuvir since this mutation was found in four of six Gt1a-infected subjects who failed therapy. Studies are being performed to determine the role of resistance mutations in treatment failure with this regimen.

HCV genotypes and subtypes can be determined by commercial and user-developed NATs based on a variety of biochemical methods (summarized in Tables 8 and 9). Assays based on 5′ UTR sequences are generally acceptable for genotype determination but must be carefully designed for subtyping due to the degree of sequence conservation between subtypes. More accurate subtype determination can be achieved through analysis of NS5b, core, and/or core-E1 genes.

As of this writing, there is one FDA-approved HCV genotyping product available in the United States, the Real-Time HCV Genotype II test (Table 8). This assay employs reverse transcription/real-time PCR using TaqMan chemistry and multiple hydrolysis probes used in three different reactions to detect all HCVs, to identify Gt1-5, and to differentiate Gt1a from Gt1b. The 5′ UTR is used as the target for genotype discrimination and NS5B sequences are used for Gt1 subtyping. The test is highly accurate compared to direct sequencing (58). Indeterminate rates of approximately 2% have been reported, with most issues arising from Gt3 determination; an inability to distinguish Gt1 subtypes was observed for approximately 5% of samples (59).

Direct sequencing is considered the gold standard in accuracy for HCV genotype and subtype determination. Sequences generated from samples are compared to reference genotype and subtype sequence libraries. Mixed genotype infections can be difficult to detect when the proportion of one genotype greatly exceeds the other. Although it is too laborious for use in clinical laboratories, amplicon cloning may help to detect minority genotype populations (below 10%); however, analysis of large numbers of clones may be required. Sequencing methods require specialized instrumentation and analysis software. These methods are generally labor-intensive and time to result is usually longer than for other methods.

Reverse hybridization is probably the most common method adopted by clinical laboratories. It is more reliable than direct sequencing for the detection of mixed infections. Low throughput formats use paper strips; higher throughput microwell plate and microarray formats have been developed (Table 8).

For tests that have received regulatory approval, laboratories must verify accuracy of test performance in-house. More extensive assay verification is required for laboratory-developed tests. From the perspective of accuracy, genotype determination of the more common viruses (Gts1–3) is fairly straightforward as these samples are readily accessible. Gts4 through 6 are more problematic, as they are found less commonly. Samples containing HCV are available commercially (Gts1–3, ProMedDx, Norton, MA; Gts 1–4, Acrometrix, Benicia, CA; Gts 1–6, BocaBiolistics, Coconut Creek, FL, and SeraCare, Milford, MA) for use in preimplementation accuracy studies. One strategy for samples found to putatively contain these less common genotypes is to refer them to a reference laboratory for genotype confirmation.

Host Genotyping Tests

IL28B haplotype testing to detect host genetic markers of interferon-refractoriness was important in Gt1-infected individuals before initiating treatment with interferon alfa-containing regimens, to determine probability of response. Real-time PCR and direct sequencing methods have been described (60–62) and testing is available at large centers and in reference laboratories. The utility of *IL28B* haplotype determination prior to treatment with interferon-free combination DAA regimens is unclear. This test likely has a limited lifespan (Table 3).

ANTIVIRAL SUSCEPTIBILITY

To date, there is limited clinical utility of antiviral susceptibility testing in the treatment of chronic hepatitis C. The viral genetic determinants associated with suboptimal response to pegylated interferon/ribavirin were poorly understood and were never directly determined. Protease resistance mutations were readily detectable after treatment with first-generation DAAs; however, testing for mutations was never used pretreatment to guide drug regimen selection in Gt1-infected patients. The findings of a number of clinical studies of resistance in treatment-naive individuals undergoing therapy with first-generation protease-inhibitors plus pegylated interferon alfa/ribavirin demonstrated that this testing was not clinically useful for

TABLE 8 Commercial HCV RNA genotyping tests[a]

Test	Method	Target(s)	Subtyping	Comment	Reference(s)
eSensor HCV Genotyping Test (GenMark)[b]	Amplicon capture with oligonucleotide probes; electrochemical detection with ferrocene-labeled oligonucleotide signal probes	5′ UTR	No	Suitable for use with Roche cobas Ampliprep/cobas TaqMan amplicons. Subtyping unreliable due to 5′ UTR conservation.	99
eSensor HCVg *Direct* Genotyping Test (GenMark)[b]	Reverse transcription/PCR with amplicon detection as per eSensor HCV Genotyping Test	5′ UTR and core	Yes	Test-specific amplicon generated and queried for genotype determination	
TruGene HCV[b] (Siemens)	Direct bidirectional sequencing using forward/reverse primers labeled with unique fluorophors	5′ UTR	No	Suitable for use with Roche Amplicor HCV or Amplicor HCV Monitor test amplicons. Subtyping unreliable due to 5′ UTR conservation. Resolution of mixed genotypes (9:1 ratio) reported.	100
Versant HCV Genotype (LiPA) 2.0 (Siemens)[c,d]	Reverse hybridization with detection on strips (line probe); automated blot processor and band interpretation instrumentation available.	5′ UTR and core gene	Yes	In contrast to direct sequencing, mixed genotype infections are readily detected. Compared to version 1.0 (5′ UTR analysis only) version 2.0 demonstrates improved genotype and subtype accuracy (particularly Gt1, 6). Some difficulty in reliably distinguishing Gt2 and Gt4 subtypes reported. Ghost bands that complicate result interpretation occur less commonly than in version 1.0.	101–103
Invader (Hologic)[e]	RT-PCR plus Invader detection	5′ UTR	No	Two genotypes detected in single well using distinct genotype-specific probes, Invader oligonucleotides and FRET probes with different fluorophores. HCV genotype determination therefore requires three wells per sample.	104
RealTime HCV Genotype II[c,f] (Abbott)	Real-Time RT-PCR	NS5b (Gt1a, 1b), 5′ UTR (Gts1-5)	Yes	96% genotype agreement with LiPA 2.0 and NS5b-based direct sequencing.	105

[a]Genotypes 1–6 can be determined with all assays.
[b]Available globally as Research Use Only product.
[c]CE-marked and approved according to European *In Vitro* Diagnostic Directive 98/79/EC.
[d]U.S. regulatory status, Research Use Only.
[e]Available outside the United States only, as Research Use Only.
[f]Approved for use by FDA.

these DAAs. For example, SVR could be achieved in treated individuals with pre-existing mutations at baseline (63). Some patients who failed therapy did not have drug resistance mutations at baseline, even with detection methods as sensitive as next-generation sequencing (64). Not all patients who failed protease inhibitor therapy had resistant virus that dominated in the posttreatment quasispecies; in fact most had wild-type virus (65). Even in those with pre-existing mutations who failed triple therapy, other more highly resistant variants emerged *de novo* (64).

Currently, baseline drug resistance testing in DAA treatment is limited to simeprevir, based on Phase III clinical trial data demonstrating that Gt1a-infected patients with the point mutation resulting in glutamine to lysine change at amino acid 80, or Q80K, had diminished cure rates to simeprevir/pegylated interferon/ribavirin compared

TABLE 9 User-defined HCV RNA genotyping tests

Test	Method (references)	Target(s)	Genotyping	Subtyping
Direct sequencing	Sanger and CLIP biochemistries (106–109)	NS5b, core, core-E1	1–6	Yes
Reverse hybridization	Amplicon detection on multiwell plates (enzyme-linked oligosorbent nucleotide assay) or microarrays (110, 111)	5′ UTR	1–6	Yes
RFLP[a]	RT-PCR with restriction enzyme digestion of amplicons; more sensitive than subtype-specific PCR (112, 113)	5′ UTR	1–6	Yes
Hetero-duplex mobility analysis	Differential electrophoretic mobility of matched versus mismatched duplexes (114, 115)	5′ UTR, NS5b	1–4, 6	Yes
PSEA[b] or PSMEA[c]	Fluorescent PCR products of different lengths detected on DNA sequencer; highly sensitive detection of mixed genotypes [level of minority genotypes detected by PSEA, ~3%; PSMEA ~1% (116, 117)]	5′ UTR	PSEA, Gt1a,b, 2a/c, 2b, 3, 4; PSMEA, Gt1a,b, 2a,b, 3a,b, 4, 6a	Partial
Subtype specific PCR	PCR with subtype-specific primers; amplicons of different size detected by gel electrophoresis (118, 119)	Core, NS5b	Gt1a,b, 2a,b, 3a, b, 4, 5a, 6a	Yes
Real-time PCR	TaqMan or FRET chemistries with specific probes or melt curve analysis (120–124)	5′ UTR	1–4	Yes

[a]RFLP, restriction fragment length polymorphism.
[b]PSEA, primer-specific extension analysis.
[c]PSMEA, primer-specific mispair extension analysis.

to patients without this mutation (66). The prevalence of this mutation varies geographically: approximately 10% of Gt1a infections in South America, 20% in Europe, and almost 50% in North America (67). Prescribing information for simeprevir therefore recommends that individuals with Gt1a infections be further tested for Q80K to determine suitability for treatment with regimens containing this protease inhibitor. Q80K resistance mutation detection is performed by direct sequencing and is available through specialty reference laboratories. The need for the development of Q80K detection tests for more general use is unclear; the role of this mutation in treatment failure with simeprevir + sofosbuvir is unclear and other combination DAA regimens that do not contain simeprevir are available (Table 1).

BLOOD SCREENING TESTS

HCV is one of many viruses that can be transmitted through blood transfusion. A safe blood supply is essential to prevent this mode of transmission. After the discovery of HCV, serologic assays were the traditional method for testing donated blood. However, NATs have become a new gold standard for blood screening. Their efficacy is evidenced by shortening of the estimated window period from 51 days by serology to 7.4 days by NAT (68). In the first decade of screening NAT use, the yield rate for HCV among donations to the American Red Cross was 1:270,000, approximately 10-fold higher than the yield rate for HIV (1:2,060,000 donations), reflecting the relative prevalence of these two infections (69). The residual risk of HCV is calculated to be 1 per 1,148,577 allogeneic blood donations (69).

Commercial NATs to detect HCV in donated blood were first developed in a singleplex format and have evolved into multiplex tests to detect HCV, HIV, and hepatitis B virus (Table 10). The indications for use of most tests are to screen blood from donors of whole blood, blood components, and source plasma; organ and tissue donors (including cadaveric and beating heart donors); donors of lymphocytes for infusion; and stem cell/progenitor cell donors. Samples for all indications except organ and tissue donations can be screened individually or as pools whose size is test- and donation-specific. Samples from organ and tissue donors must be tested individually. When mini-pools are tested, positive pools are resolved by retesting pool components individually. Virus(es) present in individual samples are then identified by subsequent testing with three different discriminatory NATs (one each for HCV, HIV, and HBV). Ultrio, Ultrio Plus, and cobas TaqScreen MPX have been shown to perform equivalently in screening for HCV from known seropositive samples and in seronegative samples collected during acute infection (70).

INTERPRETATION OF RESULTS

Interpretation of Results in Acute Hepatitis C

Establishing the diagnosis of acute hepatitis C can be challenging. Most cases of acute hepatitis C are asymptomatic; therefore serum aminotransferase elevations can be helpful if present, but should not be relied upon as the sole indicator of infection. Additionally, seroconversion can be delayed and viremia can be sporadic. Serology and RNA assessment should therefore both be performed over a 6-month period to optimally detect infection. Diagnostic accuracy also requires testing at multiple time points. Single HCV NAT results do not reliably predict exposure outcome.

HCV RNA Quantification in Chronic Hepatitis C

Reporting Quantitative Data

Linearity is one of the important parameters used to define the measurable range of a quantitative assay. With

TABLE 10 Blood screening NATs for HCV

Blood screening assay (manufacturer)	Method/ instrument	Detected analytes	Licensed sample/ minipool size	HCV limit of detection[a], IU/ml (95% confidence interval)	Comments
Ultrio (Grifols/ Hologic)	Transcription-mediated amplification/ Tigris	Multiplex screen for HCV, HIV-1, HBV	Plasma and serum/ up to 16	3.0 (2.7–3.4)	
Ultrio Plus (Grifols/ Hologic)	Transcription-mediated amplification/ Tigris	Multiplex screen for HCV, HIV-1, HBV	Plasma and serum/ up to 16	4.4 (3.7–5.6)	Sensitivity of HBV detection improved over Ultrio
Ultrio Elite (Grifols/ Hologic)	Transcription-mediated amplification/ Panther	Multiplex screen for HCV, HIV-1 (M, N, O subgroups), HIV-2, HBV	Plasma and serum/4, 8, 16	3.0 (2.5–3.9)	Not yet licensed by FDA
TaqScreen MPX (Roche)	Real-time PCR/ s201	HCV, HIV-1 (M and O subgroups), HIV-2, HBV	Plasma/IDT, MP (up to 6 for all donations except source plasma; up to 96 for source plasma donations)	11 (7.0–21.7)	
AmpliScreen HCV v2.0 (Roche)	Conventional PCR/cobas	HCV	Plasma/up to 24 for all donations except source plasma; up to 96 for source plasma donations)	Multiprep[b], 28.8 (20.5–85.8) Standard[b], 41.9 (28.0–111.8)	Used to identify HCV in samples found to be positive by TaqScreen MPX
TaqScreen MPX 2.0 (Roche)	Real-time PCR/ s201	HCV, HIV-1 (M and O subgroups), HIV-2, HBV	Plasma/up to 6 for all donations except source plasma; up to 96 for source plasma donations)	~6.8 (5.8–8.3)	Simulataneously detects and discriminates HCV/ HIV/HBV; detects but does not discriminate between HIV-1 (M and O) and HIV-2

[a]Data from package inserts.
[b]Multiprep protocol: virus concentrated by centrifuging plasma prior to nucleic acid extraction; standard protocol: nucleic acid extracted directly from plasma.

currently available quantitative tests, linearity can only be achieved using logarithmically transformed (base 10) data. HCV RNA levels should therefore be reported and interpreted in $\log_{10}$ format. This requirement is fairly unique to viral load measurement and is difficult to grasp by care providers since most quantitative interpretations are performed with arithmetic data. Laboratorians should nonetheless strive to educate care providers regarding the need for logarithmic interpretation, to faciliate proper patient management. Quantitative data and assay precision can be reported in logarithmic and arithmetic formats to assist with this educational effort.

Sources of Variability in HCV RNA Quantification

Despite the implementation of an international standard for calibration of quantitative assays, HCV RNA measurement is not equivalent between different assays (71–73). The factors that contribute to interassay quantification variability are complex. The WHO international standard and the properties of individual assays, such as inherent genotype bias, are likely contributors. The development of a WHO standard that functions as a common calibrator has been extremely useful in improving the comparability of different assays. However, it is an imperfect calibrator from the perspective of physical standardization since it is a single genotype (Gt1a) and its value represents a consensus concentration obtained by diverse assays in numerous laboratories rather than a concentration that is traceable to a physically quantifiable substance such as phosphorus content.

Sequence diversity between HCV genotypes can also affect HCV RNA quantification, resulting in genotype-dependent quantitative bias. For example, assays based on primers and probes that have varying, genotype-dependent degrees of complementarity may favor the quantification of one genotype over another, or extraction methods may favor the amplification of certain genotypes. Evidence of such genotypic bias in quantification has been reported and has been serious enough to require assay reformulation. Genotype-dependent quantification variability has been reported in multiple studies comparing the performance of different quantitative tests (51, 74–78).

Interpreting Changes in Viremia

Changes in viremia must be interpreted in the context of assay precision and variability in viremia over time in untreated chronic infection. Using a quantitative assay with excellent precision, viral load was observed to vary up to 10-fold in most individuals with stable, untreated chronic hepatitis C (21). During treatment, a viral load change of at least this magnitude must therefore occur in order to be biologically significant.

Using HCV RNA Quantification To Define Therapeutic Response

Determination of response to treatment has evolved over time as more is understood regarding response to a given regimen, as more sensitive tests with broad measurable ranges have become available, and as these newer tests are incorporated into clinical trials of new drugs. Testing during treatment became an essential feature of treatment with pegylated interferon alfa/ribavirin as clinical studies determined that response guided therapy (successfully shortening treatment in Gt1-infected patients who responded rapidly) and futility rules (early treatment discontinuation when certain quantitative milestones were not achieved) were effective in therapeutic management. Phase III clinical trials of first-generation DAAs defined response guided therapy criteria and futility rules that were unique to each drug. Testing during treatment was therefore an essential management tool as soon as these drugs were approved. In the future, the need for on-treatment testing is likely to be highly variable and drug-dependent. For the new, highly potent next-generation DAAs, testing during treatment is becoming less necessary as inhibition of replication and virus elimination are rapid. For example, directions for simeprevir use include stopping rules but not response-guided therapy and no testing is indicated in sofosbuvir prescribing information. Prescribing information should be consulted to determine the need for testing during treatment with future drug regimens. If on-treatment testing is applicable, the assay used to determine response during clinical trials should be noted, performance should be compared to the laboratory's test of record, and interpretive guidance should be provided if differences exist. Such appraisals are necessary because disparate results can be obtained with tests that vary in performance, which can lead to inappropriate management decisions. Clinical samples with low HCV RNA concentrations near the limits of detection can be particularly problematic since assays that have comparable analytical sensitivity can have differing sensitivity in clinical samples that manifests as variable HCV RNA detectability (79). For example, thresholds and definitions of rapid response to pegylated interferon alfa/ribavirin and to first-generation DAA regimens were defined with one real-time PCR test; a significant proportion of subjects who met qualifications for shortened therapy during the trials failed to qualify when on-treatment samples were retested retrospectively with a different, more sensitive, assay (80, 81). Interestingly, almost all of these subjects attained SVR after shortened therapy, as dictated by initial testing, demonstrating that individual tests can greatly impact management.

Definitions of end-of-treatment response and SVR have also changed over time, according to drug regimen and available tests. For pegylated interferon alfa/ribavirin, conventional qualitative PCR tests with a limit of detection of ≤50 IU/ml were suitable to determine treatment efficacy; end-of-treatment response and SVR were defined as "HCV RNA undetectable" (<50 IU/ml). Many DAA clinical trials were performed exclusively with one real-time PCR test, the cobas TaqMan assay using High Pure extraction. This test has a limit of detection that is less than its lower limit of quantification (Table 7). For first-generation DAAs, responses at end of treatment and SVR were defined as "HCV RNA <25 IU/ml," or less than the lower limit of quantification of the monitoring assay. Reanalysis of several early trials of first-generation DAAs demonstrated higher cure rates among individuals who had undetectable HCV RNA (<25 IU/ml, HCV RNA not detected) at key decision points in treatment, compared to those with low levels of HCV RNA (<25 IU/ml, HCV RNA detected), suggesting that distinguishing between HCV RNA detected and not detected was clinically important (82). For this reason, "<25 IU/ml, HCV RNA undetected" is now used to define end-of-treatment response and SVR.

HCV NAT QUALITY ASSURANCE (QA)

Preanalytical QA Parameters

Specimen collection, handling, transport, and storage parameters are part of the preanalytic phase. In most cases, plasma is used for HCV NATs; serum may also be an option, depending upon the test. Maximum elapsed time between blood collection and plasma processing and acceptable storage conditions of processed specimens can vary between assays. Package inserts should therefore be followed to ensure accurate results. Specimens should be rejected if (i) they are unlabeled or mislabeled, (ii) they are collected in improper containers, or (iii) transport time has exceeded maximum limits.

Analytic Phase QA

QA programs during the analytic phase consist of selection and monitoring of the performance of controls and calibrators, preventing contamination, and proficiency testing. Quality control in the form of positive and negative controls performed on each run is required for HCV NATs. Positive and negative control materials are supplied with FDA approved assays. Negative controls should consist of test matrix without HCV RNA template present. The positive control for qualitative NATs serves as a sensitivity control for the test method. For quantitative NATs, controls at high and low ends of the measurable range should be included within each batch. Controls for genotype methods ensure accurate type and subtype determination. These controls can either be purchased from commercial sources or can be derived from archived samples that have been confirmed to contain a known concentration, genotype and subtype of HCV (if subtype has been obtained with an appropriate assay).

All quantitative assays are calibrated internally or externally as discussed above (see "HCV NAT"). Calibration accuracy should be assessed periodically regardless of the calibration method by testing samples with HCV RNA levels near the upper and lower limits of quantification as determined with a different reagent lot or quantification method. Some laboratory accreditation agencies also require a specimen of intermediate concentration. Results should fall within each assay's accepted precision or within the expected range of inter-assay variability.

Contamination prevention is an important part of quality assurance, especially for laboratories using HCV

NATs based on target amplification methods (RT-PCR and TMA) that are particularly prone to contamination with extraneous nucleic acids. Several commercial and in-house developed HCV NATS have incorporated uracil *N*-glycosylase into PCR master mixes for contamination prevention. The nucleotide triphosphate, dTTP, is replaced with dUTP during amplification. Uracil *N*-glycosylase degrades any dUTP-containing amplicon prior to amplification of true target. A contamination event may be detected by obtaining a positive signal with a negative control or with a control where no reverse transcriptase enzyme is added. Signal amplification (branched DNA) is less prone to contamination.

Internal and external proficiency testing is important for HCV NATs. HCV proficiency panels from the College of American Pathology (www.cap.org) in the United States and Quality Control for Molecular Diagnostics (www.qcmd.org) in Europe are designed for broad spectrum testing of commercial and in-house developed HCV methods. Proficiency panels for testing across HCV platforms are also available from commercial sources. In addition, test-specific proficiency panels such as the Aptima HCV proficiency panel for the Aptima HCV RNA Qualitative Assay are commercially available.

Postanalytic Phase: Accuracy of Results

Assuring accurate, reliable test results is part of the postanalytic phase of quality assurance. Observing a patient's quantitative results longitudinally for discrepancies (so-called "delta check") is a useful method for detecting errors. Delta checks can be performed for a single assay (for example a sudden positive result when previous results have been negative, or vice versa) or between assays (for example, an undetectable viral load with a positive genotype determination).

FUTURE PERSPECTIVES

Future innovation in HCV is likely to occur in the arenas of drugs and NATs. With the approval of the currently available drug regimens, the goal of highly effective, oral, interferon-free regimens has been reached. Further development is likely to center on drug combinations that are truly pan-genotypic in the sense that every genotype can be treated effectively with the same regimen.

As for NATs, the types that are required during therapeutic management may change depending upon the evolution of drug regimens. Pan-genotypic regimens could end the need for baseline genotype determination, effectively eliminating the market for genotype tests. In their place, tests to detect viral mutations that lead to antiviral resistance tests could emerge if increased antiviral resistance is observed as DAA regimens transition from use in highly selected clinical trial subjects to the broader population of chronically infected individuals.

For quantitative NATs, further improvements in performance characteristics appear to be unnecessary. The analytical sensitivity of current tests is sufficient for optimal clinical performance (clinical sensitivity and clinical specificity) in the diagnosis of acute/chronic HCV and therapeutic management of chronic infection. Instead, innovations in platform design are likely to occur. On one end of the testing spectrum, instrumentation changes will improve functionality of centralized, mid/large volume laboratories, and on the other end, they will allow for point-of-care or point-of-service testing. For centralized laboratories, sample-in/answer-out instruments with continuous feed capacity and random access to test menu will become available. The need for these will be driven by escalating test volumes as birth cohort screening recommendations are fully implemented, resulting in the identification of an increased number of chronically infected patients, and the number of chronically infected individuals requiring therapeutic management-associated monitoring expands as use of combination DAA regimens increases. Domestic and global markets for rapid, random-access single-test instruments for use at point-of-service or point-of-care are also likely to develop out of the need to bring health care to HCV-affected populations that are unlikely to reliably access conventional clinic environments. Additionally, it is possible that these instruments will be useful in filling the need for on-site testing capacity in ambulatory care clinics, to provide the type of rapid results required for securing timely insurance authorization, and thereby facilitating continuous, uninterrupted treatment. Taking all of this into consideration, the observation that the only constant is change, made by Herakleitos almost 2,500 years ago (and probably thought for eons before), is the best assessment of the current state and foreseeable future of HCV diagnostics.

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Molecular Detection and Characterization of Hepatitis B Virus

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32

Hepatitis B virus (HBV) infection is one of the most widespread infectious diseases in the world. Serological evidence suggests that up to one-third of the global population has been infected, with current estimates of up to 240 million chronic infections worldwide and more than 780,000 HBV-related deaths annually (http://www.who.int/mediacentre/factsheets/fs204/en/). Despite the availability of an effective vaccine, enhanced blood screening strategies, and new therapeutics, the burden of chronic HBV infection (CHB), including cirrhosis and hepatocellular carcinoma (HCC), continues to affect populations worldwide, especially in the developing world (1, 2).

Following the landmark discovery of the Australia antigen in 1965 (3), visualization of the Dane particle by electron microscopy in 1970 (4), and molecular cloning of the virus from HBV-infected serum in 1979 (5–7), there has been an explosion in knowledge regarding HBV, including a deeper understanding of the virus, its life cycle, and pathogenesis. While these important discoveries have resulted in the development of sensitive serologic assays, an effective vaccine, and potent antiviral therapies, clinicians have continued to rely on a combination of serologic and biochemical assays for the diagnosis of acute HBV infection (8–11). However, availability of sensitive and reliable molecular assays, including HBV DNA detection, quantification, and sequencing methods, has revolutionized the clinical management, diagnosis, and treatment of CHB.

DESCRIPTION OF THE VIRUS

Human HBV is the prototype member of the family *Hepadnaviridae*. Members of this family are small, partially double-stranded, enveloped DNA viruses that share a unique replication strategy and organ tropism (11, 12). Their genomes encode necessary viral transcripts and proteins in multiple overlapping reading frames, making them remarkably compact. Members of *Hepadnaviridae* possess a narrow host range based on initial viral attachment and entry into the host cell, and they are divided into two genera, *Orthohepadnavirus* and *Avihepadnavirus*, that infect mammals and birds, respectively.

Infectious hepatitis B virions are double-shelled particles that are 40 to 42 nm in diameter, with an outer lipoprotein envelope and an inner nucleocapsid or core (4, 13). The core contains a 3.2-kb partially double-stranded viral genome and a viral polymerase responsible for synthesis of viral DNA within the host cell (12, 13). The partially double-stranded DNA genome assumes a relaxed circular state in the mature virion (Fig. 1). It consists of a full-length negative strand covalently linked at its 5′ end to the viral DNA polymerase and a shorter positive strand of variable length with a capped oligoribonucleotide at the 5′ end (12, 13). Regions containing direct repeats (11 nucleotides) are located near the 5′ ends of both the negative and positive strands (12, 14).

Four partially overlapping open reading frames (ORFs) of the genes *pre-C*/C, *pre-S*/S, *P*, and X are located on the negative strand, with four promoters initiating transcription of these regions and leading to the translation of seven viral proteins. Two nucleocapsid core proteins, hepatitis B core antigen (HBcAg) and hepatitis B e antigen (HBeAg), are encoded by the pre-C/C ORF. The pre-S/S ORF encodes three viral surface antigens, including a 24-kDa protein designated as hepatitis B surface antigen (HBsAg), while ORF P encodes the viral DNA polymerase. ORF X encodes the X protein (HBx), which has been characterized as an essential transcriptional transactivator (11, 12, 14). Of note, ORF S encoding HBsAg and ORF P encoding the polymerase share a significant overlap but differ in reading frames. This organization has important consequences for mutational selection and maintenance of these proteins, including the YMDD motif, an important mutational site located in the coding region of the RNA-dependent viral DNA polymerase that confers resistance to nucleos(t)ide analogs (15). From patient care and diagnostic laboratory perspectives, the most relevant viral proteins are HBsAg, HBeAg, and HBcAg.

The viral replication cycle begins with the attachment of HBV virions to host cell surface receptors, followed by endocytosis, uncoating, and release of the viral capsids into the cytoplasm (Fig. 2). However, these early events of the viral life cycle are not well understood in part because of a lack of cell lines that are susceptible to hepadnavirus infection (16). Following translocation to the nucleus of the host cell, viral capsids dissociate and relaxed circular DNA (rcDNA) is transformed into covalently closed circular DNA (cccDNA) by host cell repair enzymes. This process involves four major steps: (i) removal of polymerase from the 5′ end of the negative strand; (ii) extension of the positive strand to full length; (iii) ribonucleotide removal from

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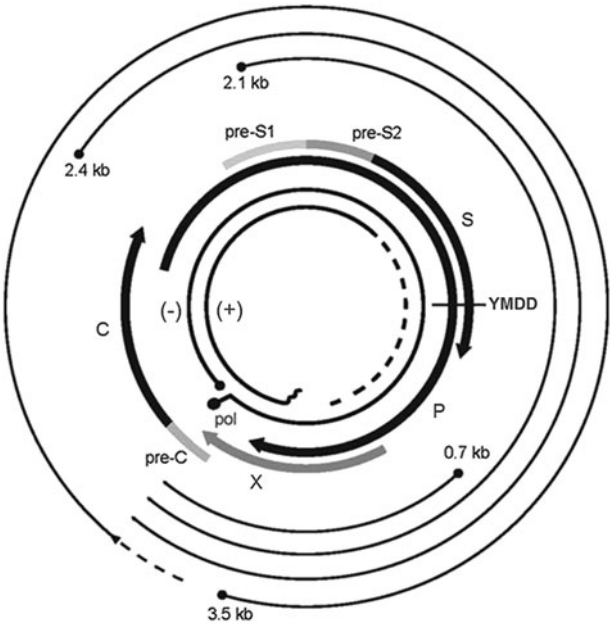

FIGURE 1 Organization of the HBV genome. The inner circles depict the plus (+) and minus (−) strands of the partially double-stranded DNA genome (3.2 kb). The overlapping ORFs pre-C/C, pre-S/S, P, and X encoded by the genome are represented by the bold arrows. Outer circles indicate the lengths of transcribed viral RNAs (3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb). The approximate location of the YMDD motif located in the reverse transcriptase domain of ORF P is also shown.

the 5′ end of the positive strand; and (iv) ligation of both the positive and negative strands followed by supercoiling of the cccDNA (12, 16). Multiple copies of cccDNA are maintained within the nucleus of the host cell as minichromosomes containing histone and nonhistone proteins, and they serve as templates for the transcription of pregenomic RNA (pgRNA) and subgenomic RNA (3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb in length) by host RNA polymerase II (17). This pool of cccDNA can also play a critical role in the persistence of drug-resistant virus during antiviral treatment by serving as a reservoir for mutant HBV (18).

Translation of viral proteins occurs in the cytoplasm following transport of viral RNA from the nucleus to the cytoplasm. In addition to serving as a template for viral genomic replication, the 3.5-kb pgRNA serves in translation of *pre-C* (HBeAg), C (HBcAg), and *P* genes. While the 2.4-kb transcript encodes L (pre-S1), M (pre-S2), and S (HBsAg) proteins, the 2.1-kb transcript encodes pre-S2 and S proteins, and the 0.7-kb transcript encodes the transcriptional transactivator X (HBx) protein.

Following encapsidation of a single copy of the 3.5-kb pgRNA along with viral polymerase, synthesis of a new viral genome occurs via reverse transcription (viral polymerase). After synthesis of the negative strand is complete, the pgRNA template is degraded, and synthesis of the positive strand occurs based on the template created by the newly synthesized negative-strand DNA. Mature viral cores either (i) bud into the endoplasmic reticulum, acquire their lipoprotein envelope (containing L, M and S proteins), and are transported to the cell surface for release; or (ii) they are directed back to the nucleus to maintain the pool of cccDNA. In addition to release of infectious viral particles, two distinct subviral particles (22-nm-diameter spheres and tubular forms) consisting of envelope glycoproteins and host lipids (HBsAg) and devoid of HBV DNA are produced at concentrations 10^3- to 10^4-fold higher than infectious virions (11, 13).

The replication cycle of HBV is unique among DNA viruses infecting humans. Unlike HIV, HBV does not require integration into the host cell genome, but its replication involves an RNA intermediate and reverse transcription step (19). This similarity between HBV and HIV replication has led to the development of effective HBV treatment strategies based on the use of nucleos(t)ide analogs (8–10). Despite the fact that HBV DNA integration is not an essential component of viral replication and that the multifactorial causes of HCC among HBV-infected individuals are not fully understood, HBV integration has been documented in a majority (80 to 90%) of HBV-infected patients with HCC and may play a role in carcinogenesis (14, 20, 21).

NATURAL HISTORY

HBV is highly infectious and can easily be transmitted through multiple routes of exposure, including perinatal, sexual, and percutaneous routes as well as other forms of mucosal contact with infected blood or body fluids. Once infected, an individual's age and immunological maturity at the time of infection become critical factors in determining the clinical course of disease (22). In the developed world, most HBV infections are acquired through sexual contact or intravenous drug use, and progression to CHB is rarely observed among immunocompetent individuals in these settings. However, in high-prevalence regions of the world such as China and parts of Southeast Asia, infection is typically acquired through perinatal transmission or as a result of close person-to-person contact at an early age. In this setting, HBV infection is associated with a much greater risk for progression to CHB. Although HBV causes both acute and chronic infections, CHB is overwhelmingly responsible for the most serious disease manifestations, including cirrhosis and HCC.

Due to the dynamic nature of HBV infection, a disease model consisting of various phases of HBV infection has been described and has become widely accepted (23). While it should be noted that not all patients progress through all four recognized clinical phases as described in this model, the approach provides a basic framework for the understanding of CHB disease progression and its associated clinical and histopathological changes. The phases include immune tolerant, immune clearance, inactive carrier, and HBeAg-negative CHB (Fig. 3). Despite some minor differences in nomenclature, this basic paradigm is used in current clinical practice guidelines for CHB (8–10).

The immune-tolerant phase typically occurs more frequently and is typically of prolonged duration among perinatally infected individuals or those persons infected in the first years of life, before the immune system is mature. This phase of disease is characterized by detectable HBsAg and HBeAg, accompanied by high levels of HBV replication (i.e., HBV DNA levels >20,000 IU/ml). Despite normal aminotransaminase levels, mild necroinflammation, and minimal or no evidence of fibrosis, these individuals can be highly contagious because of their high-level viremia. This phase is further characterized by its low rate of anti-HBe seroconversion along with a low incidence of

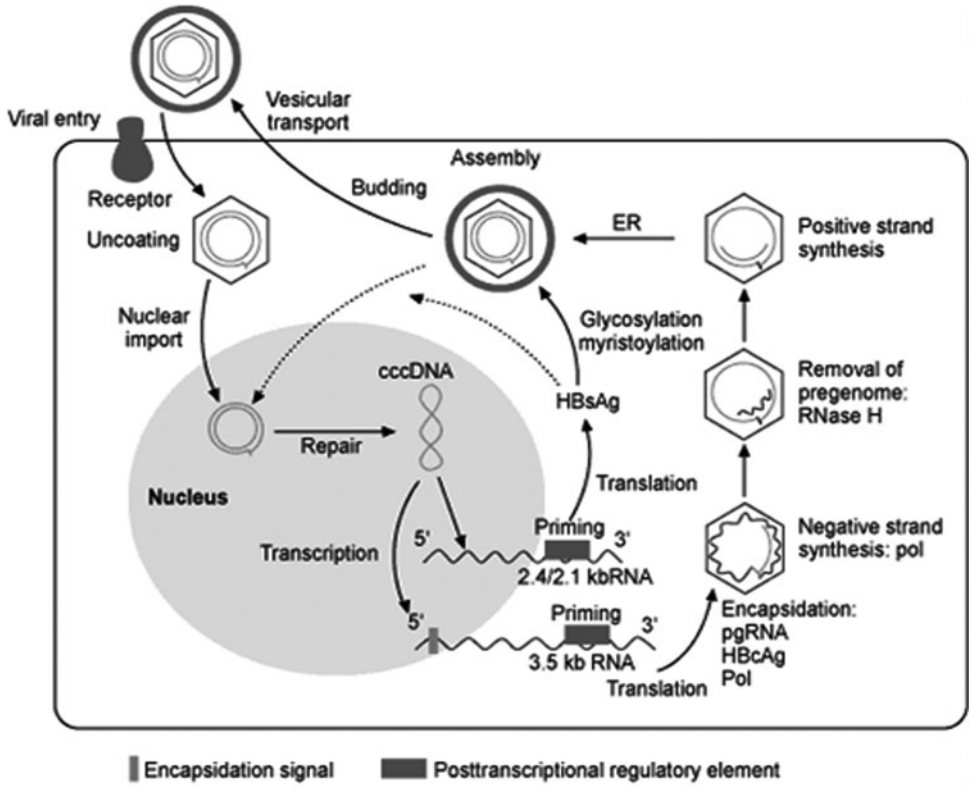

FIGURE 2 Replication cycle of HBV. Following entry of the virion into the host hepatocyte via receptor-mediated endocytosis and shedding of the envelope, viral DNA is translocated to the nucleus where it is processed to produce cccDNA, which serves as a template for the transcription of pgRNA and subgenomic RNAs (3.5 kb, 2.4 kb, 2.1 kb, and 0.7 kb). These viral RNAs exit the nucleus and are translated to produce viral proteins. The pgRNA serves as a template for reverse transcription to negative-strand DNA, which in turn serves as a template for positive-strand DNA synthesis. Newly synthesized genomic DNA is either packaged into complete virions for eventual release or directed back to the nucleus to maintain the cccDNA pool. Reprinted from Buti (128) with the permission of the publisher (© Clinical Care Options, LLC, 2009).

cirrhosis and HCC (24, 25). In the case of perinatal infection, this phase can last for decades.

The immune-clearance or immune-active phase signals an increased immune-mediated cytotoxic response to HBV-infected hepatocytes. It is generally characterized by HBeAg positivity, variable HBV DNA levels in the blood, fluctuating aminotransaminase levels, and moderate to severe necroinflammation with increased progression toward fibrosis. While the intensity and duration of the inflammatory response are variable, the resulting hepatic damage typically manifests itself in an increased potential for cirrhosis and HCC (26). This inflammatory phase may last from several weeks to several years and typically culminates in anti-HBe seroconversion.

The immune control phase, sometimes referred to as the inactive carrier state, is characterized by the absence of HBeAg and the appearance of circulating HBe antibody. Most individuals in this phase have normal aminotransferase levels and very low (i.e., <2,000 IU/ml) or undetectable HBV DNA levels. Some individuals may have higher levels of HBV DNA, but these levels typically remain below 20,000 IU/ml. Immunological control of the virus during this inactive carrier state is accompanied by mild necroinflammation, a decrease in the progression toward fibrosis, and reduced potential for development of cirrhosis and HCC. If the intensity of the inflammatory response was severe during immune clearance, evidence of inactive cirrhosis may be present. The long-term outcome for individuals in this phase is generally favorable, with up to 70% of individuals maintaining this inactive state, despite detectable HBV DNA being present in many individuals, and the anti-HBs seroconversion rate is just 1 to 2% annually (27, 28). Progression to HBeAg-negative CHB can also occur among individuals after years or even decades in this phase.

The final phase, immune escape or HBeAg-negative CHB, is characterized by the lack of detectable HBeAg despite the presence of detectable HBV DNA in blood. This phase of CHB can immediately follow spontaneous seroconversion occurring during the immune clearance phase, or it can arise after a prolonged period of immune control (27, 29). This disease state results from (i) mutations (A1762T and G1764A) in the replicating HBV basal core promoter (BCP) that can down-regulate production of HBeAg (30) and/or (ii) a precore mutation (G1896A) that results in a stop codon at codon position 28 (31). During this phase, episodic reactivation of the immune response can result in fluctuating aminotransferase and HBV

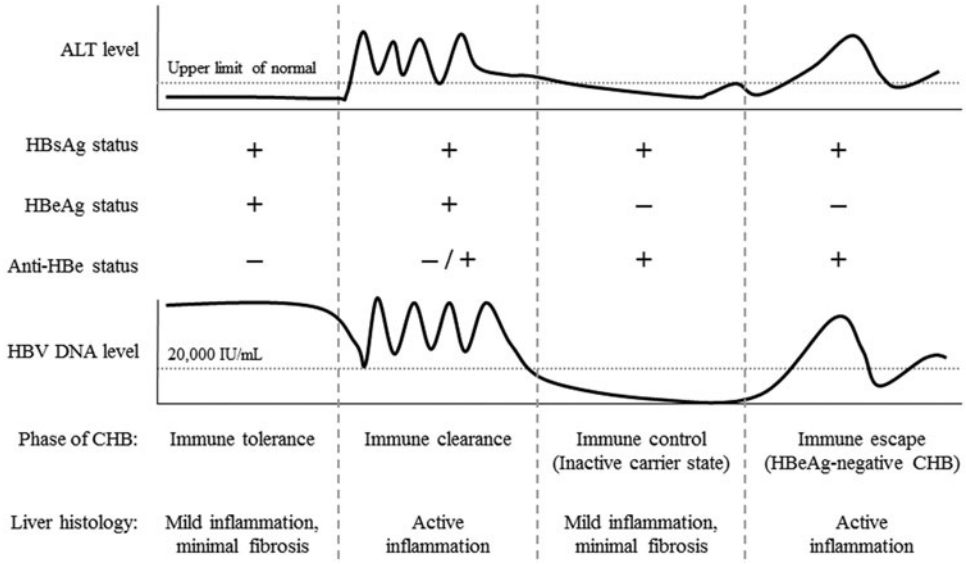

FIGURE 3 Natural history of CHB. The various recognized phases of HBV infection are depicted along with associated histopathological features, HBV serologic status, and relative HBV DNA levels.

DNA levels accompanied by active necroinflammation and an increased risk of disease progression.

Although "occult HBV" is not part of the four-phase disease model, it is a widely accepted condition in which HBsAg-negative individuals with or without HBc and HBs antibodies exhibit evidence of low-level HBV replication (32, 33). This condition appears to be more prevalent in areas of HBV endemicity and high-risk populations and in individuals with evidence of HBc antibody (32, 34, 35). The long-term outcome of occult HBV infection remains unclear, but individuals with advanced CHB prior to loss of HBsAg may remain at increased risk for development of HCC (36–38). Occult HBV is typically confirmed through the detection of low levels of HBV DNA in the blood or liver by sensitive PCR-based methods. However, current clinical practice guidelines for CHB do not specifically address the diagnosis and treatment of occult HBV (8–10).

CLINICAL APPLICATIONS

Although HBV causes both acute and chronic infection, the overwhelming burden of serious HBV-related disease is due to CHB, with its attendant risks of cirrhosis and HCC (39, 40). Although acute hepatitis B may cause serious transient illness in adults, this phase of infection is rarely fatal, and excellent laboratory tools are available for its diagnosis and management (41). However, except for screening of blood donors and resolution of atypical serologic patterns, molecular tests are not routinely used for the diagnosis or management of acute hepatitis B. Therefore, the focus of molecular testing is the characterization, treatment, and management of CHB, understanding that laboratory testing relies on an integrative use and interpretation of both serologic and molecular test results (42).

Among their many roles, serologic markers are used to establish the diagnosis of both acute and chronic hepatitis B, monitor the course of infection, and determine immune status. Compared to antigen-based detection methods for other viruses, serologic detection of HBV is relatively sensitive due to overexpression of HBsAg as noninfectious subviral particles in plasma and serum. Because its expression is variable, HBsAg is not a reliable indicator of viral replication. Therefore, HBeAg in plasma or serum is used clinically as a marker for viral replication. However, this viral protein is not a structural component of the virion, and it is also present in a variable ratio to infectious particles. Moreover, HBeAg may be undetectable serologically in patients with ongoing infection by virus with mutations in the core or precore regions of the HBV genome (43, 44). The propensity of HBV to mutate may also confound serologic detection. Mutations within the protective "a'" epitope of HBsAg arise under the selection of natural infection, repeated administration of hepatitis B immune globulin, or vaccination, leading to detection failure with many commercially available HBsAg assays (45, 46). Quantitative HBsAg and HBeAg measurements have been advocated for monitoring progression of CHB and response to antiviral therapy (47, 48). However, these quantitative assays are not widely available, and the test results have not been calibrated to the respective World Health Organization (WHO) international standards for these two viral markers. Their use in managing patients with CHB also has not been validated in large clinical trials of antiviral agents for CHB.

The rationale and advantages for molecular testing derive from its capabilities for sensitive detection, accurate quantification of infectious viral particles, and the usefulness of HBV genome sequence information for phylogenetic genotyping and prediction of antiviral drug resistance. While the early laboratory tests for HBV DNA were relatively insensitive with limited quantification ranges, availability of new molecular methods has dramatically improved the analytical sensitivity and quantification range of these assays. These new molecular diagnostic assays play a critical role in our understanding of the biology of CHB during each phase of the disease (Fig. 3).

Qualitative Molecular Testing

Prior to the availability of qualitative nucleic acid amplification tests (NATs) for detection of HBV, the prevention

of transfusion-associated HBV infection mainly relied on the use of highly sensitive HBsAg serologic screening assays. The success of NATs for screening HIV and HCV has been the major impetus for the development and application of such molecular screening tests for HBV in blood donors. According to the WHO, availability of automated and semiautomated instrument platforms developed for use with current NATs has resulted in 100% of the blood supply in U.S. and Western countries now being screened for HBV DNA (http://www.who.int/worldblooddonorday/media/who_blood_safety_factsheet_2011.pdf).

However, the complexity and cost of NATs favor the use of pooled samples for testing, instead of individual donor testing, thereby reducing the effective sensitivity of NATs. In contrast, serologic testing for HBsAg and hepatitis B core total antibodies (anti-HBc total) is performed without pooling of samples. Due to this concern of potentially false-negative NAT results in pooled sample testing, serologic testing for HBsAg and anti-HBc total continues to be part of the blood donor screening tests today (49). Nondonor NATs with high sensitivity have been developed by clinical laboratories as well as various commercial assay manufacturers for diagnostic use purposes, especially to resolve discordant serologic test results. However, use of these qualitative assays has been supplanted now by highly sensitive HBV DNA quantification assays.

Quantitative Molecular Testing

As an indirect marker for viral replication in patients with CHB, HBV viral load in plasma or serum plays a key role in patient management during all stages of the disease. Individuals with low but persistent HBV viral loads remain at risk for development of HCC and cirrhosis, and increasing levels of viremia have been associated with this increased risk (50, 51). HBV DNA levels provide the best overall assessment of disease prognosis, including monitoring progression to cirrhosis and HCC (50, 52–55). Since quantitative molecular methods such as real-time PCR provide the most accurate quantitative measurement of infectious viral particles, they have also become the basis for assessing viral burden during treatment.

Criteria for establishing the diagnosis of CHB include persistence of HBsAg for more than 6 months, persistence or intermittent elevation of alanine aminotransferase and aspartate transaminase levels in serum, biopsy evidence for hepatic necroinflammation, and elevated HBV DNA levels in plasma or serum (8–10, 56). Depending on the phase of disease, considerable variation is observed in various laboratory markers, including HBV DNA level. The diagnostic threshold of ≥20,000 IU/ml for HBeAg-positive CHB in current practice guidelines reflects the lower limits of quantification for early-generation qualitative assays. Patients with HBeAg-negative CHB generally have lower DNA levels (2,000 to 20,000 IU/ml), associated with presence of HBV basal core promoter and precore mutations. In both patient groups, viral load serves as the only direct marker of viral replication. The value of using viral load thresholds to discriminate between active versus inactive HBV disease has been confirmed by using quantitative assays that are more sensitive (57, 58). However, individual and population variations significantly complicate the assessment of viral load associations. HBV DNA testing with highly sensitive viral load assays is integral to detection of occult HBV infection (59, 60) and has also revealed the occurrence of low-level viremia in patients with cirrhosis and HCC.

HBV viral load in plasma or serum is the primary tool used to monitor patients during antiviral therapy, with the goal of eliminating or suppressing viral replication (8–10, 56). Since durable seroconversion from HBsAg-positive to HBsAg-negative status occurs in only a minority of patients treated with interferon, and even fewer patients treated with nucleos(t)ide analogs, HBV viral load is the only reliable marker for assessing response to antiviral treatment. Both the absence of a rapid drop in viral load following initiation of therapy and persistent viremia are predictors for emergence of antiviral resistance. Current clinical practice guidelines recommend monitoring HBV viral load every 3 to 6 months while on antiviral therapy for CHB, with different monitoring and treatment endpoints for HBeAg-positive and HBeAg-negative patients treated with nucleos(t)ide analogs (8–10, 56).

Genotyping

HBeAg-negative CHB is characterized by a lack of detectable HBeAg despite the presence of detectable HBV DNA in blood. This disease state results from mutations (A1762T and G1764A) in the replicating HBV basal core promoter that can down-regulate production of HBeAg (30), and/or a precore mutation (G1896A) that results in a stop codon at codon position 28 (31). During this phase of CHB, episodic reactivation of the immune response can result in fluctuating serum aminotransferase and HBV DNA levels accompanied by active necroinflammation and an increased risk of disease progression. The prevalence of these mutations can vary by geographic region, with the basal core promoter mutation more common in Asia (77%) and the stop codon at position 28 most commonly found in the Mediterranean region (92%) (61). Despite their potential clinical significance, routine testing for these mutations is not recommended in current clinical practice guidelines.

Eight HBV phylogenetic genotypes (A to H) are recognized and defined currently by intergroup sequence divergence of >8%, with prevalence of individual genotypes varying among worldwide geographic regions. Data suggest that HBV genotype may influence disease outcome and treatment success. In Asia, genotype C has been associated with a higher risk of reactivation and disease progression than genotype B (62, 63). Other studies related to disease outcome have associated the stop codon mutation at precore codon 28 with stabilization of a stem loop structure found in sequences of HBV genotype B, D, E, G, and some strains of C (64). Several studies have also shown higher rates of HBeAg seroconversion in response to interferon alpha therapy for CHB due to genotypes A and B compared to genotypes C and D (65–68). Despite such evidence suggesting potential clinical utility of HBV genotype determination, the role of HBV genotype in predicting response to antiviral therapy and clinical outcomes remains unclear. Current clinical practice guidelines do not recommend routine HBV genotype determination in the management of patients with CHB.

Selective pressure from active or passive immunization can lead to mutations in the HBV surface antigen gene, which can in turn result in altered binding affinity for neutralizing antibodies (anti-HBs). Emergence of these HBV immune-escape mutants has been described both in infants born to HBV-infected mothers and in liver transplant recipients who have received long-term treatment with HBV immune globulin to prevent HBV reinfection of the graft (69–73). While the occurrence of these HBV

immune-escape mutants remains relatively low in infants born to HBV-infected mothers in regions of endemicity, the occurrence can be as high as 40% among liver transplant recipients receiving HBV immune globulin (74). Despite additional concerns that mutations arising from immune selection or nucleos(t)ide analog therapy could confound HBsAg detection assays or effectiveness of antiviral therapy due to the overlap in the HBV surface antigen and polymerase genes, these concerns do not play a clinically significant role at present.

Drug Resistance Testing

Current antiviral therapy for CHB aims to permanently suppress viral replication; reduce inflammation to delay or prevent progressive fibrosis, liver failure, and development of HCC; and prolong patient survival. Despite such long-term outcome objectives, treatment typically focuses on near-term goals such as reductions in HBV DNA and aminotransferase levels, HBeAg seroconversion (HBe-positive patients), HBsAg seroconversion (HBe-negative patients), and improvements in hepatic inflammation and histology (10). Treatment efficacy is commonly monitored through the use of HBV viral load, HBeAg, and HBsAg as surrogate laboratory markers of success of antiviral therapy. Although viral breakthrough often results from a patient's noncompliance with antiviral therapy, HBV is prone to develop adaptive mutations in its genome resulting in reduced susceptibility of the virus to the antiviral agents during treatment, mainly because of its typically high rate of replication ($>10^{11}$ viral particles per day) and the error-prone reverse transcription process in its replication cycle (75, 76).

As of 2015, seven therapeutic agents are approved for antiviral therapy of CHB in the United States. They include the immunostimulatory agents interferon alpha and pegylated interferon alpha-2a, and the nucleos(t)ide analogs lamivudine, adefovir, entecavir, telbivudine, and tenofovir. In addition, the L-nucleoside analog emtricitabine, approved for antiretroviral therapy, has activity against HBV and is used off-label in combination with tenofovir (Truvada) for treatment of CHB. These nucleos(t)ide analogs can be classified structurally into three groups: L-nucleosides, acyclic phosphonates, and D-cyclopentanes (Table 1). These analogs exert their antiviral activity by inhibiting HBV polymerase. Although interferons have both direct antiviral activity and immunostimulatory effects, their exact mechanisms of antiviral action remain poorly understood. Overall efficacy of these antiviral agents, as measured by surrogate markers, liver histology, and durability of therapeutic response in various patient populations, is summarized in current clinical practice guidelines (8–10, 56).

Resistance to nucleos(t)ide analogs arises from mutations in one or more domains of the gene encoding the HBV polymerase, and rates of resistance vary among the different drug classes (77). In general, antiviral resistance in HBV occurs with prior use of low-potency drugs with low barriers to resistance. In recognition of the advantages of potent antiviral agents with high genetic barriers to development of drug resistance, entecavir and tenofovir are recommended now in all current clinical practice guidelines as first-line monotherapy for long-term treatment of CHB (8–10, 56). Amino acid substitutions and major mutational pathways associated with resistance to nucleos(t)ide analogs are summarized in Table 1.

AVAILABLE LABORATORY ASSAYS

Numerous commercially available molecular assays for the detection and characterization of HBV have been developed and are now widely used in clinical practice worldwide. In particular, these recent advances in technology have produced a number of highly automated instrument systems for the sensitive detection and standardized quantification of HBV DNA in serum and plasma. Additional assays also have been developed for the detection of mutations in the basal core promotor and precore regions, phylogenetic genotype assignment, and detection of mutations associated with resistance to nucleos(t)ide analogs, and they are available in convenient formats suitable for use in clinical laboratories. Finally, specialized assays designed to quantify cccDNA in liver tissue for the study of viral replication and persistence as well as evaluation of the effectiveness of new antiviral therapies have been described, but these investigational assays are not currently commercially available (78).

Qualitative Molecular Tests

Whether using individual or pooled samples, the benefits of blood and organ donor screening for HIV-1 RNA and HCV RNA by NATs are widely recognized. However, the potential advantages of HBV screening by NATs in

TABLE 1 Antiviral drug classes and antiviral resistance in HBV[a]

Drug class	Amino acid substitution(s)	Susceptibility to antiviral agent				
		LAM	LdT	ETV	ADV	TDF
L-Nucleoside (LAM, LdT)	M204I	R	R	I	I	S
	L180M + M204V	R	R	I	I	S
Acyclic phosphonate (ADV, TDF)	A181T/V	R	R	S	R	I
	N236T	S	S	S	R	I
	A181T/V + N236T	R	R	S	R	R
D-Cyclopentane (ETV)	L180M + M204V/I ± I169T ± V173L ± M250V	R	R	R	S	S
	L180M + M204V/I ± T184G ± S202I/G	R	R	R	S	S

[a]LAM, lamivudine; LdT, telbivudine; ETV, entecavir; ADV, adefovir dipivoxil; TDF, tenofovir disoproxil fumarate; I, intermediate; R, resistant; S, sensitive. Adapted from EASL (8) and APASL (9) CHB clinical practice guidelines.

TABLE 2 Select commercially available HBV assays for screening of donors of blood, human cell, tissue, and tissue products

Manufacturer	Assay method	Assay name	Regulatory status[a]	Limit of detection (IU/ml)[b]
Grifols, S.A.	Transcription-mediated amplification	Procleix Ultrio Assay	CE-IVD, US-IVD	10.4
		Procleix Ultrio Plus Assay	CE-IVD, US-IVD	3.4
		Procleix Ultrio Elite Assay	CE-IVD	4.3
Roche Molecular Systems, Inc.	End-point PCR	cobas AmpliScreen HBV Test	CE-IVD, US-IVD	4.4
	Real-time PCR	cobas TaqScreen MPX Test	CE-IVD, US-IVD	3.8
		cobas TaqScreen MPX Test, v2.0	CE-IVD, US-IVD	2.3
		cobas MPX (for use on the cobas 6800/8800 Systems)	CE-IVD	1.4

[a]CE-IVD, Conformité Européenne-marked; US-IVD, U.S. FDA approved.
[b]All values are based on information contained in the manufacturer's product insert for individual donor screening.

addition to screening for serological markers have been somewhat more controversial (49). Despite this debate, assay manufacturers have included HBV in their most recently developed, automated, multiplex NAT assays, and recent studies have shown the value of routine NATs for HBV (79–81). The detection limits of these various NAT assays (based on testing of individual donor samples) along with their regulatory status (e.g., CE-marked, FDA-approved) are presented in Table 2.

The Procleix Ultrio and Ultrio Plus Assays (Grifols, S.A.) utilize transcription-mediated amplification for detection of HIV-1 RNA, HCV RNA, and HBV DNA in donor serum or plasma. While testing with the Procleix Ultrio Assay is performed using a semiautomated assay format (Procleix system), the Procleix Ultrio Plus Assay also can be fully automated by the Procleix Tigris System (Procleix Ultrio Assay, package insert rev. A, 02/2012, Gen-Probe Inc.; Procleix Ultrio Plus Assay, package insert rev. A, 07/2012, Gen-Probe Inc.).

More recently, the Ultrio Elite Assay has been developed for detection of HIV-2 RNA in addition to HIV-1 RNA, HCV RNA, and HBV DNA in donor serum or plasma, and it has been designed for use with the automated Procleix Panther System (Procleix Ultrio Elite Assay, package insert rev. 001, 01/2014, Gen-Probe Inc.). With the exception of HIV-1 and HIV-2, supplemental discriminatory testing can be performed to identify the specific viral target contained in individual samples initially generating reactive results. Donor testing can be performed with individual samples or pools of up to 16 samples.

The Cobas AmpliScreen HBV Test (Roche Molecular Systems, Inc.), which received approval from the FDA in 2005, is based on end-point PCR and is intended only to be used for the qualitative detection of HBV DNA in donor plasma. The assay procedure is semiautomated through the use of the Cobas Amplicor Analyzer, and donor testing can be performed with individual samples or pools of up to 24 samples (Cobas AmpliScreen HBV Test, package insert rev. 7, 01/2010, Roche Molecular Systems, Inc.).

The real-time PCR-based cobas TaqScreen MPX Test and cobas TaqScreen MPX Test, version 2.0 (Roche Molecular Systems, Inc.), are fully automated multiplex assays designed for the simultaneous amplification and detection of HIV-1 RNA, HIV-2 RNA, HCV RNA, and HBV DNA in donor plasma (cobas TaqScreen MPX Test for use on the cobas s 201 system, package insert rev. 6, 01/2013, Roche Molecular Systems, Inc.; cobas TaqScreen MPX Test, version 2.0 for use on the cobas s 201 system, package insert rev. 1, 01/2011, Roche Molecular Systems, Inc.). Such fully automated testing is performed with the cobas s 201 system, which utilizes the Cobas AmpliPrep and Cobas TaqMan instruments for sample extraction and real-time PCR amplification, respectively. The cobas TaqScreen MPX Test can be performed with individual donor samples or pools of up to six samples, but it does not include discriminatory testing for specific viral targets. In contrast, the cobas TaqScreen MPX Test, version 2.0, can be performed with individual donor samples or pools of 6, 24, 48, or 96 individual samples. With the exception of HIV-1 and HIV-2, this assay can discriminate among the specific viral targets without the need for supplemental testing (80).

The new cobas MPX multiplex HIV, HCV, and HBV nucleic acid test for use on the cobas 6800/8800 Systems (Roche Molecular Systems, Inc.) is CE-marked and currently available commercially for use in Europe. This assay was designed for the qualitative, multiplex detection of HIV-1 (groups M and O) RNA, HIV-2 RNA, HCV RNA, and HBV DNA in human serum or plasma using either the cobas 6800 or cobas 8800 instrument systems. The assay can be performed with individual donor samples or pooled samples, and the cobas p 630 instrument (Roche Molecular Systems, Inc.) has been configured for preanalytical pooling of samples prior to testing. Target detection and discrimination are designed to occur simultaneously, without further differentiation between HIV-1 (groups M and O) and HIV-2 (cobas MPX, package insert rev. 2.0, 10/2014, Roche Molecular Systems, Inc.).

Quantitative Molecular Tests

When combined with highly automated instrument platforms, real-time PCR has dramatically improved the HBV DNA quantification range and reduced the need for sample dilution, while improving ease of use and overall reliability of HBV viral load testing. Due to these improvements, real-time PCR has rapidly become the preferred method for HBV DNA quantification in clinical laboratories (82–84). Details of several widely used and commercially available HBV DNA quantification assays are provided in Table 3.

TABLE 3 Select commercially available assays for HBV viral load testing

Manufacturer	Assay method	Assay name	Regulatory status[a]	Quantification range (IU/ml)[b]
Abbott Molecular Inc.	Real-time PCR	Abbott RealTime HBV	CE-IVD, US-IVD	10 to 1.0×10^9
Cepheid AB	Real-time PCR	SmartHBV	CE-IVD	172 to 1.72×10^8
Hologic, Inc.	Transcription-mediated amplification	Aptima HBV Quant Assay	In development	
Qiagen Inc.	Real-time PCR	Artus HBV LC PCR Kit	CE-IVD	20 to 4.0×10^9
		Artus HBV QS-RGQ Kit	CE-IVD	26 to 4.21×10^8
		Artus HBV RG PCR Kit	CE-IVD	1 to 4.0×10^9
		Artus HBV TM PCR Kit	CE-IVD	9 to 5.6×10^9
Roche Molecular Systems, Inc.	Real-time PCR	Cobas TaqMan HBV Test for use with the High Pure System	CE-IVD, US-IVD	29 to 1.1×10^8
		Cobas AmpliPrep/Cobas TaqMan HBV Test, v2.0	CE-IVD, US-IVD	20 to 1.7×10^8
		cobas HBV (for use on the cobas 6800/8800 Systems)	CE-IVD, US-IVD	10 to 1.0×10^9 (500 µl input)
			CE-IVD	25 to 1.0×10^9 (200 µl input)
Siemens Healthcare Diagnostics, Inc.	Real-time PCR	Versant HBV DNA 1.0 Assay (kPCR)	CE-IVD	13 to 7.0×10^8

[a]CE-IVD, Conformité Européenne-marked; US-IVD, U.S. Food and Drug Administration approved; US-RUO, research use only in the United States.
[b]All values are based on information contained in the manufacturer's product insert.

The Versant HBV DNA 3.0 Assay (bDNA) (Siemens Healthcare Diagnostics, Inc.) is a third-generation signal amplification assay for quantification of HBV DNA in human serum or plasma. The capture and target probes used in this assay bind to conserved regions throughout the entire HBV genome. Testing was performed in microwell plate format with either the semiautomated System 340 bDNA Analyzer or the fully automated Versant 440 Molecular System. The assay was shown to be both linear and precise over a relatively broad quantification range (357 to 17,900,000 IU/ml) (85). Limitations of bDNA chemistry restricted the ability of this assay to reliably and specifically detect low-level viremia relative to real-time PCR-based methods (86, 87). Production of this assay was discontinued by the manufacturer in 2014.

The Cobas Amplicor HBV Monitor Test (Roche Molecular Systems, Inc.) amplified the highly conserved precore/core region of the HBV genome and utilized end-point PCR in conjunction with enzymatic detection performed on the Cobas Amplicor Analyzer. Despite its improvements over earlier assay designs, the relatively narrow quantification range of this assay (i.e., 60 to 38,000 IU/ml) limited its use in clinical laboratories (88–90). Production of this assay was discontinued by the manufacturer in 2010.

Several different versions of the Cobas TaqMan HBV Test (Roche Molecular Systems, Inc.) have been released in the United States and Europe. While these assays amplify and detect the same conserved DNA sequence located in the precore/core region of the HBV genome, changes in sample processing steps have led to these various versions of the assay. The Cobas TaqMan HBV Test for use with the High Pure System was the first version available in Europe and the United States. This assay is suitable for testing human serum or plasma, and it relies on manual sample preparation with the High Pure System Viral Nucleic Acid Kit, followed by amplification and detection performed on the Cobas TaqMan 48 Analyzer (91).

The initial version of the fully automated assay (Cobas AmpliPrep/Cobas TaqMan HBV Test) is no longer in production and was never available commercially in the United States (92, 93). The Cobas AmpliPrep/Cobas TaqMan HBV Test, version 2.0, is currently available both in Europe and the United States. Automated sample processing is performed on the Cobas AmpliPrep instrument, while real-time amplification and detection are performed on the Cobas TaqMan analyzer (Roche Molecular Systems, Inc.), with the option of operating these instruments in either a docked or undocked configuration. Unlike the earlier version of this assay, the version 2.0 assay is suitable for use with both serum and plasma, requires a smaller sample volume (0.65 ml versus 1.0 ml), and has demonstrated amplification equivalency among HBV genotypes A to H (94, 95).

All versions of the Cobas TaqMan HBV assays utilize a competitive quantitation standard (internal control) added to each sample prior to sample processing to monitor for extraction and amplification inhibition and compensate for their effects on quantification accuracy. Without relying on assay calibration performed by the laboratory, quantification of HBV DNA is determined through the use of lot-specific calibration coefficients that have been predetermined by the assay manufacturer. Published reports of the evaluation of these assays have shown good performance relative to other commercially available assays (90, 96–100).

The cobas HBV quantitative nucleic acid test for use on the cobas 6800/8800 Systems (Roche Molecular Systems, Inc.) is available commercially for clinical use in Europe and the United States. This assay is suitable for testing human serum or plasma on either the cobas 6800 or cobas 8800 instrument. Quantification of HBV DNA is determined through the use of lot-specific calibration coefficients

that have been predetermined by the assay manufacturer. The assay includes a noncompetitive quantitation standard added to each sample prior to processing to monitor for extraction inefficiencies or amplification inhibition and to compensate for their effects on quantification accuracy. The reportable range of this assay spans nearly 9 logs, and there are two different sample input volume options available (200 µl and 500 µl), with minor differences in analytical sensitivity and quantification range (cobas HBV, package insert rev. 1.0, 01/2015, Roche Molecular Systems, Inc.).

The Abbott RealTime HBV assay (Abbott Molecular Inc.) uses real-time PCR and hydrolysis probe chemistry to amplify and detect the highly conserved N-terminal region of the S gene, which is required for assembly and secretion of subviral particles (101). The internal control is a noncompetitive target sequence that is amplified from a plasmid coextracted along with the serum or plasma sample. Automated DNA extraction and amplification are performed on the Abbott *m2000sp* and Abbott *m2000rt* instruments, respectively, in a 96-well plate format. The assay can be performed with DNA extraction protocols using either 0.2-ml or 0.5-ml sample input volumes with minimal change in the lower limit of quantification (15 IU/ml versus 10 IU/ml, respectively), and results can be reported in either IU/ml or copies/ml. Assay calibrators and controls are supplied separately by the manufacturer. Comparative studies reported generally good correlation of quantitative results with other real-time PCR methods (82, 102, 103).

The artus HBV assays (Qiagen Inc.) are CE-marked and available commercially for clinical use in Europe but not in the United States. Currently there are four different real-time PCR kit formats available: artus HBV LC PCR Kit, artus HBV QS-RGQ PCR Kit, artus HBV RG PCR Kit, and artus HBV TM PCR Kit. While the manufacturer's directions for use in three of the assays are based on manual sample processing with the QIAamp DSP Virus Kit, the artus HBV QS-RGQ PCR Kit uses the QIAsymphony RGQ system for automated sample processing and assay setup. The artus HBV LC PCR Kit uses dual-hybridization fluorescent resonance energy transfer probes and is designed for use with LightCycler instruments (Roche Diagnostics). The artus HBV RG PCR Kit and artus HBV QS-RGQ PCR Kit are based on hydrolysis probe chemistry and are designed for use with the Rotor-Gene Q instrument (Qiagen Inc.), while the artus HBV TM PCR Kit, also based on the hydrolysis probe chemistry, is designed for use with the ABI Prism 7000/7700/7900HT Sequence Detection System instruments (Life Technologies). Individual kits include a set of five quantitation standards calibrated to the First WHO International Standard (NIBSC code 97/746), a heterologous internal control, and all required amplification and detection reagents. Several studies have compared the performance characteristics of these assays to those of other commercially available real-time PCR assays (82, 102).

The Versant HBV DNA 1.0 Assay (kPCR) (Siemens Healthcare Diagnostics, Inc.) is a relatively new real-time or kinetic PCR assay for quantification of HBV DNA in serum or plasma. Testing is performed on the Versant kPCR Molecular System consisting of the Sample Preparation Module (SP Module) and the Amplification and Detection Module (AD Module). The assay can be performed with either serum or plasma, with a minimum sample volume requirement of 0.7 ml. Assay calibrators and controls are supplied separately by the manufacturer. This assay is calibrated to the Second WHO International Standard (NIBSC code 97/750), and results can be reported in either IU/ml or copies/ml. To date, there are no published reports comparing correlation of quantitative results generated by the Versant HBV DNA 1.0 Assay (kPCR) with other real-time PCR assays.

The recently introduced SmartHBV assay (Cepheid AB) uses real-time PCR and Scorpion chemistry performed on the SmartCycler II System to detect and quantify HBV DNA in plasma. The kit contains all required reagents for real-time PCR assay setup along with high and low calibrators and a noncompetitive internal control designed to be coextracted along with sample DNA and calibrated to the First WHO International Standard (NIBSC code 97/746). When the assay is performed as recommended by the manufacturer using the affigene DNA Extraction Kit with 0.2 ml of plasma, the manufacturer claims a quantification range of 6 $\log_{10}$ IU/ml and a lower limit of detection of 46 IU/ml (SmartHBV User Manual, 71001274 en-a278, 06/2008, Cepheid AB).

Additional assays for the quantification of HBV DNA are expected to be commercially available in the near future. One of these assays currently under development is the Aptima HBV Quant Assay (Hologic, Inc.). This assay is based on transcription-mediated amplification, designed for use with the automated Panther System, and is anticipated to have a quantification range of nearly 8 $\log_{10}$ IU/ml.

Genotyping and Antiviral Drug Resistance Tests

HBV genotyping and antiviral drug resistance testing has been successfully performed using a variety of technologies ranging from direct sequencing to laboratory-developed methods based on restriction fragment length polymorphism analysis, sequence-specific amplification, and mass spectroscopy (104–110). Although new and promising techniques are under development, commercial assays currently available in Europe and the United States for interrogation of the HBV genome rely on either target-specific hybridization or direct DNA sequencing (Table 4).

Assays designed specifically for HBV genotypic analysis other than antiviral drug resistance include the Inno-LiPA HBV PreCore and Inno-LiPA HBV Genotyping assays (Fujirebio Europe N.V.). The Inno-LiPA HBV PreCore assay is designed to detect the G1896A mutation resulting in a premature stop codon at amino acid position 28 that prohibits production of HBeAg and the dual mutation A1762T/G1764A located within the basal core promoter region that is associated with reduced HBeAg production. The Inno-LiPA HBV Genotyping assay is designed to provide an HBV phylogenetic genotype result (A to H). Both assays are based on reverse hybridization using a line probe assay format. Following PCR amplification of the target sequence, biotinylated amplification products are denatured and hybridized to specific oligonucleotide probes immobilized on nitrocellulose strips. Blots are incubated with a streptavidin–alkaline phosphatase conjugate and a chromogenic substrate to allow visualization of bound amplification products on the strip. Processing of the strips can be performed in a semiautomated format on the Auto-Blot 3000H or Auto-LiPA 48 instrument, with strip reading performed either manually or with the aid of LiRAS for LiPA HBV V1.01 software.

Commercially available assays designed for antiviral drug resistance testing and currently available in Europe and the United States include the Inno-LiPA HBV Multi-DR assay (Fujirebio Europe N.V.) and the Abbott HBV Sequencing Assay (Abbott Molecular Inc.). Production of

TABLE 4 Select commercially available HBV assays for genotyping and antiviral resistance testing

Manufacturer	Assay method	Assay name	Target region	Regulatory status[a]	Intended use
Abbott Molecular Inc.	Sanger sequencing	Abbott HBV Sequencing Assay	Polymerase, surface[b]	CE-IVD, US-RUO	Detection of genotypic antiviral resistance mutations[b]
Fujirebio Europe N.V.	Reverse hybridization	Inno-LiPA HBV PreCore	Basal core promoter, precore	RUO	Detection of basal core promoter dual mutation A1762T/G1764A and precore mutation G1896A (codon 28)
		Inno-LiPA HBV Genotyping	Surface	CE-IVD, US-RUO	Genotype determination
		Inno-LiPA HBV Multi-DR	Polymerase	CE-IVD, US-RUO	Detection of genotypic antiviral resistance mutations

[a]CE-IVD, Conformité Européenne-marked; RUO, research use only; US-RUO, research use only in the United States.
[b]Genotype determination and detection of surface mutations possible with user-defined software modifications.

the TruGene HBV Genotyping Kit (Siemens Healthcare Diagnostics, Inc.), which utilized proprietary bidirectional CLIP sequencing in conjunction with the OpenGene DNA Sequencing system, was discontinued by the manufacturer in 2014. A notable advantage of the TruGene assay was its capability to assign a phylogenetic genotype and detect mutations relevant to antiviral drug resistance and HBsAg alteration in a single assay (111).

The Inno-LiPA HBV Multi-DR assay (Fujirebio Europe N.V.), consisting of separate Inno-LiPA HBV DR v2 and v3strips, is the latest version of this assay available in Europe and the United States. Like the Inno-LiPA HBV PreCore and Inno-LiPA HBV Genotyping assays, this assay is based on reverse hybridization in a line probe assay format, and it is capable of detecting mutations associated with antiviral drug resistance and key compensatory mutations located within the POL/RT domain of the *P* gene. The codons interrogated by this assay include 80, 173, 180, 181, 184, 194, 202, 204, 236, and 250 of the POL/RT domain.

The Abbott HBV Sequencing Assay (Abbott Molecular Inc.) is a direct DNA sequencing assay designed for interrogation of the clinically relevant segments of the overlapping *P* and *S* genes for detection of mutations associated with antiviral drug resistance and for HBV genotype assignment, respectively. Automated DNA extraction and amplification are performed on the Abbott *m2000sp* and Abbott *m2000rt* instruments in a 96-well plate format followed by analysis of sequencing reactions on either an Applied Biosystems 3130 or 3130xl Genetic Analyzer (Life Technologies). Sequence data are assembled with the aid of an assay-specific project template created in Applied Biosystems SeqScape software and exported for interpretation by a web-based interpretation tool, SeqHepB (http://www.evivar.com/seqhepb.html), with complimentary access provided by the assay manufacturer. Alternatively, the sequence data can be submitted for interpretation via various open-access, web-based tools (see "Genotyping and Antiviral Drug Resistance Tests," below).

INTERPRETATION OF TEST RESULTS

Introduction of the First WHO International Standard for HBV DNA Nucleic Acid Amplification Techniques in 1999 was a significant advance in standardization of commercially available molecular assays for HBV because assay manufacturers were able to calibrate their assays to a common standard for improved assay comparability and standardized reporting (112). The previous lack of standardization among HBV viral load assays had been a long-standing concern prior to the development of this internationally recognized HBV DNA standard. This was particularly true as clinical practice guidelines for CHB began to recommend the use of viral load cutoffs for disease classification and routine viral load monitoring during antiviral treatment.

The first WHO standard (NIBSC code 97/746) consisted of HBV whole virus (genotype A) obtained from a single donor, diluted in plasma, and lyophilized for stability. Each vial was assigned a potency of 1,000,000 IU/ml based on testing with a variety of commercially available and laboratory-developed assays (112). A second WHO standard (NIBSC code 97/750) consisting of the same-source material and prepared in parallel with the first standard was subsequently released in 2006 (113) followed by release of a third standard (NIBSC code 10/264) in 2011, which consisted of the same-source material with an assigned potency of 850,000 IU/ml (114).

The development and widespread availability of standardized, highly sensitive, and specific molecular assays have contributed greatly to our understanding of CHB disease progression and its associated clinical and histopathological changes, especially our understanding of HBeAg-negative CHB and occult HBV infection. As a result, detection and quantification of HBV DNA in plasma or serum using real-time target amplification techniques have become standard of care, and they play an important role in the management of HBV-infected individuals. Despite these advances, the intrinsic variability of HBV genome and the extreme range of HBV DNA levels found among clinical serum and plasma samples continue to pose challenges to accurate detection, quantification, and characterization of HBV in clinical samples.

Refinements in assay performance with the latest generation of qualitative and quantitative molecular assays have resulted in assay detection capabilities that approach the theoretical limits of sensitivity. These improvements have set the stage for future assay improvement efforts focusing on ensuring reliable detection and accurate quantification of all strains of HBV. In the case of HIV-1 and HCV, similarly focused efforts have led to the implementation of novel real-time PCR amplification and detection strategies (e.g., partially double-stranded probes, dual probes, and dual-target amplification) that have successfully improved detection capability and quantification accuracy (115–117).

Despite the increased use of automation and closed-tube target amplification techniques in the current commercially available assays, the risk of sample-to-sample

contamination remains higher for HBV molecular assays than with other viral assays, mainly due to the very high viral loads (>10^8 IU/ml) frequently encountered in clinical plasma or serum specimens from patients with untreated CHB. Although this risk potential is widely recognized among laboratorians (82, 99), rates of false-positive results attributable to contamination during routine testing are difficult to assess and the risks remain poorly defined. While continued adherence to a unidirectional workflow and good laboratory practices remains critically important for successful molecular testing, an appreciation of these potential risks by patient care providers is essential for optimal patient management.

Qualitative Molecular Tests

The widespread adoption of real-time target amplification and detection techniques has not only resulted in dramatic increases in the quantification range of HBV viral load assays, it also has led to significant improvements in assay sensitivity. The evolution of these sensitive HBV viral load assays has for all practical purposes eliminated the need for companion qualitative molecular assays for use in diagnosing and monitoring CHB. Thus, the primary role of commercially available qualitative HBV molecular assays has been in blood and tissue donor screening.

The development of fully automated testing platforms designed specifically for donor screening and featuring multiplex NAT assays targeting HBV in addition to HIV and HCV has made routine donor screening of individual or pooled donor specimens a reality. The challenge of occult HBV infections characterized by extremely low levels of HBV DNA in blood and occurring with some regularity in areas of HBV endemicity also has been addressed by the development of a new generation of extremely sensitive NAT assays. However, resolution of reactive samples can sometimes be complicated when supplemental discriminatory testing cannot be successfully performed as a result of low viral load. Such situations highlight the advantages of using assays that do not require separate discriminatory testing (80).

Quantitative Molecular Tests

While standardized calibration and reporting among HBV DNA quantification assays have enabled direct comparisons of accuracy and sensitivity among different assays and resulted in generally improved assay concordance, limited availability of the WHO standard dictates the use of secondary standards for routine assay calibration. Unfortunately, even minor differences in the genetic makeup and stability of these secondary standards can result in substantial inaccuracies. Differences in assay chemistry and design also can impact accuracy and agreement of viral load measurements, even though many assays utilize internal controls or quantitation standards to monitor and in some cases compensate for inefficiencies in DNA extraction and amplification. It is also important to recognize that unexpected DNA sequence variation remains a potential cause of HBV DNA underquantification or detection failure (101, 118). Such assay-specific differences serve as the basis for continued recommendations calling for the use of a single assay during sequential viral load monitoring of HBV-infected patients despite the introduction of standardized reporting units (82, 83).

Current clinical practice guidelines for CHB recommend the standardized reporting of HBV viral load measurements in international units (IU/ml). However, the broad range of HBV viral loads frequently found among clinical samples better lends itself to reporting of $\log_{10}$-transformed results. Thus, many laboratories report $\log_{10}$-transformed values (i.e., $\log_{10}$ IU/ml) in addition to arithmetic values, which are commonly cited in clinical practice guidelines and peer-reviewed publications. Even though the development of a WHO international standard and the subsequent transition to standardized reporting units were welcomed by the vast majority of clinicians, laboratorians must continue to encourage the use of standardized reporting units and exercise discretion when asked to convert and report results in copies/ml.

Genotyping and Antiviral Drug Resistance Tests

While there is growing evidence for the consideration of HBV phylogenetic genotype in both prognostic and therapeutic decision making, there are no specific recommendations for routine HBV genotyping included in current clinical practice guidelines (8–10, 56). Due to this lack of consensus regarding the utility of HBV genotyping assays and the complexities of routinely performing them, clinical laboratories have been slow to adopt such assays.

Despite the effectiveness of nucleos(t)ide analogs for the treatment of CHB, the emergence and characterization of mutations associated with antiviral resistance have been well documented. Even with general agreement on the relatively small number of primary mutations predictive of antiviral resistance in HBV, progress in the development of interpretive guidelines has been slow. In particular, progress has been hampered by lack of data regarding the significance of novel mutations occurring at previously identified resistance sites, basic questions regarding viral fitness and the clinical impact of compensatory mutations, and the recognition of various multi-drug resistance pathways. Viral load monitoring has become widely used for assessing the effectiveness of nucleos(t)ide analog therapy and indirectly monitoring for emerging antiviral resistance. The widespread adoption of entecavir and tenofovir, both potent analogs with high genetic barriers for development of resistance, as first-line monotherapy for long-term treatment is also likely to have reduced the need for combination therapy and an increased demand for HBV resistance testing (119, 120). However, specific recommendations for HBV resistance testing and interpretation are now included in all current clinical practice guidelines for CHB (8–10, 56).

Commercially available genotyping assays based on reverse hybridization (e.g., Inno-LiPA HBV PreCore and Inno-LiPA HBV Genotyping assays) offer several advantages over methods utilizing direct sequencing. The primary advantages of these assays include ease of use and enhanced capability to detect mixed genotypes within a given sample (43, 121). However, it is important to consider the potential for indeterminate results as a consequence of unexpected sequence variation in flanking regions and an assay failure rate of up to 5% among clinical samples (121). There is also a potential for sample contamination associated with the use of these assays due to the extremely high viral loads present in some clinical specimens, optional use of nested PCR, and open-tube analysis of amplification products.

Since the Inno-LiPA HBV Multi-DR assay is based on reverse hybridization, it shares many of the performance characteristics of the Inno-LiPA HBV PreCore and Inno-LiPA HBV Genotyping assays. In fact, the Inno-LiPA

HBV Multi-DR assay can be performed efficiently in conjunction with the Inno-LiPA HBV Genotyping assay, since both assays share a common target region due to the overlap of *P* and *S* genes in the HBV genome. The Inno-LiPA HBV Multi-DR assay reportedly can generate valid results down to an HBV DNA level of 3.0 $\log_{10}$ copies/ml, with the ability to detect mutant subpopulations of HBV comprising as little as 5% of total virus population at HBV DNA levels of ≥4.0 $\log_{10}$ copies/ml (122). Finally, in addition to the previously discussed limitations of reverse hybridization, this assay may exhibit limited discriminatory capability at resistance-associated sites located in close proximity to one another (e.g., rt180 and rt181) (122).

The Abbott HBV Sequencing Assay, like other population-based direct sequencing assays, has limited capability to detect minor viral subpopulations, which can occur in clinical specimens with regularity (123). As is the case with other molecular assays, including the Inno-LiPA HBV Multi-DR assay, results may reflect preferential PCR amplification of select strains from a larger and more diverse pool of virus, and the sequence variations identified in any given sample may represent the sum of all sequence variants occurring in a heterogeneous population rather than in a single strain of HBV. The potential for sample-to-sample contamination associated with high viral load also remains a concern with this assay. However, the optional use of uracil-*N*-glycosylase can reduce the potential for contamination of unprocessed samples with exogenous HBV DNA sequences.

While data analysis for the Abbott HBV Sequencing Assay can be complex and somewhat labor intensive and require the use of either user-defined or web-based interpretive software, a major advantage of this sequencing assay is its versatility (124). It can identify novel mutations at either new or existing sites, and the interpretive software can be easily updated if the need arises. The overlapping nature of the HBV genome also permits the simultaneous interrogation of the *P* and *S* genes for mutations associated with antiviral drug resistance and genotype assignment, respectively, from a single assay.

Numerous web-based resources are currently available to aid in HBV sequence analysis, including genotyping and antiviral resistance mutation analysis. Available software programs include SeqHepB (http://www.seqhepb.com/login.html; fee-based access), Genafor/Arevir HBV Drug Resistance Interpretation Tool (http://www.genafor.org/hbv/hbvpredict.php), HIV-Grade HBV Resistance Interpretation Tool (http://www.hiv-grade.de/hbv_grade/deployed/grade.pl?program=hbvalg), Geno2Pheno HBV 2.0 (http://hbv.geno2pheno.org/index.php), and HBVseq (http://hivdb.stanford.edu/HBV/HBVseq/development/HBVseq.html). Additional web-based software programs designed specifically for HBV genotyping and subtyping include NCBI Viral Genotyping Tool (http://www.ncbi.nlm.nih.gov/projects/genotyping), HBV Star (http://www.vgb.ucl.ac.uk/starn.shtml), and Oxford HBV Subtyping Tool (http://www.bioafrica.net/subtypetool/html/subtypinghbv.html).

FUTURE DIRECTIONS

Despite widespread adoption of molecular tests for use in the diagnosis and management of CHB, many practical needs remain unmet. Current quantification assays for HBV DNA in plasma or serum have been designed for use on various medium- to high-throughput platforms with limited capability to accommodate other analytes (e.g., HIV, HCV) or variable run sizes. While future assay improvements are likely to address some of these needs, the design goals for the next generation of automated instrument platforms should include a clear focus on broader test menus and expanded capability for random access testing or use of variable testing batch sizes. Additional improvements aimed at further minimizing the potential for sample-to-sample contamination will also be critical to the success of future automated molecular assay platforms designed for HBV molecular testing due to the frequent presence of specimens with extremely high HBV viral loads from patients with untreated CHB. Such improvements in platform design would provide the necessary quality and efficiency of molecular diagnostic testing and welcome cost savings to clinical laboratories with differing operational needs.

The limits of detection and quantification for the current generation of HBV viral load assays are similar to those of qualitative HBV DNA detection assays for a given processed sample volume, thus negating the need for separate qualitative molecular assays that add little value to the diagnosis and monitoring of CHB. However, continued improvements in quantification accuracy and reproducibility, especially at the lower limit of quantification, should remain important goals for those involved in future assay development. Such needs are particularly relevant for the determination of cure or relapse of disease in patients receiving potent antiviral agents. Developers of these molecular assays must also do more to demonstrate their assay's ability to amplify and quantify equivalently all known genotypes and variant strains of HBV.

Deep sequencing and next-generation sequencing methods have been developed for potential clinical applications, such as to help guide antiviral therapy of CHB (125–127). However, many of the genotypic mutations found in the HBV polymerase and surface gene sequences of clinical strains of HBV have yet to be characterized or correlated with phenotypic resistance and/or viral fitness. In particular, additional data and consensus are necessary to interpret the clinical significance of atypical mutations at resistance-associated codons and compensatory mutations. Furthermore, these new sequencing technologies are currently lacking user-friendly bioinformatics tools that can quickly analyze the large amount of complex sequence data generated and automatically provide clinically relevant interpretations regarding antiviral drug resistance. Availability of new informatics tools and comprehensive sequence databases correlating genotypic and phenotypic characteristics and interpretive guidelines will be essential and of increasing practical importance for the optimal management of antiviral therapy for CHB in the future.

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Molecular Detection of Human Papillomaviruses

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33

The importance of human papillomaviruses (HPVs) in biology and medicine was highlighted by the award of the 2008 Nobel Prize in Medicine to Harald zur Hausen in recognition of his discovery of the oncogenic role of HPV in cervical cancer (1). Numerous international epidemiologic studies confirmed this association, which ultimately led to the successful development of vaccines that prevent HPV infection and tests that provide another tool for cervical cancer control.

The purpose of this chapter is to provide a brief overview of the biology and natural history of the virus and a description of current clinical applications, laboratory methods, and possible future directions. For more-detailed information on HPV biology and pathogenesis, the interested reader is referred to one of several excellent reviews (2–4).

BRIEF DESCRIPTION OF THE VIRUS

HPVs are in the *Papillomaviridae* family. Papillomaviruses (PVs) are widespread in the animal kingdom and, by way of explanation for their name, have been recognized to cause benign warts, or papillomas. Infection is generally species specific. HPV has been the subject of the most intensive research and study, but animal PVs, in particular bovine PVs, have served as useful models in studying basic mechanisms of PV replication and transcription.

All PVs are very similar in their genetic organization and their appearance by electron microscopy. The virus is composed of an 8-kb double-stranded circular genome enclosed in a capsid shell. The open reading frames (ORFs) of HPV are illustrated in Fig. 1, using HPV16 as an example. Only one strand is transcribed, and transcriptional products are polycistronic. The ORFs and corresponding protein products are referred to as early (E) or late (L), again based on homology to bovine PV and their presumed function in the viral life cycle. The long control region, also known as the upstream regulatory region, functions in the initiation of replication and regulation of transcription.

The major (L1) and minor (L2) capsid proteins make up the viral protein coat. L1 alone will self-assemble to form empty shells that resemble virus, called virus-like particles. While the L1 ORF is the region of the genome that is most conserved across all HPV types, L1 virus-like particles retain type-specific conformational epitopes and are the basis of current vaccine formulations.

As one would predict from the limited number of viral genes, the virus is dependent on the host cell's replication, transcription, and translational machinery. The E1 protein is essential for viral replication, and in conjunction with E2, it maintains the viral episomes during cell division. The E2 protein also functions in transcriptional regulation. The E6 and E7 proteins interact closely with cellular replication proteins to achieve viral replication. The E6/E7 ORFs are the least conserved across types.

The abbreviation "HPV" masks the fact that HPV is not a single virus, but a family of more than 150 closely related viral types (5). Types are distinguished based on the sequence of the L1 region and are numbered based on their order of discovery, and so the numbering system appears confusing from a biologic perspective. Sequence divergence of more than 10% corresponds to a new PV type. Lesser divergence defines subtypes (2 to 10%) or variants of a type (<2%). A phylogenetic tree based on the genetic relatedness of L1 has been used to provide a taxonomic classification of the *Papillomaviridae* family (5, 6) (Fig. 2). The term "genus" is used for the higher-order clusters that are named using the Greek alphabet. Within a genus, the smaller clusters are referred to as "species" that are named by number. A bioinformatics resource, The Papillomavirus Episteme, hosts curated information related to PV genomics (7).

HPVs have also been grouped based on the type of epithelium that they preferentially infect. The cutaneous group includes types that are associated with common hand and foot warts as well as other lesions of keratinized epithelium such as skin. Most of these are in the *Beta-* and *Gammapapillomavirus* genera. The other group is referred to as mucosal or genital, reflecting their predilection for nonkeratinized squamous epithelium and the anogenital region. This group includes about 40 types, most in the *Alphapapillomavirus* genus. Mucosal HPVs are grouped into so-called high-risk (HR) and low-risk (LR) types based on their epidemiologic association with cancer. While complete agreement is missing as to which types should be considered HR, the International Agency for Research on Cancer currently designates 12 as HR (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59) with an additional two as having limited evidence of carcinogenicity (HPV 68 and 73), although there is evidence that some rare types may play a role in oncogenesis (8, 9).

Based on a combination of animal PV studies and observations of HPV-associated lesions, the general features

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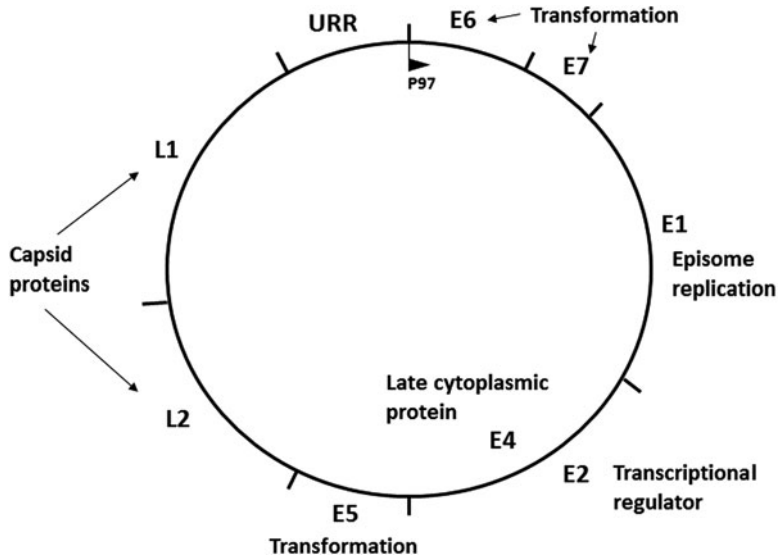

FIGURE 1 Schematic diagram of genomic organization of the open reading frame of human papillomavirus 16. P97 refers to dominant promoter. All papillomaviruses are similar in their genetic organization and appearance by electron microscopy. URR, upstream regulatory region.

of the PV life cycle are understood. PVs infect the undifferentiated basal layer of the epithelium. The viral DNA is maintained in the nucleus of the basal cells as a circular episome at low copy number. Basal cells are accessible through microabrasions or at regions of squamocolumnar junction where one type of epithelium is replaced by another. This is why the cervix transformation zone is preferentially targeted by HPV.

Viral genome amplification occurs in differentiating cells using the cellular replication machinery, and the production of virus is critically dependent on cellular proteins expressed during keratinoctye differentiation. The viral proteins E6 and E7 allow cellular replication to occur in differentiating cells that normally would have exited the cell cycle. The prolonged proliferating phase of these cells results in a thickened epithelium visible as an exophytic wart (papilloma) or flat plaquelike area of whitening. The virus is released as cells exfoliate from the epithelium and remains cell associated. The nonlytic HPV life cycle is confined to the epithelium, and as a consequence, the virus has minimal exposure to the host immune system.

Oncogenesis can be thought of as an abnormal viral life cycle, although clearly oncogenesis is a result of viral and cellular interactions. With neoplastic progression, there is little virion production. The viral genes that are responsible for prolonging cell replication, *E6* and *E7*, also contribute to genomic instability, viral integration, and stepwise progression to malignancy.

The predominant mode of transmission of mucosal types is through sexual contact. HPV is prevalent in the general population and is the most common sexually transmitted infection in the United States, with an estimated 14 million new infections each year (10). The vast majority of these infections are transient and asymptomatic; approximately 70% of new infections clear within 1 year and 91% within 2 years (11). HR types are more persistent than LR types. Consistently, persistent infection with HR HPV is associated with an increased risk of neoplastic progression. In population-based studies the prevalence of cervical/vaginal HPV is highest in young women shortly after the onset of sexual activity and decreases gradually with age (12–14), with a similar pattern in males (15).

LR HPV types, particularly 6 and 11, are associated with genital warts (condylomas) and recurrent respiratory papillomatosis (warts or papillomas in the upper respiratory tract). A wider variety of LR types are also associated with low-grade cervical cytology abnormalities. HR types are associated with low- and high-grade cervical cytology abnormalities; cervical cancer precursors; and invasive cervical, vaginal, vulvar, anal, penile, and oropharyngeal cancers. The relation of HR HPV infection to cervical cancer is somewhat unique in that it is a necessary but not sufficient cause of neoplastic progression. Cancer is a very rare outcome of a very common infection. Types 16 and 18 predominate in cancers; they are associated with approximately 70% of cervical cancers worldwide. Current HPV vaccines are designed to prevent infection and therefore eliminate the requisite first step toward malignancy.

CLINICAL APPLICATIONS

In most instances microbiology laboratories are called upon to test samples from a patient with overt illness in order to detect an infectious cause for that illness. Interventions are subsequently directed toward elimination or control of the identified organism(s). For HPV, this paradigm is completely reversed. HPV testing is performed in asymptomatic individuals in order to indicate the presence of a disease. HPV infection itself is not treated; rather, treatment is directed to the HPV-associated disease. Because HPV tests detect virus and not disease, careful distinction must be made between the analytic performance of an assay (i.e., detection of HPV) and clinical performance (i.e., detection of disease). A test that is analytically more sensitive for detection of HPV may actually

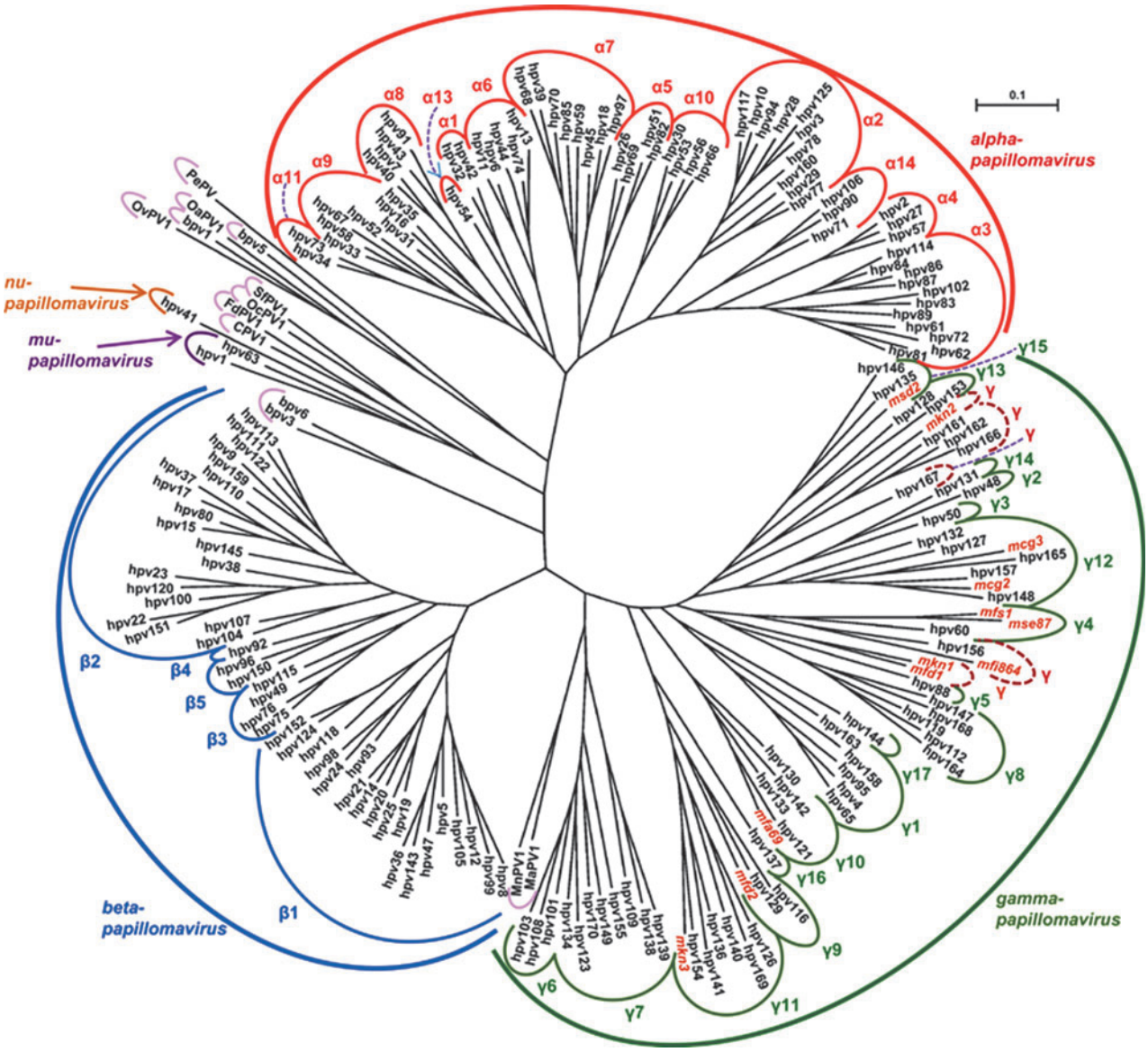

FIGURE 2 Phylogenetic tree based on the L1 open reading frame sequences of 170 HPV types, single animal papillomaviruses, and newly identified HPVs using metagenomic sequencing. The maximum likelihood method was used for the analysis and the tree was constructed using the MEGA5.1 program. The numbers at the ends of each of the branches identify an HPV type; m-numbers refer to metagenomics. All other abbreviations refer to animal PV types. The outermost semicircular symbols identify PV genera (e.g., the genus *Alphapapillomavirus*). The number at the inner semicircular symbol refers to PV species. To give an example taken from the upper part of the figure, the HPV types 33, 58, 67, 52, 31, 16, and 35 together form the HPV species 9 in the genus *Alphapapillomavirus*. Reprinted from de Villiers (5) with permission of the publisher.

have significantly poorer clinical relevance due to detection of clinically irrelevant levels of virus (16).

Cervical Cancer Screening

Once the etiologic link between HR HPV and cervical cancer was established, it was logical to think that HR HPV testing could be used to improve the sensitivity of cervical cancer screening; that is, HPV could be used as a biomarker for cervical cancer and cervical cancer precursors. Since the introduction of cervical cytology, the Pap test has been the basis for cervical cancer screening in countries that have sufficient trained personnel and infrastructure for systematic screening, recall, evaluation, and treatment of women with abnormal screening results. Cytology interpretation is subjective, and even though good specificity can be achieved, the sensitivity of a single test is limited. Pap tests have been effective in dramatically reducing cervical cancer because the long interval for progression from cancer precursor to invasive disease allows for multiple rounds of screening. HPV testing has the advantage of being objective and more readily standardized than cytology.

The literature consistently supports the strong negative predictive power of HR HPV testing; that is, women testing negative are extremely unlikely to have clinically significant cervical disease currently or within the next 3 to 5 years. Unfortunately, the high prevalence of completely

asymptomatic infection in the general screening population results in a relatively low positive predictive value for detection of cervical disease requiring treatment. Use of HPV testing as a primary screen for cervical cancer risk has been a long-debated topic with proponents citing the increased sensitivity of HPV testing over traditional cytology and the potential for reduced health costs (17–19). Cotesting with cervical cytology and HPV testing adds little added protective value over HPV testing alone (17). HPV testing may be an appropriate first-line test, but additional laboratory tests such as cytology, genotyping, gene methylation, or immunohistochemical stains will be needed to guide referral for follow-up (18). Those who are not advocates for primary HPV screening are concerned about lack of specificity and potential over treatment of young women who often carry the virus but are not at high risk due to frequent transient infections. An additional concern of primary HPV testing is choice of HPV detection assay, as one report highlights disagreement among results of different HPV detection assays, although reassuringly assay performance in screening settings is comparable (20, 21).

Clinical Testing Guidelines

While the FDA does not issue cervical cancer screening guidelines, it does influence practice by review and approval of HPV tests for specific indications. Currently there are four FDA-approved HPV tests, three that target HPV DNA and one that targets E6/E7 message (see following sections for test descriptions). All are approved for the indications of determining the appropriate clinical follow-up for equivocal cytology results in a woman of any age, or in conjunction with a routine Pap test, for cervical cancer screening in women 30 years of age or older. In March 2014 the FDA approved primary cervical cancer screening as an indication for one test, the cobas HPV Test (Roche Diagnostics, Indianapolis, IN), which detects 14 HR HPV types including specific detection of types 16 and 18 (see assay description below).

In 2012 the American Cancer Society (ACS), the American Society for Clinical Pathology (ASCP), and the American Society for Colposcopy and Cervical Pathology (ASCCP) published updated guidelines for cervical cancer screening that included discontinuation of screening for women over age 65 with adequate prior negative screening and cotesting for women ages 30 to 65 years at 5-year intervals instead of 3-year intervals. HPV 16/18 genotyping can be used to triage follow up for HPV HR positive/cytology negative cotest results (22). In the same year, the United States Preventive Services Task Force (USPSTF) and American Congress of Obstetricians and Gynecologists released similar cervical cancer screening guidelines (23, 24). The guideline statements endorse continued age-appropriate screening for individuals having received the HPV vaccine. The ACS, ASCP, and ASCCP guidelines also cited insufficient evidence to recommend for or against the use of HPV testing as a primary screen in the absence of Pap smear analysis. The recent FDA approval of Roche's HPV test as a primary screening tool has not yet been incorporated into screening guidelines (25). There are no guidelines for other testing indications such as marker of residual or recurrent disease posttherapy or testing for other, noncervical cancer indications.

Other Applications under Investigation

HPV has been implicated as the causative agent in cancers other than cervical, particularly in oropharyngeal cancer (26, 27). While head and neck cancers have historically been associated with tobacco use and alcohol consumption, recent increases in oropharyngeal cancer have been attributed to HPV, particularly HPV 16 (28–30). Studies have indicated that HPV-positive oropharyngeal cancers are genetically distinct from HPV-negative tumors and generally have a better prognosis (31, 32). HPV testing of oropharyngeal tumors is being investigated as a method to guide therapy and provide prognostic information (33). There is no role for HPV testing in screening for oropharyngeal cancer.

HPV has also been implicated as the causative agent in anal cancers, associated with the same HR types as cervical cancer, especially types 16 and 18 (34–36). In recent years, the incidence of anal cancer has increased, particularly in HIV-infected individuals and men having sex with men (MSM) (37, 38). In the general population, women have a higher incidence for anal cancer than do men (39). Although no formal recommendations or guidelines exist for anal cancer screening, some institutions have developed anal cancer screening algorithms similar to cervical cancer screening algorithms for individuals at high risk. These algorithms include a combination of cytology, histology, high-resolution anoscopy, and HPV testing and primarily target HIV-positive individuals and MSM (40–42). Studies have shown that HPV status is a significant risk factor for development of high-grade anal dysplasia (40–42).

Women treated for cervical precancers have a high risk of recurrence, reported as high as 15% (43). HPV testing is being investigated for use as "test of cure" in these women either alone or with cytology cotesting. Initial results indicate HPV has a higher sensitivity than cytology in detecting high-grade cervical disease post therapy, with some variation by assay (44, 45).

HPV ASSAYS

Numerous HPV assays that vary widely in their test characteristics and sample preparation requirements have been reported in the literature and continue to be developed. This chapter will primarily focus on those assays that have been FDA approved for clinical use, with mention of other commercial assays that have been compared with FDA-approved assays in published studies. With these restrictions, some new assays may not be covered. Serologic assays, useful in epidemiologic surveys of HPV exposure, have no recognized role in individual clinical evaluation and are not included.

Commercially Available Clinical Assays

Multiple laboratory methods have been developed for detection of HR HPV (Table 1). There has been no regulatory designation as to which HPV types should be included as HR, and assay choice is largely based on timing of assay integration into the laboratory, regulatory approval, equipment, overall cost, and requirement for reporting format (overall or type specific). Assays tend to detect the same 14 HR types most frequently associated with cervical cancer (HPV 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68). At least one assay is being developed for point-of-care testing (46). Many of these assays have been CE marked (Conformité Européenne marked, indicating that they meet requirements of all relevant European Directives) for clinical use outside the United States.

TABLE 1 Summary of clinical tests for high-risk human papillomavirus detection discussed in this chapter

Test	Manufacturer	Method	Types detected (molecular target)	Genotyping	Internal control included	FDA approved
Hybrid Capture 2	Qiagen (Valencia, CA)	Signal amplification	13 HR types (genomic)	No	No	Yes
Invader HPV	Hologic (Marlborough, MA)	Probe amplification	14 HR types (L1 and E6/E7)	Yes, separate reaction	Yes	Yes
cobas HPV	Roche Diagnostics (Indianapolis, IN)	PCR	14 HR types (L1 consensus region)	Yes, simultaneous	Yes	Yes
Aptima HPV	Hologic/Gen-Probe (San Diego, CA)	Transcription-mediated amplification	14 HR types (E6/E7 mRNA)	Yes, separate reaction	No, but has process control	Yes
Real Time High Risk HPV	Abbott Molecular GmbH & Co. KG, Wiesbaden, Germany	PCR	14 HR types (genomic)	Yes, simultaneous	Yes	No
BD Onclarity HPV	Becton Dickinson and Company (Franklin Lakes, NJ)	PCR	14 HR types (E6/E7)	Yes, simultaneous	Yes	No
Xpert HPV	Cepheid, Sunnyvale, CA	PCR	14 HR types (E6/E7)	Yes, simultaneous	Yes	No
PreTect HPV-Proofer	NorChip (Klokkarstua, Norway)	Nucleic acid-based sequence amplification	5 HR types (E6/E7 mRNA)	No	Yes	No

FDA Approved

There are currently four assays that have been approved by the FDA for clinical diagnostic use; three detect HPV DNA and one targets E6/E7 mRNA. Each of the assays is widely used in the United States.

Digene Hybrid Capture 2

The Hybrid Capture 2 method (Qiagen, Valencia, CA) is a liquid-based assay that utilizes genetically complex RNA probes representing nearly the whole viral genome to detect viral DNA. The probe-virus complexes are detected by antibodies specific for RNA-DNA hybrids, while alkaline phosphatase-conjugated antibodies with the same specificity are employed for chemiluminescent signal generation. The HR HPV Hybrid Capture test targets 13 HR types noted above (excluding type 66). The cutoff value for positive results is 1 pg DNA, which corresponds to a sensitivity of approximately 5,000 copies of virus per sample input to the assay. Results are generally reported as negative or positive for HR HPV, although the chemiluminescent signal does roughly correlate with viral load. Individual types cannot be distinguished. There is no internal control to validate quality or quantity of sample. The manufacturer provides detailed information on appropriate sample collection and storage. The assay is suitable for clinician-collected exfoliated cervical cells from the transformation zone, but biopsy material can also be used. The Digene collection kit includes a brush targeting the transformation zone and specimen transport media. Cells collected in PreservCyt (Cytyc, Marlborough, MA) are also suitable for testing. While other liquid-based cytology collection media have been used, these sampling methods must be validated by the laboratory. The assay has good interlaboratory reproducibility (47).

The Hybrid Capture 2 test has been FDA approved for cervical HPV detection since 1999, thus it is often considered the gold standard HPV detection assay. Although the assay has good interlaboratory reproducibility (47), cross-hybridization with HR types not included in the probe set (particularly HPV66) as well as with LR types does occur (47–51). One disadvantage of the Hybrid Capture 2 assay is the lack of an internal control for sample adequacy. The assay cannot distinguish specific HPV genotypes nor is there an available adjunct genotyping test.

Cervista HPV HR and Cervista HPV 16/18

The Cervista HPV HR assay (Hologic, Marlborough, MA) obtained FDA approval in 2009 for detection of 14 HR HPV types along with the Cervista HPV 16/18 test (Hologic) for HPV16/18 genotyping. The Cervista assays are approved for cervical cytology samples collected in PreservCyt fixative, require DNA extraction, and use probe hybridization and specific cleavage of a fluorescent resonance energy transfer cassette for fluorescent signal generation and amplification. The probes target multiple sites within the HPV genome including the *L1*, *E6*, and *E7* genes. Simultaneous detection of human genomic DNA serves as a control, designed to assess adequacy of the sample. For Cervista HPV HR results are represented as negative or positive for each of three probe pools that detects phylogenetically related types. The Cervista 16/18 assay yields a genotype result based on fluorescent signal generation in each of two genotype-specific reactions.

The Cervista assays were initially quite labor intensive because the FDA-approved method required a specific manual DNA extraction method and pipetting of the

HPV detection reagents. Automated solutions for the DNA extraction step, sample preparation, and HPV detection reactions have since been developed on a Tecan-based platform. In addition, sample aliquoting from the primary PreservCyt vial can be automated using the Sample Transfer System (Hologic). The Cervista HPV HR assay has shown comparable results to the Digene Hybrid Capture 2 method and may show increased sensitivity (52, 53). One criticism of the Cervista HR HPV assay is the preponderance of false-positive "triple-positive" results (54). Such samples show increased fluorescence signal for each of the three phylogenetically related probe pools.

cobas HPV Test

The cobas HPV Test (Roche Diagnostics, Indianapolis, IN) is the first FDA-approved PCR-based assay for detection of the 14 most common HR HPV types with simultaneous genotyping of HPV types 16 and 18. In April 2014, the assay was the first test to receive FDA approval for use as a primary screening testing for cervical cancer. The method utilizes a consensus pool of PCR primers to amplify an approximately 200-bp region of the L1 region. Type-specific fluorescently labeled oligonucleotide probes detect L1 region amplification. HR HPV types 16 and 18 are each detected with a dedicated fluorescent dye, while the remaining 12 HR types are detected via the same fluorophore. Simultaneous amplification and unique detection of β-globin, a human genomic DNA target, serves as a control for sample adequacy, successful DNA extraction, and PCR amplification. The assay utilizes TaqMan technology to cleave double-stranded amplicon-probe hybrids, releasing the fluorescent reporter dyes from the probe containing quenchers and allowing for fluorescence detection. Results are represented as HPV HR positive (types 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68), HPV type 16 positive and/or HPV type 18 positive based on specific wavelength of detected reporter dyes. The test is approved for cervical cytology specimens collected in PreservCyt fixative and requires DNA extraction prior to the PCR amplification. Specimen extraction is fully automated and no pipetting is required to proceed to PCR on the cobas 4800 system (Roche).

The ATHENA (Addressing THE Need for Advanced HPV Diagnostics) study showed that the cobas HPV test performed comparably to the Hybrid Capture 2 test and that (simultaneous) HPV 16/18 genotyping is useful for risk stratification in patient management (55). The cobas HPV test allows for simultaneous, specific detection of HPV types 16 and 18 without the need for a separate stand-alone genotyping assay. The inclusion of an endogenous positive control to monitor specimen and assay adequacy is particularly important if the assay is used as a primary screening test. It has been suggested that the L1 region targeted by the assay has the potential to be disrupted by viral integration into the host genome. However L1 assays have been used to evaluate HPV in cancers (e.g., 34, 56) and assay performance has been comparable to assays targeting other regions (57).

Aptima HPV Assay

The Aptima HPV Assay from Hologic/Gen-Probe (San Diego, CA), FDA approved in 2011, is the only FDA-approved HPV assay that is designed to detect HPV mRNA. It is designed for liquid-based cervical cytology samples that are transferred to specimen transport media that mediate cell lysis and protect released mRNA from degradation. The Aptima HPV assay targets the E6/E7 mRNA of the same 14 HR HPV types listed above and incorporates an internal process control to monitor nucleic acid capture, amplification, and detection, as well as operator error. The method involves three steps, target capture followed by transcription-mediated amplification of mRNA targets and analyte detection based on the hybridization protection assay technique. Target capture is accomplished via magnetic microparticle linked oligomers complementary to mRNA targets. The transcription-mediated amplification step involves generation of a DNA copy of the mRNA targets from which multiple copies of an RNA amplicon are created. RNA amplicons are detected using chemiluminescent labeled DNA probes creating RNA-DNA hybrids based on hybridization protection. The assay is designed to be performed in a single reaction tube and fully automated on Gen-Probe's Tigris or Panther instrument.

In cross-sectional studies the Aptima HPV assay has performed similarly to other clinical HPV assays (48, 58). Since HPV integration into the host genome followed by E6/E7 mRNA expression are necessary steps in progression of precancer and cancerous lesions, mRNA-based HPV detection is assumed to be a more specific method for women at high risk compared with DNA-targeted HPV screening. Indeed, studies comparing methods have demonstrated greater assay specificity for cytology positive samples and cell lines (21, 57, 59). The Aptima HPV assay is often used as an adjunct test along with DNA-based HPV screening for further triage. Since HPV screening guidelines have generally been predicated on DNA-based screening methods, updated guidelines may be needed to suggest adjunct testing options and/or alterations to screening intervals based on methods utilized.

Non-FDA Approved

Real Time High Risk HPV

The Real Time High Risk HPV assay (Abbott Molecular GmbH & Co. KG, Wiesbaden, Germany) is a multiplex real-time PCR-based assay that simultaneously detects the 14 common HR types while specifically genotyping types 16 and 18. Although not FDA approved, the assay is CE marked for clinical use in Europe. The method utilizes homogeneous target amplification and detection and is compatible with liquid-based cytology specimens collected in PerservCyt solution, SurePath Preservative Fluid, or an assay-specific Abbott Cerv-Collect Specimen collection kit. Beta-globin is utilized as the internal control for assay reliability and sample adequacy. The assay is automated on the Abbott m2000 real-time PCR system. In a head-to-head comparison study the Real Time High Risk HPV assay showed high concordance with the Roche cobas HPV assay. It also showed a lower positivity rate compared with the Roche assay, but a high specificity (21).

BD Onclarity HPV Assay

The BD Onclarity HPV Assay (Becton Dickinson and Company, Franklin Lakes, NJ) is a DNA-based PCR method that targets the HPV *E6/E7* genes. The assay simultaneously detects the 14 HR types and discretely genotypes six types (HPV 16, 18, 45, 31, 51, and 52) with the remaining types being reported in three pooled groups (HPV 33 and 58; HPV 35, 39, and 68; and HPV 56, 59, and 66). The enhanced genotyping capacity may prove useful for more accurate clinical triage. The Onclarity method incorporates detection of the beta-globin gene as

an internal control for sample and method adequacy and is automated on the BD Viper LT System (Becton Dickinson and Company). The assay is pending certification in the European Union and is not yet available in the United States. The assay performed similarly to both the Roche cobas HPV and Digene Hybrid Capture 2 assays (21, 60).

Xpert HPV

Being marketed as a point-of-care HPV test with a 1-h turnaround time, the Xpert HPV test (Cepheid, Sunnyvale, CA) was designated as a CE-marked product in April 2014. The assay, which detects the 14 HR types with simultaneous specific reporting of types 16 and 18/45, is processed in a single-use cartridge on the random access GeneXpert system. The method is PCR-based targeting the HPV *E6/E7* genes and the hydroxymethylbilane synthase (*HMBS*) gene as an internal control. The HPV specific types or pooled types and internal control are reported in different fluorescent channels. The Xpert HPV test showed comparable sensitivity and specificity to both the Roche cobas HPV and Digene Hybrid Capture 2 assays (46). Potential advantages of this point-of-care test format include same day turnaround time for rapid clinical management and reduced risk for lost follow-up. In addition, the cartridge system uses sample without processing so that specialized laboratory skills are not required to perform the test. Cepheid sells kits for multiple analytes on the GeneXpert platform.

PreTect HPV-Proofer

The PreTect HPV-Proofer assay for detection of HPV mRNA is available from NorChip (Klokkarstua, Norway). The test is CE marked and uses multiplex nucleic acid-based sequence amplification to amplify mRNA and real-time detection of the products with molecular beacon technology to detect expression of *E6* and *E7* genes for five HR HPV types (HPV 16, 18, 31, 33, and 45) and a genomic control gene to assess RNA quality. This approach is purported to be more specific than DNA-based strategies because detection of gene expression may distinguish active HPV infection from simple DNA presence. Thus, an RNA-based detection approach may be more predictive of the risk for neoplastic progression (61, 62). In a recent study the PreTect HPV-Proofer showed reduced sensitivity for high-grade lesions compared with other DNA and an RNA based HPV detection assays (21).

Indirect Adjunct Assays

HPV neoplastic progression has been associated with changes in protein expression and methylation that can be useful as adjunct assays of HPV. For example, p16 overexpression (a cyclin-dependent kinase) occurs as a result of HPV oncogene expression and can be detected by immunohistochemistry in tissues and cytology specimens to improve diagnostic accuracy (63). A combined p16 and Ki-67 immunohistochemical (IHC) test is CE marked for use in cytology (CINtec PLUS, Roche and Ventana Medical Systems, Tucson, AZ). The IHC platform does not require molecular facilities and can be performed in most histopathology laboratories. Interpretation requires evaluation in the context of morphology (cytology or histology) and uniform standards have not been developed. Methylation of cellular genes has shown promise as a triage test in cervical cancer screening, but standardized approaches for testing and setting thresholds for referral have not been established (64).

LABORATORY ISSUES

Sample Collection

Commercial tests specify sample collection methods and transport media that are compatible with the assay, and FDA approval requires use of the manufacturer's guidelines. This is because changes in collection devices can affect numbers and types of cells that are included and transport media affect extraction, yield, and inhibitors. Even when guidelines are followed, appropriate validation studies are necessary for each specimen type that will be processed. Conditions for storage prior to testing must also be within guidelines for the test.

Testing personnel should be cognizant of sample adequacy, particularly sample cellularity, not just volume. While most HPV tests include an internal control target for sample adequacy, the Digene Hybrid Capture 2 and Aptima tests do not have this control. Detection of an internal control is useful to help verify sample adequacy and absence of significant inhibition. However, it should be kept in mind that the internal control only indicates the presence of cells, not the type of cell. Internal control probes would not detect a problem with inflammatory samples that contain minimal epithelial cells. If inadequate specimens are routinely received from a particular client or clinic, the laboratory should attempt to communicate with the client regarding sample collection.

Assay Selection

Selection of the appropriate HPV detection assay is largely dependent on the laboratory type and work flow. Regulatory approval of the assay is another important indication. Use of tests that are not FDA approved for clinical diagnosis requires extensive laboratory validation. Other issues to consider are coordination of reflex testing with cytology, utilization of existing equipment, and expertise of staff. Nucleic acid detection assays are often performed in molecular diagnostic or cytology laboratories and necessitate specific training. Many methods are performed as open assays with potential for cross-contamination of detected products. Laboratories need to have adequate space to develop unidirectional work flow practices, including separate areas for sample preparation and analyte detection, to minimize potential contamination.

The testing laboratory must consider which assay type can be accommodated by current equipment or fits well within the anticipated equipment list and testing menu. Assay selection may be dictated by equipment already present within the laboratory. However, if HPV testing requires acquisition of a new testing platform, the laboratory would benefit from utilizing a platform that can accommodate additional assays. Besides maximizing use of equipment, redundancy will allow for backup in the event of instrument failures. The laboratory must also consider the expertise of current staff and attempt to select a testing methodology that fits well within the skill set of testing personnel and requires little additional training.

The anticipated number of specimens and the ability to automate may be an important factor in assay selection. Most assay platforms are essentially automated, although some manual steps are often still required. Pipetting of specimens from the primary collection container has largely not been addressed. This may be an important consideration in assay selection. A fully automated system for sample processing from collection vial to result would be ideal for high-throughput laboratories.

Quality Control

Quality control measures for HPV testing need to be monitored regularly to ensure that the assay is working correctly. The prevalence of HR HPV in specimens with a cytological diagnosis of atypical squamous cells of undetermined significance (ASCUS) ranges between about 35 and 45% depending on the testing method (65, 66). Monitoring the prevalence of HPV positivity in ASCUS samples, as well as other cytological diagnoses, is a useful measure of assay accuracy and performance. For laboratories processing specimens for screening, HPV prevalence should also be monitored. The prevalence of HPV infection correlates with patient age (67, 68), so monitoring of screening results may be most accurate if delineated by age group. Besides ensuring that the HPV incidence falls within an acceptable range, laboratories should also look for trends in HPV prevalence over time to confirm consistent assay performance. Positive and negative controls should also be observed to confirm that they fall within the acceptable range and to monitor for drift. Additional quality control parameters to monitor may include incidence of assay or sample failure, turnaround time, and incidence of insufficient samples, particularly by ordering provider or facility.

To assist in standardization and harmonization of HPV assays the World Health Organization (WHO) has established International Standards for HPV 16 and 18 DNA (69). These are available from NIBSC (National Institute for Biologic Standards and Controls, UK) and are not intended for use as working standards; they are to be used in calibration of secondary standards. They are plasmid based and designed to be applicable for a wide variety of clinical and research assays. Additional controls are needed to monitor all aspects of sensitivity and specificity of HPV assays, notably standards for additional HPV types and challenges of extraction from clinical samples. Importantly these controls address analytic performance, not clinical performance.

Proficiency Testing

Proficiency testing samples for HPV detection are available through the College of American Pathologists. The survey samples are designed to simulate cervical specimens and are available in several different types of collection media. Participants should choose the survey that best represents the specimen collection media(s) that are processed in their laboratory and should participate in genotyping if applicable. In Europe, External Quality Assessment programs and Proficiency Testing for HPV are offered by QCMD (Quality Control for Molecular Diagnotics, Glasgow, Scotland) and UKNEQAS (United Kingdom National External Quality Assessment Service, Sheffield, UK). Proficiency testing should be performed by laboratories releasing clinical HPV results in accordance with appropriate regulatory organizations.

FUTURE DIRECTIONS

Incorporation of HPV testing and genotyping into clinical guidelines has made HPV assay development economically attractive to manufacturers. It is likely that new tests with expanded genotyping options and greater flexibility in sample handling, to address needs of point-of-care testing as well as high-throughput laboratories, will become available. It remains to be seen how HPV testing as a primary screen for cervical cancer will be incorporated into guidelines for cervical cancer screening in the United States. Patterns of practice have shown that adding HPV to cytology screening (cotesting) has been slowly adopted and was not always used according to recommended guidelines (70). Regardless of recommendations, it can be anticipated that adoption will be slow. Clinical experience with cotesting results may help clinicians and patients feel comfortable with omitting cytology during the primary screen. Reduced costs for testing may occur as additional assays receive FDA approval for this indication. Increased study of methods to triage women who test positive for HPV should lead to improved methods for follow-up and should help mitigate concerns about overreferral to colposcopy and loop electrosurgical excision procedure.

Three HPV vaccines are licensed for use in the United States. Cervarix (GlaxoSmith Kline, Rixensart, Belgium), Gardasil, and Gardasil 9 (Merck and Company, Inc., Whitehouse Station, NJ) all target HPV 16 and 18, the two HR HPV types associated with approximately 70% of cervical cancers worldwide. Gardasil also targets two LR types (HPV 6 and 11) that are associated with over 90% of genital warts. Gardasil 9 targets the four types in Gardasil and an additional five HR types (31, 33, 45, 52, and 58). Routine HPV vaccination has been recommended by the Advisory Committee on Immunization Practices (ACIP) for girls age 11 to 12 years since 2006 and for boys age 11 to 12 years since 2011 (71). Recommendations including the 9-valent vaccine were issued in 2015 (72). All three vaccines are licensed for use in girls; only Gardasil and Gardasil 9 are licensed for use in boys. Clinical trials show the vaccines have high efficacy for the prevention of high-grade cervical cancer precursors associated with types targeted by the vaccine. Cancer precursors were used as a surrogate marker for cervical cancer since the standard of care is to identify and treat cervical precancer. In addition, the time to development of cervical cancer makes this endpoint infeasible. While it is expected that universal vaccination will prevent essentially all cervical cancer associated with HPV 16 and 18, cervical cancer screening will still be required since about 30% of cases of cervical cancer are associated with other types. It is estimated that the additional five HR types targeted by Gardasil 9 could prevent an additional 15% of cervcial cancer cases in the US (73).

The introduction of HPV vaccines also brings the need for vaccine surveillance (74, 75). Sensitive HPV detection and typing will play a role in monitoring short-term end points of vaccine efficacy by determining the type distribution in the general population as well as in lesions. The optimal HPV testing strategy for public health surveillance will differ from that used in the clinical setting because high type-specific analytic sensitivity will be required in very-high-throughput testing formats in order to monitor changes in HPV types. Currently vaccine uptake in the United States has been slow. According to the most recent National Immunization Survey-Teen, only 37.6% of U.S. girls aged 13 to 17 were fully vaccinated (76). Despite suboptimal implementation, a reduction in vaccine-targeted HPV in young women (ages 14 to 19) was seen in data from the National Health and Nutrition Examination Surveys in postvaccine years (2007 to 2010) compared with prevaccine years (2003 to 2006) (77). Systematic evaluation of health care claims in private health care plans in the United States in 2003 to 2010 demonstrated reductions in the diagnosis of anogenital warts in young women (ages 15 to 24), suggesting an impact of HPV vaccination

(78). Finally, a significant reduction in HPV 16/18-related cervical precancers was identified in women who were vaccinated at least 24 months prior to screen-detected abnormality (79). These monitoring systems will continue with the expectation that the continuing data on HPV vaccine safety combined with reductions in cervical disease will foster increased uptake of the vaccine.

Currently guidelines for cervical cancer screening do not recommend changes based on HPV vaccination status. Widespread vaccination is expected to impact the efficiency of cervical cancer screening, particularly with cytology, as the number of cervical precancers decreases (80). The positive predictive value of HPV testing will also be impacted as the prevalence of disease decreases. Changes to cervical cancer screening guidelines, in terms of age of initiation, interval of testing, and type of test, will continue to be revised as data are gathered. Laboratories can anticipate that HPV testing and genotyping will remain important tools for at least the next decade.

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Molecular Diagnostics for Viral Infections in Transplant Recipients

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34

Viral infections are some of the most common complications in transplant recipients as a result of these patients' immunocompromised status. The ability to accurately detect and identify viral pathogens in transplant patients is important to tailor therapy and improve outcomes. In this chapter, we review the role of molecular tests in the diagnosis and management of human adenovirus (HAdV), BK virus (BKV), cytomegalovirus (CMV), Epstein-Barr virus (EBV), human herpesvirus 6 (HHV-6), herpes simplex virus (HSV), parvovirus B19, and varicella-zoster virus (VZV) in hematopoietic stem cell transplant (HSCT) and solid-organ transplant (SOT) recipients.

ADENOVIRUS

HAdVs are nonenveloped, double-stranded DNA viruses associated with a wide variety of clinical syndromes. There are over 55 recognized serotypes of HAdV that are grouped into seven species (A to G) based on the level of DNA homology, hemagglutinin properties, and pathogenicity. Among these seven species, six (A to F) are known to be a common cause of disease in humans. Adenoviruses establish latent infection in lymphocytes, but unlike the herpesviruses, HAdV does not appear to yield lifelong latency in the host, but rather, latency over a period of several months to several years. This has important implications in transplant recipients, as reactivation can occur when an individual becomes immunosuppressed; however, detection of HAdV may not always be associated with clinical disease.

In immunocompetent individuals, adenoviruses typically cause mild, self-limited respiratory, gastrointestinal, or conjunctival disease. However, in transplant recipients and other highly immunosuppressed patients, HAdV can cause severe, life-threatening disease. Pediatric HSCT recipients are at highest risk for disseminated HAdV disease, although high morbidity and mortality may also occur in adults and in SOT recipients. Interestingly, HAdV species C types have been shown to be associated with more severe disease among immunosuppressed patients (1, 2). The incidence of adenovirus disease in transplant recipients is not well defined because available studies have: (i) used different definitions of disease, (ii) employed different diagnostic methods, and (iii) evaluated diverse patient populations. Furthermore, large prospective studies have not been performed (3).

In bone marrow or HSCT recipients, HAdV may cause upper and lower respiratory tract infection, gastrointestinal disease, hepatitis, or cystitis (4). Progression to disseminated disease occurs in 10 to 20% of patients, especially those with severe lymphopenia and those receiving intense immunosuppression for graft-versus-host disease (3). Respiratory tract disease can range from a mild, nonspecific upper respiratory illness, to severe pneumonia. Gastrointestinal tract disease ranges from mild diarrhea to hemorrhagic colitis and is often associated with HAdV species B and C. Hemorrhagic cystitis (HC) is frequently associated with adenovirus type 11, and less commonly with the other species B viruses. Detection of adenovirus in the urine by real-time PCR is frequently associated with HC.

The onset of HAdV disease usually occurs within the first 100 days following HSCT, although disease onset may be later in adults. Overall, the mortality rate in HSCT recipients for untreated adenovirus disease is estimated to be 25%; it can be as high as 50% and 80% for pneumonia and disseminated disease, respectively (3).

In SOT recipients, adenovirus-associated HC, nephritis, pneumonia, hepatitis, enterocolitis, and disseminated disease have been described (3). Adenovirus serotype 5 is typically associated with hepatitis in liver transplant recipients, whereas serotypes 1 and 2 are more commonly associated with pneumonia. Enterocolitis occurs more commonly in small-bowel transplant recipients and may mimic rejection. Adenovirus pneumonia is associated with graft loss, death, or progression to obliterative bronchiolitis in lung transplant recipients. In pediatric heart transplant recipients, detection of adenovirus DNA in myocardial biopsies may predict adverse clinical events including coronary vasculopathy and graft loss.

Diagnosing adenovirus disease is often problematic because detection of the virus does not always correlate with clinical symptoms and other co-pathogens may contribute to the clinical syndrome. A definitive diagnosis of HAdV disease is accomplished by visualizing adenovirus nuclear inclusions on routine histopathologic examination and/or detecting the virus by culture or real-time PCR from biopsy specimens (5). Treatment options for HAdV are limited and may include (i) reduction in immunosuppression and/or (ii) the use of antiviral therapy. Currently, the only

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antiviral agent with potential clinical efficacy is cidofovir, a cytosine analogue that inhibits DNA polymerase.

Evaluation of transplant recipients for adenovirus infection can include culture or real-time PCR of blood, plasma, tissue, respiratory, stool, or urine specimens. The virus is found at the highest levels in liver tissue, but is also predominant in the bone marrow, lung, and pancreas. Inoculation of clinical samples into routine viral cell culture has been used for many years, but this approach requires the observation of viral cytopathic effect, which may take up to several weeks to develop. Centrifugation culture (rapid shell vial) using either single cell lines or a mixture of cell lines provides more rapid results, but is not as sensitive as conventional tube culture. Real-time PCR assays for HAdV have shown improved sensitivity over culture methods, provide rapid results, and may be used for quantitative purposes (6–8). Due to the potential for HAdV to cause latent infection, quantitation of the virus in clinical samples in order to document high levels of viral replication has diagnostic, clinical, and prognostic significance (9–11).

Genetic variability is high among adenoviruses, and consequently, more than one set of primers and probes is often necessary to detect all serotypes. The transactivating region of the E1A gene and the 3′ end of the hexon gene are the most conserved target regions among the different serotypes (12, 13), but there is no universally accepted target region or primer-probe combination. The lack of a universal adenovirus DNA standard complicates real-time PCR assay calibration and comparison of results between laboratories.

Blood specimens should be obtained for quantitative real-time PCR as soon as adenovirus disease is considered, and viral loads should be monitored weekly. Plasma or serum, rather than whole blood, should be tested due to the low positive predictive value of detecting HAdV in whole blood. Patients with persistently high viral load measurements (e.g., of $>10^4$ copies/ml) are at greater risk for severe end-organ or disseminated adenovirus disease, and viral load measurements that exceed $>10^6$ copies/ml have been associated with a higher likelihood of death (9, 14).

Although the positive predictive value of detecting HAdV in whole blood is low, detection of adenovirus by real-time PCR in blood (or stool) has been suggested as a potentially effective screening tool to identify HSCT recipients at risk for progression to adenovirus disease (4). However, the outcomes of asymptomatic patients with adenovirus viremia are variable, with patients often clearing the viremia without specific antiviral therapy or reduction in immunosuppression. Screening of asymptomatic SOT recipients for adenovirus is generally not helpful, because progression to disease is infrequent (3).

Adenovirus infections are increasingly recognized as a significant cause of morbidity and mortality in transplant recipients. However, much remains to be learned about the natural history of adenovirus infections in HSCT and SOT recipients. Molecular diagnostics will play a major role in increasing our understanding of the disease processes by providing sensitive and specific tools for diagnosing and managing patients with adenovirus infections.

BKV

BKV is a member of the *Polyomaviridae* family, which also includes JC virus and simian virus 40 (SV40). It is an enveloped, double-stranded DNA virus that shares approximately 70% sequence homology with JC virus and SV40. The seroprevalence to BKV typically exceeds 90% by early childhood, with most healthy individuals experiencing an asymptomatic, primary infection (although fever and nonspecific upper respiratory tract symptoms may occur) (15). After primary infection, the virus can remain latent in many sites, most notably the epithelium of the urinary tract and lymphoid cells. In transplant recipients and other immunosuppressed hosts, reactivation of BKV can occur with subsequent active replication of the virus. Reactivation of BKV in immunocompromised hosts may be asymptomatic or cause organ dysfunction, affecting the kidney, bladder, or ureter. BKV disease in the urinary system manifests as HC or nonhemorrhagic cystitis and ureteric stenosis in HSCT and SOT recipients (16). BKV infection may also cause polyomavirus-associated nephropathy (PVAN) in renal transplant recipients (17).

HC is a cause of morbidity and occasional mortality in HSCT recipients (18). The manifestations range from microscopic hematuria to severe bladder hemorrhage leading to clot retention and renal failure. The incidence of HC varies from 7 to 68% following HSCT. Although mild HC usually resolves with supportive care, severe HC may require bladder irrigation, cystoscopy, and cauterization (19). BKV was observed in early studies to be associated with the development of HC after HSCT; however, subsequent studies using sensitive PCR assays have shown that BKV DNA can be detected in the blood and urine of patients with or without HC (20–22). Recently, quantitative assays for BKV DNA in urine have demonstrated that patients with HC have higher peak BK viruria and larger total amounts of BKV excreted following HSCT compared with asymptomatic patients (23, 24).

Although BKV was first isolated from the urine of a renal transplant recipient in 1971 (25), the association between nephropathy and BKV in renal transplant recipients was not reported until 1995 (26). BKV replication in renal allografts can lead to progressive graft dysfunction and, potentially, graft failure and loss. The risk factors for development of PVAN have not been elucidated, but are likely a function of an over-immunosuppressed status (27). The prevalence of PVAN ranges from 1 to 10% in kidney transplant recipients, with loss of allograft function in about one-third to one-half of these cases if pharmacologic immunosuppression is not minimized (27). The disease appears to result most commonly from reactivation of BKV infection in the donor allograft.

The signs and symptoms of PVAN are mild and nonspecific, often with only a gradual increase in serum creatinine levels over weeks, as the allograft loses function (28, 29). This is often misdiagnosed clinically as allograft rejection. A definitive diagnosis of PVAN is obtained through histopathology of the biopsied kidney, with characteristic patterns showing viral cytopathic changes in epithelial cells, and interstitial inflammation and fibrosis. However, these changes are not pathognomonic for PVAN, and most centers use immunohistochemical staining with antibodies specific for polyomavirus proteins or *in situ* hybridization to confirm the diagnosis (27). Because of the focal nature of the nephropathy, and the possibility of sampling error, negative biopsy results do not rule out PVAN. Biopsy of the kidney is an invasive procedure that is impractical for serial monitoring and clinical management of patients with PVAN. Other less invasive diagnostic methods for PVAN have also been assessed. Urine cytology may reveal renal epithelial cells with intranuclear viral

inclusion bodies, termed decoy cells (30, 31). The sensitivity and specificity of decoy cells for diagnosing PVAN have been estimated at 99% and 95%, respectively; however, the positive predictive value varies between 27% and 90% (30, 31). Quantification of BKV in urine by nucleic acid amplification methods has been proposed as a method to monitor trends in BKV viral load (29, 32–34). However, asymptomatic shedding of the virus in the urine occurs naturally, and may complicate the interpretation of the results. Moreover, physiological changes of urine and the use of different urine fractions may yield considerable variation in viral load values, and this has complicated the establishment of diagnostic thresholds and interpretation of results (17). PCR methods for detection and quantitation of BKV in a sterile site, such as blood (e.g., BKV viremia), have emerged as clinically useful tools in the diagnosis and management of PVAN, as viremia precedes the development of nephropathy by up to 5 weeks in most cases (31, 34–38).

In 2005, an expert interdisciplinary panel recommended the use of either urine cytology or nucleic acid amplification tests (NAATs) to screen renal transplant recipients: (i) every 3 months up to 2 years posttransplant, (ii) whenever allograft dysfunction occurs, or (iii) when an allograft biopsy is performed (27). Those patients with a positive screening test should have an adjunct quantitative NAAT performed using urine or plasma. Patients with a urine BK viral load of $>10^7$ copies/ml, or plasma viral load of $>10^4$ copies/ml that persists for more than 3 weeks, are considered to have presumptive PVAN and should have a renal biopsy performed to confirm the diagnosis.

Reducing the intensity of immunosuppression is the primary intervention in patients with PVAN. No effective antiviral agents for BKV are available but low-dose cidofovir has been used for treatment of those cases that are either not amenable, or are clinically refractory to, reduction in immunosuppression (27). The BK viral load in urine or plasma should be monitored every 2 to 4 weeks to monitor the effectiveness of the intervention.

Currently, real-time PCR is the method of choice for BKV DNA quantification due to its sensitivity and wide dynamic range. High viral loads (e.g., $>10^7$ copies/ml) in plasma are very uncommon, but comparatively, levels in the urine often exceed 10^{12} copies/ml. Although BK viral load tests have become a standard of care for diagnosis and monitoring of patients with PVAN, there is neither consensus in the design of PCR assays nor recognized standard reference material. As a consequence, most clinical laboratories rely on laboratory-developed tests (LDTs) that may yield markedly different results between institutions. The lack of a standardized assay for detection and quantification of BKV causes individual laboratories to establish and verify their own clinical threshold values (39, 40). A real-time PCR analyte-specific reagent for quantification of BKV DNA is available (Elitech Group, Puteaux, France), but published data on the performance characteristics of this assay are limited (41).

PCR assay design is complicated by the high degree of homology between the genomes of the different human polyomaviruses. Gene targets for BKV-specific assays include coding sequences for VP1, large T antigen, and Agno protein, since these sequences are sufficiently variable among human polyomaviruses (36, 42–44).

Only in the past few years have a large number of full-genome BKV sequences been published (45–47). As a consequence, the extent of BKV genetic diversity is not reflected in the design of many commonly used PCR assays. BKV has been classified into six subtypes based on phylogenetic analysis of full-genome sequences: subtypes Ia, Ic, III, IV, V, and VI (46). Hoffman et al. (48) compared seven TaqMan real-time PCR primer-probe sets in conjunction with two different reference standards to measure BKV DNA levels in urine samples. The authors observed substantial disagreement among assays attributable both to features of the primer and probe design, and to choice of reference material. The most significant source of error was primer and probe mismatches due to BKV subtype polymorphisms, primarily among subtype III and IV isolates. However, the authors found less subtype bias among the seven assays for the more common subtypes Ia, V, and VI. The assay that provided the most reliable measure of all subtypes included a mixture of primers and probes that targeted both the VP1 and large T antigen sequences.

Molecular diagnostics play a central role in the diagnosis of HC in HSCT patients and in the diagnosis and management of PVAN in renal transplant recipients. Increased knowledge about the genetic heterogeneity of BKV should lead to improvements in assay design. The limited availably of analyte-specific reagents, absence of standard reference material, and lack of FDA-cleared test kits contribute to wide interlaboratory variation of results, and make it difficult to establish interpretive guidelines that can be applied broadly in clinical practice.

CMV

CMV is a common pathogen, with seroprevalence rates of 40 to 70% among healthy adults in the United States and rates potentially exceeding 90% in developing nations. As a result, it is one of the most frequent causes of disease in SOT and HSCT recipients. CMV may be transmitted via infected donor organs, cells, or cellular blood products. Furthermore, the virus may reactivate following transplantation in seropositive patients. Primary infection may occur when a seronegative individual receives a transplant from a seropositive donor. Secondary infection develops when endogenous (latent) virus is reactivated in a CMV-seropositive individual posttransplantation. Superinfection (or reinfection) occurs when a seropositive recipient receives latently infected cells from a seropositive donor, and the virus that reactivates posttransplantation is of donor origin. Community-acquired infection is also possible. Following primary infection, long-term cellular and humoral immunity develops, but CMV remains latent within the host indefinitely.

Following the establishment of a latent infection, periods of intermittent viral reactivation are usually controlled by an intact, cellular immune system. However, high levels of immunosuppression following transplantation may lead to uncontrolled viral replication, and consequently, CMV disease. SOT recipients without prior immunity to CMV (seronegative recipients) who receive a transplant from a seropositive donor are at the highest risk of uncontrolled viral replication and subsequent disease. In contrast, CMV-seropositive recipients of hematopoietic stem cells from CMV-seronegative donors are at highest risk of CMV disease after HSCT. In addition to the serostatus of the donor and recipient, other factors that impact the risk of CMV disease following transplantation are the type of transplant (e.g., HSCT or lung versus other solid organs) and the degree of pharmacologic immunosuppression. Certain

compounds, such as antithymocyte globulins, alemtuzumab, and mycophenolate mofetil, can markedly impair the function of the immune system and increase the risk for CMV disease.

CMV infection in transplant recipients can cause a wide range of clinical manifestations, from asymptomatic infection to severe, life-threatening disease. Most cases of CMV disease following transplantation are mild to moderate in severity and those infected may present with fever, malaise, and decreased neutrophil counts (often referred to as "CMV syndrome"). Myalgias and arthralgias often occur. In SOT recipients, organ involvement correlates with the organ transplanted, with hepatitis occurring most frequently in liver transplant recipients, pancreatitis occurring most frequently in pancreas transplant recipients, and pneumonitis occurring most frequently in lung transplant recipients. Involvement of the gastrointestinal tract is the most common organ involved in tissue-invasive CMV disease [49, 50]. CMV can affect any segment of the gastrointestinal tract, including the esophagus, stomach, and small and large intestines. Symptoms may include dysphagia, odynophagia, nausea, vomiting, abdominal discomfort, gastrointestinal hemorrhage, and/or diarrhea. Intestinal perforation may also occur. Endoscopic findings may show erythema and diffuse, shallow erosions or localized ulcerations; however, these findings are nonspecific and biopsy is needed to confirm the diagnosis. CMV pneumonitis presents as fever, dyspnea, and cough, with findings of hypoxemia and pulmonary infiltrates. Radiographic appearances include bilateral interstitial, unilateral lobar, and/or nodular infiltrates. CMV retinitis is rare in transplant patients and usually presents very late in the posttransplant period. Patients may be asymptomatic or may experience blurring of vision, scotoma, or decreased visual acuity. The diagnosis of CMV retinitis is often supported by funduscopic examination. CMV may also cause disease of the central nervous system resulting in encephalitis or polyradiculopathy.

For transplant recipients that develop CMV disease, intravenous ganciclovir or oral valganciclovir is the treatment of choice. Foscarnet and cidofovir are also treatment options, but these are typically reserved for patients who are intolerant to or who have failed ganciclovir as they have significant side effects, including nephrotoxicity and electrolyte disturbances.

Methods of preventing CMV disease in transplant recipients involve two major strategies: (i) antiviral prophylaxis and (ii) preemptive therapy. In addition, CMV-seronegative, filtered, or leukocyte-reduced blood products should be used when needed. Passive immunization with immunoglobulins is less commonly used as it is expensive and less effective than antiviral drugs. In many transplant programs, especially SOT programs, the prevention strategy of choice is antiviral prophylaxis with oral valganciclovir, and it is given for at least 3 months among CMV-seropositive SOT recipients, and at least 6 months for CMV D+/R- SOT recipients. Preemptive therapy, which involves serial monitoring for CMV replication and administering of antiviral treatment upon detection of CMV, is also used in many transplant programs, particularly among HSCT recipients. As antiviral therapy for CMV disease becomes more common, the potential exists for emergence of resistance to ganciclovir or other antivirals (51). Antiviral resistance has been shown to be associated with mutations in the genes *UL97* (phosphotransferase) and *UL54* (DNA polymerase). Mutations in *UL97* generally occur first and confer resistance to ganciclovir, while subsequent *UL54* mutations can confer cross-resistance to cidofovir and foscarnet (52). Combined resistance to ganciclovir and foscarnet due to dual-strain CMV coinfection has been reported (53).

Laboratory methods for CMV include serology, viral cell culture, antigenemia, histopathology, and real-time PCR. Serology is useful in determining the serostatus of recipients and donors prior to transplantation; however, this method has no role in the diagnosis of active CMV disease following transplantation. Viral culture has limited clinical utility as the recovery of CMV in blood specimens has poor sensitivity (e.g., low negative predictive value) and the growth of CMV from urine has been associated with poor specificity (e.g., low positive predictive value). The detection of CMV antigenemia (i.e., pp65) was used for years in the diagnosis and management of disease; however, pp65 antigenemia is labor intensive, requires a subjective interpretation, and is not standardized among testing laboratories. Due to the limitations of conventional methods, molecular tests (e.g., real-time PCR) have become the method of choice for the diagnosis and management of CMV in the transplant population. The qualitative detection of CMV from certain sample types (e.g., biopsy tissue, sterile body fluids) by real-time PCR may assist in the diagnosis of CMV disease (54). However, quantitative methods are frequently required due to the possibility of detecting virus that is not associated with disease. Quantitative real-time PCR from plasma or whole blood is now commonly used to diagnose CMV disease and to monitor the response to antiviral therapy (55). Until recently, clinical laboratories have relied on LDTs or nonstandardized commercial assays for the detection and quantitation of CMV. Therefore, the quantitative threshold at which a diagnosis is made or preemptive therapy initiated has varied between institutions and transplant populations (e.g., HSCT versus SOT). For example, Gerna et al. showed that in SOT recipients, a cutoff of 300,000 copies/ml was useful to guide preemptive therapy of both primary and reactivated CMV infection (56). Interestingly, the same group proposed a cutoff of 10,000 copies/ml for adult HSCT recipients (57). Humar et al. demonstrated by receiver operator curve analysis that among 97 liver transplant patients, a viral load of 2,000 to 5,000 copies/ml showed a sensitivity and specificity of 85.7% and 86.6%, respectively, for detecting CMV disease (58). The results of these studies underscore the need for a standardized approach to diagnose and monitor CMV in the transplant population.

In July 2012, the first FDA-approved assay (cobas AmpliPrep/cobas TaqMan [CAP/CTM] CMV; Roche Diagnostics, Indianapolis, IN) for the detection and quantification of CMV in plasma became available in the United States. This real-time PCR assay is calibrated against the WHO CMV standard, and has a reportable range of 137 to 9,100,000 international units (IU)/ml. Hirsch et al. demonstrated that the precision of quantitative CMV viral loads was significantly better among laboratories performing the FDA-approved CAP/CTM assay compared to laboratories performing nonstandardized tests (59). Other commercial assays for the detection and quantification of CMV are available, some of which (e.g., artus CMV RGQ MDx; Qiagen, Valencia, CA) have also been approved by the FDA (60). The broad implementation of standardized, FDA-approved assays for the quantification of CMV should assist in the establishment of clinical thresholds for the diagnosis and management of CMV. However,

establishing a universal "cutoff" for defining CMV disease will be difficult, and health care providers should rely on the trend of viral loads over time, rather than using a result obtained at a single point in time (61). Current recommendations are to perform baseline viral load testing on the day CMV treatment is initiated and repeat quantitative real-time PCR every 5 to 7 days for at least 3 months (62).

EBV

EBV is a member of the *Herpesviridae* family and has a worldwide seroprevalence of >95% among adults over the age of 40. Primary infection with EBV is followed by lifelong latency, with reactivation being possible in immunocompromised hosts. In transplant recipients, EBV infection may cause malaise, fever, headache, and sore throat, but may also result in posttransplant lymphoproliferative disorder (PTLD). PTLD is a spectrum of lymphocytic proliferation that ranges from benign lymphocytic hyperplasia to potentially fatal malignant lymphoma. It is associated with significant morbidity and mortality and may involve the central nervous system (CNS), eyes, gastrointestinal tract (with bleeding and perforation), liver, spleen, lymph nodes, lungs, allograft, oropharynx, and other organs. Clinical presentations vary and include adenopathy, fever (including fever of unknown origin), abdominal pain, anorexia, jaundice, bowel perforation, gastrointestinal bleeding, renal dysfunction, liver dysfunction, pneumothorax, pulmonary infiltrates or nodules, and weight loss.

The pathogenesis of PTLD involves the exponential proliferation of B cells as a result of uncontrolled EBV infection. Risk factors for PTLD include a donor and recipient EBV seromismatch status (e.g., D+/R−), high degree of immunosuppression (especially the administration of antilymphocyte therapy for rejection), and a high EBV viral load (63). Most cases of PTLD occur during the first year posttransplant, and the cumulative incidence ranges from 1 to 2% in HSCT and liver transplant patients, up to 11 to 33% in intestinal or multi-organ transplant recipients (64).

Treatment of EBV-related PTLD involves the reduction in immunosuppression and the use of anti-CD20 monoclonal antibody. Once PTLD is established, antiviral treatment is not effective and the level of immunosuppression must be reduced. Rituximab is the chimeric anti-CD20 monoclonal antibody that is used for treatment of most cases, while in those with refractory disease, combined chemotherapy may be required, with or without radiation therapy. Adoptive immunotherapy using donor-derived, cloned EBV-specific cytotoxic T cells may be useful for prophylaxis and treatment of lymphoproliferative disease in allogeneic HSCT (and SOT) transplant recipients, but their use is not yet widespread in the clinical arena.

The laboratory diagnosis of EBV-related PTLD is established through a combination of radiology (i.e., computed tomography and positron emission tomography), excisional tissue biopsy with review of stained sections by a hematopathologist, and quantitative real-time PCR. Tissues from patients with PTLD may show monoclonal, oligoclonal, or polyclonal lesions. *In situ* hybridization targeting *EBER1* and/or *EBER2* is the gold standard assay for determining whether a lymphoproliferative process is EBV-related. Commercial systems for *EBER in situ* hybridization are available from Ventana (Tucson, AZ), Leica (Bannockburn, IL), Dako (Glostrup, Denmark), Invitrogen (Carlsbad, CA), and Biogenex (San Ramon, CA) (65).

Although biopsy is required to establish a definitive diagnosis, quantitative real-time PCR is important in identifying potential cases of PTLD as well as monitoring a patient's response to rituximab treatment. Increases in EBV viral load by nucleic acid amplification of peripheral blood (whole EDTA blood, peripheral blood lymphocytes, or plasma [66]) may be detected in patients before the development of EBV-associated PTLD (67–69), and high viral loads typically decrease with effective therapy. Whereas high EBV DNA viral loads are strong predictors for the development of PTLD, low-level elevated EBV viral loads occur relatively frequently, and may resolve without intervention (70, 71). To complicate the matter, some pediatric liver and heart transplant recipients may exhibit chronic EBV DNAemia (72, 73) in the absence of a lymphoproliferative disorder.

Due to the lack of an FDA-approved test for the quantitation of EBV nucleic acid, clinical laboratories have relied on either LDTs, or modified, commercially available assays. This approach lacks standardization, and the optimal assay and sample type (i.e., whole blood, lymphocytes, or plasma) are not well defined. Nevertheless, a number of laboratory-developed and commercial EBV viral load assays have been developed and have demonstrated good performance characteristics (65, 74, 75). Although a quantitative threshold for predicting PTLD has not been established, persistently detectable levels of EBV DNA (cutoffs vary between programs) typically result in a thorough evaluation for PTLD (e.g., computerized axial tomography of the chest, abdomen, and pelvis). Commercial primers and probes are available from Roche Diagnostics targeting *LMP2*; Qiagen, Bioactiva Diagnostica (Bad Homburg, Germany), and Amplimedical (Milan, Italy) targeting *BKRF1* (*EBNA1*); Elitech (Puteaux, France) targeting a major tegument protein (*BNRF1*); and Argene (Varilhes, France) targeting thymidine kinase (*BXLF1*) (65, 76, 77).

HHV-6

HHV-6 infection can present as primary or reactivated disease in HSCT and SOT recipients. It may be transmitted via infected donor organs, cells, or cellular blood products, or the recipient's own virus may reactivate. HHV-6 may result in fever, bone marrow suppression, CNS dysfunction, pneumonitis, and/or hepatitis. Ganciclovir or foscarnet is used for treatment in some cases.

HHV-6 can be cultured from peripheral blood mononuclear cells (and other clinical specimens). However, isolation is labor-intensive and can take up to 21 days (although the detection time can be shortened to 1 to 3 days with the use of the shell vial assay). In addition to a long processing time, culture-based assays suffer from poor sensitivity. HHV-6 antigens can be demonstrated by *in situ* hybridization tests in formalin-fixed, paraffin-embedded tissues.

The most commonly used molecular test for the laboratory diagnosis of HHV-6 is real-time PCR, which can be performed on a variety of sample types (78–80). Qualitative real-time PCR does not differentiate between primary and latent infection and therefore quantitative testing may be needed. This is especially important given that a small percentage of patients (<1%) have chromosomally integrated HHV-6 (CIHHV-6) (81, 82). The presence of CIHHV-6 will cause positive PCR results from clinical samples, although the clinical significance of these results is not well defined. Lee et al. showed that liver transplant

patients with CIHHV-6 had a significantly higher risk of bacterial infection and allograft rejection compared to patients without CIHHV-6 (83). These results suggest that the ability to differentiate between active and CIHHV-6 may be important in the transplant population. A droplet digital PCR assay was recently shown to detect CIHHV-6 with a sensitivity and specificity of 100% and 82%, respectively (82). This was accomplished by determining the ratio of HHV-6 to cellular DNA in patient samples, with a ratio of "1" indicating the presence of CIHHV-6. Continued research in this area will be important as the role of HHV-6 and CIHHV-6 in transplant recipients is further defined.

HSV

Like other members of the human *Herpesviridae* family, HSV types 1 and 2 (HSV-1 and HSV-2) establish life-long latency following initial, primary infection. In transplant recipients, HSV most commonly causes reactivated disease, although primary infections can occur in seronegative individuals. Orolabial infections are most commonly caused by HSV-1 and may be asymptomatic to mild in severity; however, significant ulceration and discomfort, which may be complicated by bacterial superinfection or esophageal involvement, are occasionally found in highly immunosuppressed patients. Anogenital infection is frequently caused by HSV-2 and usually presents as ulceration that may or may not be accompanied by characteristic herpetic vesicles. Treatment of HSV infection is accomplished using acyclovir or valacyclovir, and resistance to these antivirals remains low (at <5%) overall.

On rare occasions, reactivated or primary HSV infection causes pneumonitis, tracheobronchitis, esophagitis, hepatitis, or disseminated infection. HSV pneumonitis usually occurs as a secondary process in intubated patients with pneumonia, due to another etiology. Due to the potential for asymptomatic oropharyngeal shedding of the virus, detection of HSV in respiratory secretions does not definitively diagnose HSV as the cause of pneumonitis. Therefore, a lung biopsy may be needed to establish the diagnosis. HSV esophagitis typically presents as dysphagia, which mimics candidal esophagitis. Another possible outcome of HSV infection is disease of the CNS, which should be diagnosed and treated immediately due to the high morbidity and mortality associated with the illness.

Historically, viral culture has been considered the gold standard method for the detection of HSV in clinical samples. However, numerous studies have shown that real-time PCR is more rapid and sensitive (84, 85). For dermal or genital disease, specimen collection consists of vigorously rubbing a culture transport swab over the suspect skin or mucosal lesion. It should be emphasized that calcium alginate-tipped swabs, wooden-shaft swabs, or transport swabs containing gel are unacceptable for PCR testing. Real-time PCR on cerebrospinal fluid is useful to diagnose HSV CNS disease, although this clinical entity is infrequent in transplant recipients. Ideally, real-time PCR assays for HSV should detect and differentiate between HSV-1 and HSV-2. A number of LDTs have been described (84–86) for the detection of HSV-1/2 in a variety of clinical samples; however, several FDA-cleared assays are now available (Table 1). Notably, the Simplex HSV-1/2 assay (Focus Diagnostics, Cypress, CA) recently gained clearance from the FDA for testing cerebrospinal fluid collected from patients with suspected HSV CNS disease. This test does not require up-front nucleic acid extraction, and results are available in approximately 60 minutes (87).

PARVOVIRUS B19

Parvovirus B19 (B19) is a small, single-stranded, nonenveloped DNA virus belonging to the family *Parvoviridae* and the genus *Erythrovirus*. The erythroviruses are now divided into three distinct genotypes with isolates closely related to B19 corresponding to genotype 1 and isolates related to V9 distributed into genotypes 2 and 3 (88). Although the genotypes differ by about 10% at the nucleotide level, they appear to have similar antigenic and pathogenic properties. B19 infections in humans are very common with a seroprevalence of 60 to 90% in adults.

Primary B19 infections, which occur most commonly in children, typically manifest as erythema infectiosum. In adults, primary B19 infection may present as arthropathy, and infection during pregnancy can lead to hydrops fetalis. B19 has a propensity to infect erythroid progenitor cells and leads to a severe anemia in individuals with hemolytic disorders. The cellular receptor for B19 is the blood group P antigen, or globoside (89). This receptor can also be found on cardiac myocytes, white blood cells, platelets, and trophoblasts and reflects the ability of B19 to infect other types of cells.

The first report of B19 infection in a transplant recipient was published in 1986 (90). Since then, a number of reports of B19 infections following SOT and HSCT have been published in the literature. Severe anemia is the most common clinical manifestation, but B19 infection has also been associated with hepatitis, pneumonitis, myocarditis, and allograft dysfunction (91). However, evidence is lacking for a causal role for B19 in these organ-specific syndromes.

Several studies have assessed the incidence of B19 infection among transplant patients and it has been estimated to be 2 to 31% (5, 91–94). A high index of clinical suspicion for B19 infection is advised when transplant patients present with unexplained refractory and severe anemia (91). Reduction in immunosuppression and the use of intravenous IgG are the only available interventions for patients with B19 infections.

Serological testing for B19-specific IgG and IgM antibodies is common for the diagnosis of infection in immunocompetent individuals (95). However, nucleic acid amplification tests are the method of choice for detecting B19 infections in immunosuppressed individuals. Plasma, serum, and bone marrow aspirates are most commonly tested, but appropriate tissue specimens from patients with organ-specific syndromes may also be submitted for analysis.

A number of laboratory-developed PCR-based assays for detection of B19 DNA have been described. The primers and probes of some of the earlier assays were designed before the amount of genetic diversity within the erythroviruses was defined. Consequently, some assays may not be suitable for detection of genotypes 2 and 3. Consensus primers and probes targeting the *NS1* gene, or VP1 unique region, have been successful in detecting genotypes 1 to 3 with equal sensitivities (88, 96, 97). Agarose gel electrophoresis with Southern blotting, dot hybridization, or enzyme-linked immunoassay were used for amplified product detection in the earliest iterations of PCR assays for B19. More recently, real-time PCR assays have been employed because of the benefits of speed, reduced risk of contamination, and ease of quantification (98, 99). The

TABLE 1 Examples of PCR assays for the detection and identification of viruses impacting transplant recipients

Virus	Technology (manufacturer)	Assay target	Specimen type(s)	FDA-approved	Reference(s)
Adenovirus	Nested multiplex PCR with endpoint melting curve analysis (BioFire FilmArray)	Hexon gene	NP swab	Yes	106
	Micro-array hybridization with solid-phase electrochemical detection (GenMark eSensor XT-8)	Hexon gene	NP swab	Yes	106
	xTAG (Luminex)	Hexon gene	NP swab	Yes	106
	TaqMan (Argene/bioMérieux)	Hexon gene	NP swab/aspirate	Yes	8
	FRET (LDT)	Penton base gene	Respiratory specimens, blood, stool, urine, ocular swabs	No	6
BKV	MGB (Elitech)	VP1 gene	Urine, plasma	No	41, 48
	FRET	VP2	Urine	No	39, 40
CMV	TaqMan (Roche)	UL54 gene	Plasma	Yes	59
	TaqMan (Qiagen)	MIE gene	Plasma	Yes	60
	FRET (LDT)	US9 gene	Respiratory specimens, urine, CSF, tissue, amniotic fluid, bone marrow	No	54
EBV	TaqMan (Argene/bioMérieux)	*bxlf1* (thymidine kinase)	Whole blood	No	74, 76
	TaqMan (Artus/Qiagen)	EBNA1	Whole blood	No	74
	FRET (Roche)	LMP1	Whole blood	No	77
	TaqMan (LDT)	BALF5	Whole blood	No	75
HHV-6	MGB (LDT)	U67 gene	Whole blood, CSF	No	80
	TaqMan (Argene/bioMérieux)	U57 gene	Whole blood, CSF	No	80
	TaqMan (LDT)	U95 gene	PBMC, plasma	No	78
	TaqMan (ddPCR)	U67 gene	PBSC, PBMC, FFPE, plasma	No	82
HSV	Scorpion (Focus Diagnostics)	DNA polymerase	CSF	Yes	87
	MultiCode-RTx (Eragen/Luminex)	Glycoprotein B gene	Vaginal swab	Yes	107
	TaqMan (Quidel)	NA	Cutaneous or mucocutaneous swab	Yes	NA
	FRET (Roche)	DNA polymerase	Genital, dermal, ocular, CSF	No	104
Parvovirus B19	TaqMan (Qiagen)	NA	Serum, plasma, CSF, bone marrow, amniotic fluid	No	100
	TaqMan (LDT)	VP1 gene	Plasma	No	108
	TaqMan (LDT)	NS1 gene	Plasma	No	109
VZV	TaqMan (Quidel)	NA	Cutaneous and mucocutaneous swabs	Yes	NA
	TaqMan (LDT)	DNA polymerase	Cutaneous and mucocutaneous swabs	No	85
	FRET	Gene 28	Cutaneous swabs	No	104

NP, nasopharyngeal swab; FRET, fluorescent resonance energy transfer; LDT, laboratory-developed test; MGB, minor groove binder; MIE, major immediate early; CSF, cerebrospinal fluid; PBMC, peripheral blood mononuclear cells; ddPCR, droplet digital PCR; PBSC, peripheral blood stem cells; FFPE, formalin-fixed paraffin-embedded tissue; NA, not available.

wide dynamic range of real-time PCR assays is also beneficial, since B19 viral loads may reach levels as high as 10^{12} genome copies/ml during the acute phase of illness, and rapidly decline to low levels with the onset of antibody production.

No FDA-cleared nucleic acid amplification test for parvovirus B19 is available, but several manufacturers produce analyte-specific reagents (96, 100). The LightCycler parvovirus B19 quantification reagent (Roche Diagnostics Corp.) is highly sensitive for detection of genotype 1, but

does not adequately detect genotypes 2 and 3. The Artus Parvo B19 PCR reagents (Qiagen) provide less genotype bias in quantification of erythrovirus DNA (96).

VZV

VZV causes two distinct clinical diseases following transplantation. Most adult transplant recipients are seropositive to VZV prior to transplantation and therefore are at risk for reactivation, most commonly in the form of zoster. Zoster has been shown to develop in ~11% of renal and other SOT recipients within the first 3 to 5 years after transplant (101). Furthermore, VZV disease occurs in 35 to 40% of allogeneic peripheral blood stem cell transplant recipients within 3 years of transplant (102). Localized dermatomal zoster may involve one or more adjoining dermatomes, and widespread dissemination with visceral and/or CNS involvement may occur (102).

Transplant recipients that are seronegative to VZV are at risk for primary infection through contact with an infected individual. Although rare, primary infection can cause life-threatening disseminated infection. Primary VZV may also cause a chickenpox syndrome. Due to the severity of primary VZV infection in transplant recipients, SOT candidates should be screened for IgG-class antibodies to VZV prior to transplantation and if found to be negative, should be considered as candidates to receive the varicella vaccine. For nonimmune HSCT or SOT recipients that are exposed to VZV, postexposure prophylaxis is recommended (103). Low-dose acyclovir may prevent VZV reactivation and primary infection, but is typically not administered for a prolonged period of time.

Treatment of localized dermatomal zoster usually involves valacyclovir, famciclovir, or acyclovir. For primary VZV infection, intravenous acyclovir is recommended and varicella-zoster immunoglobulin may also be considered.

As with HSV, the most sensitive and specific test for diagnosis of VZV-associated skin lesions is real-time PCR. A culture transport swab is vigorously rubbed over the suspect skin lesion, placed in viral transport media, and submitted to the testing laboratory. Calcium alginate-tipped swabs, wood swabs, or transport swabs containing gel are unacceptable for PCR testing. VZV PCR can be performed on cerebrospinal fluid as an aid to the diagnosis of VZV CNS infection, which is occasionally diagnosed in transplant recipients. A number of LDTs have been developed for the detection of VZV in clinical samples (85, 86, 104), and the Quidel Lyra Direct HSV 1+2/VZV multiplex assay was approved by the FDA in 2014 for testing cutaneous and mucocutaneous lesion samples (Table 1).

CONCLUSIONS AND FUTURE DIRECTIONS

Molecular diagnostics play an important role in the diagnosis and management of transplant recipients with viral infections. These methods have greatly enhanced the ability of health care providers to rapidly diagnose patients with suspected viral infection. In addition, routine monitoring of transplant recipients for CMV, EBV, and BKV viremia using these methods has become the standard of care at many transplant centers and has facilitated earlier clinical interventions that have dramatically reduced the morbidity caused by these viruses.

The major challenges facing both laboratorians and clinicians are the lack of standardization of molecular assays and the limited availability of FDA-cleared tests. A large number of the assays currently used by clinical laboratories are LDTs, and consequently, the results may vary between institutions, which complicates the establishment of interpretive guidelines that can be applied broadly in clinical practice.

Future work should be focused on the continued development of standardized protocols calibrated against international standards. This will assist in the interpretation of results between transplant centers, and will be critical in the generation of clinical thresholds for differentiating active disease from transient viral shedding. Standards have been developed by the WHO for CMV, EBV, and parvovirus B19. Implementation of these standards in clinical laboratories has the potential to standardize results between testing laboratories, and allow for comparison of results between transplant centers. Additionally, future studies should be directed at improving the ability to detect and quantify multiple viruses in a single test. A recent study showed that a multiplex viral transplant panel could test for five viruses (BKV, CMV, EBV, HHV-6, and HHV-7) in one assay (105). Interestingly, the multiplex assay detected mixed viral infections in 5.7% (16/280) samples tested, indicating that coinfections with multiple viruses may be common in transplant recipients. This will be an exciting area for further study in the years to come.

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section VII

Fungi and Protozoa

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Molecular Detection and Identification of Fungal Pathogens

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35

Fungi are a diverse group of eukaryotic organisms, most of which are found as saprophytes in soil and on decomposing organic material. Of the more than 100,000 fungal species, fewer than 500 have been implicated in human disease. Most fungi are opportunistic pathogens that only cause disease in (severely) immunocompromised patients. Many fungal diseases have a worldwide distribution, but some are restricted to specific geographical regions (e.g., endemic mycoses). Fungi cause a broad spectrum of diseases, ranging from superficial mycoses (infection of skin, hair, nails, and/or mucosal membranes), to allergic diseases, to life-threatening invasive diseases. Most dermatophytes are not able to survive as free-living saprophytes and are obligate pathogens of animals and humans that cause contagious infections. Millions of individuals worldwide are affected by superficial mycoses, mainly dermatophytoses and candidiasis (1, 2). An increase of invasive infections has been observed over the past decades due to the ever-increasing size of the population at risk for these infections (3) as well as the availability of better diagnostics.

The need for fast and accurate laboratory diagnosis is obvious for life-threatening invasive diseases but also for the diagnosis of dermatophytoses where a rapid and precise diagnostic test is needed to guide optimal treatment (4). The isolation of a fungus from a clinical sample does not necessarily imply that the patient is suffering from fungal disease, given the ubiquitous nature of fungi. In addition, some fungi are (persistently) present in the human microbiota. Hence, differentiating contamination or colonization from infection is of great importance.

Due to the varied and nonspecific clinical signs of a fungal infection and the limited sensitivity of conventional diagnostic assays (microscopy and culture), research has been directed toward the detection and identification of biomarkers (mainly antigens and nucleic acids) for fungal infections. The huge diversity of fungal pathogens sometimes complicates correct species identification, and the limitations of the classical phenotypic identification methods are becoming more and more clear through the application of molecular techniques.

Molecular tests are not widely implemented yet in clinical laboratories for the diagnosis of fungal infections, although PCR-based diagnostics is a fast-growing segment in this domain.

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Two main applications of the use of molecular tests in the microbiology (mycology) laboratory will be discussed in this chapter, namely, the detection (and identification) of fungal pathogens directly in clinical samples and the identification of fungal isolates cultured from clinical samples.

MOLECULAR DETECTION OF FUNGAL PATHOGENS DIRECTLY IN CLINCAL SAMPLES

First, we will discuss some general issues to be considered for the development of molecular fungal detection tests.

Molecular techniques can be applied to the detection and identification of virtually any fungus involved in human disease in many different sample types. In this section, we will discuss the role of molecular tests optimized for the detection of a broad range of fungi (called "panfungal" tests). Next, we will discuss specific tests developed for the diagnosis of invasive aspergillosis (IA), invasive candidiasis, and *Pneumocystis jirovecii* pneumonia (PCP) (or fungus-specific tests). For guidelines regarding the use of molecular tests for the diagnosis of mucormycosis, we refer to the recent European Society of Clinical Microbiology and Infectious Diseases guidelines (5).

General Issues Concerning the Development of Molecular Tests for the Detection of Fungi

Since the 1990s, many studies of molecular diagnostics in the field of medical mycology have been published, but results were often difficult to compare due to variations in testing parameters, differences in the definition of invasive fungal disease, and the amount and quality of technical information and data provided. In 2009, guidelines for minimum information for publication of quantitative real-time PCR experiments (MIQE) were published (6) to ensure the quality of real-time PCR evaluation manuscripts.

A characteristic feature of fungal cells is the presence of a rigid cell wall consisting mainly of polysaccharides (glucan, mannan), chitin, and glycoproteins. Hence, efficient extraction of fungal DNA is difficult and appears to be the rate-limiting step in the development of a fungal molecular detection assay. Because the fungal load in clinical samples (e.g., blood or bronchoalveolar lavage [BAL] samples) from patients with invasive mycosis is usually low and often close to the detection limit of the most sensitive molecular detection techniques currently available, efficient

extraction of fungal DNA is essential (7). Multicopy targets such as the rRNA gene targets (18S, 28S, and internally transcribed spacer [ITS] regions) are preferred to enhance performance and sensitivity. Of course, obtaining sufficient amounts of fungal DNA of good quality is less critical for the development of a fungal identification test performed on clinical isolates.

The ubiquitous nature of fungi is another complicating feature, since it poses a high risk of contamination at several levels, i.e., during the sampling process and during the extraction phase but also during the PCR due to fungal-DNA-contaminated reagents such as enzyme preparations. One should ensure that only verified fungal-DNA-free reagents are used for DNA extraction and PCR setup. Inclusion of one or more control samples to detect contamination is necessary in each test run.

Mrazek and Lass-Flörl (8) have reviewed various procedures (percutaneous, bronchoscopic, open surgery, or video-assisted thorascopic surgery) for obtaining biopsies for molecular tissue diagnosis of invasive fungal infections.

PCR-based assays are currently the most common molecular-based approach for the diagnosis of fungal infections, and most PCR amplification systems perform with excellent efficiency. Agarose gel electrophoresis is not recommended for fungal detection (as it is for other microorganisms) because of the lower sensitivity compared to probe-based detection systems and the relatively low specificity. Due to contamination risk, nested PCR formats are no longer recommended in this setting. The introduction of real-time amplification PCR greatly advanced the field of molecular microbiology in general and of medical mycology in particular. Furthermore, identification of the fungus can also be realized by amplicon sequencing (9). Microarray technology can be used as an alternative for amplicon characterization (10). However, the preferred method depends also on the extraction and amplification platforms available to the user. Few publications report results of RNA detection techniques such as the nucleic acid sequence-based amplification (NASBA) technique (11, 12).

Broad-Range Detection of Fungi

For particular clinical settings and diagnostic-based strategies, assays may be designed to detect a broad range of fungal pathogens. Such assays can be implemented to screen patients at high risk for invasive fungal disease. In addition, they can be used on sterile-site samples (e.g., blood, cerebral fluids, tissue biopsies) in which fungal elements were detected by microscopy/histology but remained culture negative.

Detection of a broad range of fungi can be achieved by a multiplex format with primers targeting a set of different species or by the design of PCR primers that target conserved regions of the fungal DNA (panfungal primers). Species identification can be achieved by the use of specifically labeled probes, hybridization to a DNA microarray, or sequencing of the PCR product to guide further diagnostic testing or antifungal therapy. The use of specific probes to further characterize the amplification product is preferred over the use of intercalation dyes such as SYBR Green and melting curve analysis (13). When these broad-range assays are used in combination with nonsterile site samples (such as bronchial aspirates or BAL fluids), the detection of clinically nonrelevant saprophytes (such as *Penicillium* species) may represent a problem. Of note, the use of panfungal primers may be limited by the fact that greater target diversity is associated with reduced sensitivity; primers that were optimized to detect specific pathogens tend to have greater sensitivity (14). As such, when a panfungal PCR is used to exclude a fungal infection, a negative test result may need to be followed by more specific PCRs designed to target the most prevalent fungal pathogens (depending on the clinical setting, e.g., an *Aspergillus* PCR) to improve the sensitivity of the detection and avoid the need for post-PCR steps.

Another limitation is the difficulty in detecting the different pathogens involved in case of a polyfungal infection. Often only the fungus with the highest load in the sample will be detected. This is of clinical relevance because polyfungal infections may be more prevalent than is generally accepted. In patients with invasive mucormycosis, for instance, surveillance studies revealed that coinfection with aspergilli is a common phenomenon (15, 16). A panfungal design also harbors the risk of coamplification of and/or hybridization with any eukaryotic DNA including human DNA, which is often present in large amounts in human samples (17).

Several authors reported excellent performance of panfungal home-brew assays (performed on blood or tissue samples) for the diagnosis of invasive fungal diseases (18–21). In the Spanish Mycology Reference Laboratory the following algorithm is applied for tissue biopsy specimens that are culture negative. A specific PCR test is performed in case of a clear clinical, epidemiological, or histopathological suspicion of a specific fungal disease such as aspergillosis, mucormycosis, scedosporiosis, fusariosis, or histoplasmosis. When no informative data about the possible or putative fungal pathogen involved is available, a panfungal assay will be performed. Also, when the initial specific PCR assay results are negative, a panfungal assay will be performed (18). With this approach, the lab was able to identify the species implicated in all 132 patients from whom they received 151 biopsy samples from 2006 to 2013. Four cases of mixed infections were detected, and they all involved *Aspergillus* spp. mixed with *Candida* spp. or *Mucorales* spp.

Commercially available broad-spectrum PCR tests were mainly designed for the detection and identification of bacterial and fungal pathogens associated with sepsis. The LightCyclerSeptiFast Test Mgrade (Roche Diagnostics, Mannheim, Germany) was the first CE-cleared PCR kit for the rapid detection and identification of 25 common bloodstream infection pathogens including five *Candida* spp. and *Aspergillus fumigatus*. The assay is based on real-time PCR targeting species-specific ITS regions. Several evaluation studies were conducted, but the total number of patients included with a fungal bloodstream infection remains limited (22). The Vyoo test (Analytik Jena, Jena, Germany) is a multiplex PCR assay that allows the simultaneous amplification and detection of 34 bacterial and 7 fungal species (*A. fumigatus*, *Candida albicans*, *Candida dubliniensis*, *Candida glabrata*, *Candida krusei*, *Candida parapsilosis*, *Candida tropicalis*), as well as five antibiotic resistance genes. The procedure involves a mechanical lysis step for whole blood (up to 5 ml), automated total DNA extraction, and a patented pathogen DNA enrichment technology. The SepsiTest from Molzym (Bremen, Germany) is a CE-IVD kit that uses broad-range primers that bind to conserved regions of 18S rRNA genes for the detection of a broad range of bacteria and fungi in whole blood. Sequencing of the amplicons allows direct identification of the pathogen detected. The RenDx Fungiplex assay (Renishaw Diagnostics, Glasgow, United Kingdom), based on

multiplex PCR and detection by surface-enhanced Raman scattering, was developed for the detection of the most common fungal pathogens. The test result is reported as *Candida* spp. (includes *C. albicans*, *C. parapsilosis*, *C. tropicalis*, *C. guilliermondii*, *C. lusitaniae*, *C. dubliniensis*), *C. krusei*, *C. glabrata*, *Aspergillus* species (includes *A. fumigatus*, *A. flavus*, *A. niger*), or *Aspergillus terreus*. Promising clinical performance was demonstrated in a recent retrospective clinical evaluation (23).

These assays have the potential to provide rapid (depending on availability during weekends and nights) detection of fungal pathogens causing invasive fungal infections, but prospective clinical evaluations are needed to better define their added value to blood cultures and other biomarkers such as *Aspergillus* antigen (galactomannan [GM]) and β-D-glucan (BDG). Because these molecular assays cannot replace blood cultures and are more expensive, it is also important to determine for which patient groups the implementation of a molecular sepsis test can be cost effective.

Diagnosis of Invasive Candidiasis

The current "gold standard" for the diagnosis of invasive candidiasis is blood culture and cultures from other sterile sites, but the slow turnaround time and poor sensitivity are important limitations. In a Danish surveillance study, the times to blood culture positivity for two blood culture systems (BacT/ALERT and BACTEC) were similar but species dependent. Blood cultures from episodes due to *C. tropicalis* or *C. krusei* infection were positive after a median incubation time of 1 day, whereas blood cultures from fungemia episodes due to *C. albicans*, *C. glabrata*, or *C. parapsilosis* were positive after a median incubation time of 3 days, with 25% of the episodes due to *C. glabrata* being positive on day 4 or later (24). In patients with autopsy-proven hepatosplenic candidiasis, premortem positivity rates of less than 50% were reported for blood cultures (25, 26).

A commercial non–nucleic acid amplification method, the Yeast Traffic Light PNA FISH (AdvanDx), was developed for the identification of *C. albicans*/*C. parapsilosis*, *C. tropicalis*, and *C. glabrata*/*C. krusei* in positive blood cultures. The assay is based on peptide nucleic acid fluorescent *in situ* hybridization technology and uses nucleic acid probes targeting the 26S rRNA region. The test is FDA- and CE-cleared, and clinical validation of this assay demonstrated high sensitivity and specificity (27). Only a limited number of *Candida* species can be identified this way, which is a limitation compared to matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF MS), which is a promising nonmolecular technique for the rapid identification of yeasts in positive blood cultures (see below). Unfortunately, this technology can rarely be used directly on clinical specimens.

Clancy and Nguyen (28) recently reviewed the performance of culture and nonculture diagnostics for invasive candidiasis with a focus on the so-called missing 50%, i.e., patients with invasive candidiasis who are not diagnosed by blood cultures. The European Society of Clinical Microbiology and Infectious Diseases has developed guidelines for the diagnosis of invasive candidiasis (29). Molecular assays are attractive because of their high sensitivity when targeting multicopy genes, their capacity for rapid speciation, and their ability to quantitate the fungal burden. A challenging aspect is the low burden of *Candida* cells during bloodstream infections. Less than 10 CFU/ml circulate in the bloodstream (30, 31). Also, the intermittent nature of candidemia due to hepatic clearance of fungal cells and/or the periodic release of cells from deep organ sites into the circulation plays a role (32). Compared to culture, PCR detects both viable and dead *Candida* cells, including freely circulating DNA that may be present after intact organisms have been eliminated from the circulation (30). In a rabbit model of disseminated candidiasis a steady increase of *Candida* DNA levels occurred in plasma as disease progressed. Phagocytic cells may play an active role in increasing this DNA release over time. Plasma is thus a suitable blood fraction for invasive candidiasis assays (32).

A comparison of PCR validation studies is complicated because of variations in testing parameters, the single-institution design of most of these studies, and differences in the definition of diseased and control populations. Avni et al. conducted a systematic review and meta-analysis to assess the diagnostic accuracy of PCR-based methods to detect *Candida* spp. directly in blood samples (25). A total of 54 studies were included, with 4,694 patients, 963 of whom had proven/probable or possible invasive candidiasis. Perfect (100%) sensitivity and specificity for PCR in whole-blood samples were calculated when the evaluation was limited to cases with candidemia and healthy controls. PCR positivity rates among patients with proven or probable invasive candidiasis were 85%, while blood cultures were positive for 38% of cases. The use of whole-blood samples, rRNA, or P450 gene targets and a PCR detection limit of ≤10 CFU/ml were associated with improved test performance. Nguyen et al. compared the performance of a validated *Candida* real-time PCR and the Fungitell BDG assay on blood samples with blood cultures from prospectively included patients with invasive candidiasis and hospitalized controls. In this study, PCR on plasma or sera was more sensitive than whole blood for diagnosing invasive candidiasis ($p = 0.008$). PCR had a sensitivity similar to BDG detection for diagnosing candidemia but a higher sensitivity for diagnosing deep-seated candidiasis. The highest sensitivity (98%) was achieved by combining blood cultures with PCR (33).

From a clinical point of view, a major unmet need is the diagnosis of blood culture-negative, deep-seated candidiasis. It will be important to include these patients in carefully designed diagnostic trials. It is expected that wide use of nonculture techniques will improve our understanding of invasive candidiasis and will lead to a knowledge base that is less skewed toward the clinical entity with candidemia.

Diagnosis of IA

Unlike invasive candidiasis, blood cultures are not useful for the diagnosis of IA, even in the case of disseminated disease. Most patients present with invasive pulmonary aspergillosis, but the sensitivity of culture of respiratory samples is at best 60%. Diagnosis of IA cannot be made on the basis of a positive laboratory test result only (except when the fungus is detected in a sterile-site sample) but should be based on the integration of clinical, radiological, and microbiological signs and data (34). GM and BDG detection is included in the revised criteria of the European Organization for Research and Treatment of Cancer for the definition of probable disease; this is not (yet) the case for molecular tests. The European Conference on Infections in Leukemia Laboratory Working Groups concluded that there is good evidence to support the use of GM detection in serum for the diagnosis of IA in adult leukemic

patients and hematopoietic SCT recipients (AII grading) and moderate evidence for BDG detection in serum (BII grading). Detection of GM in BAL fluid samples of patients at risk of invasive pulmonary aspergillosis was shown to have excellent diagnostic accuracy (35, 36).

A major obstacle to the design of molecular assays for the diagnosis of IA is the very low fungal load in clinical specimens, especially in blood. During the past decades, numerous in-house assays with many different target sequences and PCR protocols have been published. In the past 8 years, great efforts have been dedicated to improvement and standardization of these assays. The first multicenter study of PCR methods for the detection of *Aspergillus* and *Candida* spp. was conducted by the United Kingdom Fungal PCR Consensus Group and can be considered the initial attempt to achieve a consensus method (37). In 2006, the European *Aspergillus* PCR Initiative (www.eapcri.eu), a working group supported by the International Society of Human and Animal Mycology, was founded. The aim was to put forward clear recommendations for the design of PCR-based techniques for the detection of *Aspergillus* DNA in clinical samples. To improve the analytical sensitivity of the detection of *Aspergillus* DNA in whole blood, the European *Aspergillus* PCR Initiative recommends the use of ≥3 ml EDTA blood specimens, a red and white cell lysis step, bead-beating to lyse the fungal elements, and elution volumes of less than 100 μl (38).

It is not yet fully understood how *Aspergillus* DNA circulates in the bloodstream: either associated with the fungal cell and/or as free DNA. As such, the optimal clinical specimen for PCR testing for IA is still subject to considerable debate. Serum is an attractive matrix because the extraction of free DNA is easy and permits full automation on the same instruments as used for the extraction of bacterial and/or viral DNA. This matrix also allows the use of a single sample for GM, BDG, and PCR analysis. In a multicenter comparison of serum and whole-blood specimens for detection of *Aspergillus* DNA in high-risk hematological patients, 803 samples from 47 cases (proven and probable IA) and 31 controls (without IA) were tested to determine the performance of whole-blood PCR, serum PCR, and serum GM testing. There was no significant difference between whole-blood and serum specimens for PCR-based diagnosis of IA. There was a trend for testing of whole blood to be more sensitive (85% versus 79%) than serum analyses, but false positivity was higher for whole-blood specimens compared to serum. The best performance was obtained by combining different assays (GM and PCR) (39). In conclusion, there is no clear evidence that whole blood or serum is better for *Aspergillus* PCR, and thus the selection of the sample type for DNA extraction may be guided by the technical platforms available at each center.

In a prospective multicenter study including 58 respiratory tract biopsy samples from patients with a proven invasive mold infection, the etiological diagnosis increased from 63% based on culture to 96% by the additional use of two seminested PCR assays (targeting mitochondrial DNA of *Aspergillus* species and the 18S ribosomal DNA of *Mucorales*, respectively) (40).

Several commercially available real-time PCR tests for the detection of *Aspergillus* DNA have contributed to the standardization of PCR testing. The MycAssay *Aspergillus* test (Trinity Biotech, Ireland) employs molecular beacon technology targeting the 18S rRNA gene and is developed for the detection of DNA from 15 *Aspergillus* spp. However, the assay cross-reacts with *Penicillium* spp. This kit was launched together with the MycXtra kit for the extraction of fungal DNA from respiratory samples and includes a bead-beating step for the disruption of the fungal cell wall. Sensitivity and specificity of the PCR on BAL fluid from high-risk patients with proven or probable IA ranged between 93.0 and 94.1%, and 82.4 and 98.6%, respectively (41, 42). Other commercial PCR-based tests specifically designed for *Aspergillus* detection are available such as the Affigene *Aspergillus* tracer kit, which is CE-labeled for analysis of whole blood, serum, and plasma samples. With this kit, there is also cross-reaction with *Penicillium* spp., and clinical validation is still lacking.

Diagnosis of PCP

P. jirovecii is a pathogen specific for humans which has not been found in the environment. Immunocompetent individuals are the most important reservoir, where the fungus lives at the surface of the alveolar cells. The best sample for the diagnosis of PCP is BAL fluid; the lowest load is found in specimens taken from the upper respiratory tract. The detection of cysts and/or trophic forms by staining is still considered the gold standard for the diagnosis of PCP, but important limitations of this detection method are suboptimal reproducibility due to subjective interpretation, the need for highly trained lab technicians, the labor intensity of the procedure, and the lack of sensitivity in non–HIV immunocompromised patients due to the low burden of *P. jirovecii*. Because of these limitations, PCR and, more recently, BDG have been implemented progressively in clinical laboratories for the diagnosis of PCP. Several authors have published home-brew PCR methods with different targets which were evaluated in two meta-analyses. This evaluation revealed a high pooled sensitivity (>98.3%) for PCP PCR performed on BAL fluid samples and a pooled specificity of >88% (43, 44). Because of the high sensitivity, a negative PCR test on BAL fluid may be used to rule out a PCP diagnosis. Quantitative PCR and the major surface glycoprotein gene target were associated with higher specificity (43). The MycAssay Pneumocystis kit (Trinity Biotech, Ireland) was the first CE-certified commercial PCR assay, and it targets *P. jirovecii* mitochondrial large-subunit DNA. Its performance was evaluated in a multicenter trial of 110 patients (14 patients with PCP). PCR sensitivity, specificity, and positive and negative predictive values were 93%, 90%, 65%, and 98%, respectively (45).

It is clear that the sensitivity of PCR detection tests for *Pneumocystis* is greater than that of staining methods and that PCR tests may render positive results in patients that are colonized but not infected by *Pneumocystis* (46). Therefore, quantitation of the fungal load is of utmost importance for the interpretation of test results and the distinction between colonization and infection. Consequently, real-time quantitative PCR is the preferred PCR method for PCP testing. Unfortunately, no international standard is available. Quantitative results are expressed in different ways, i.e., as copy numbers per volume unit, as numbers of microorganisms (for example, trophic form equivalents) per volume unit, or as the cycle threshold, which is defined as the number of cycles required for the fluorescent signal to cross the threshold. Botterel et al. (47) evaluated the clinical significance of quantifying *P. jirovecii* DNA by real-time PCR in BAL fluid from immunocompromised patients. With immunofluorescence as the gold standard, PCR assay sensitivity was 100% applying

a threshold ≥2.6 $\log_{10}$ copies/µl, and specificity reached 100% when the threshold was raised to ≥4 $\log_{10}$ copies/µl. The relevant threshold remains to be determined and may vary according to the underlying disease of the patient. Combination of BAL PCR and serum BDG results may aid in the differentiation between PCP and colonization as suggested by Damiani et al. (48). In the future there may also be a role for the detection of circulating *Pneumocystis* DNA in blood samples, but this needs further evaluation (49).

MOLECULAR IDENTIFICATION OF FUNGAL PATHOGENS

Beside the development of sophisticated methods for the detection of nucleic acids or circulating antigens, culture-based methods are still the cornerstone for the diagnosis of fungal infections. Culture is particularly appropriate for the diagnosis of superficial mycosis. In this setting both the clinical sample and fungal growth can easily be obtained. Fungal culture is also an essential investigation for the diagnosis of invasive fungal infections. The isolation of a fungus from a sterile site sample provides the proof of an invasive fungal infection and should be attempted whenever possible (34) . Furthermore, if the fungus is isolated by culture, antifungal susceptibility testing can be performed and treatment adapted if necessary. Sabouraud dextrose agar supplemented with antibiotics is the reference medium generally used by labs for a fungal culture, but most of the fungi can even grow on routinely used bacteriological culture-enriched media (22). The incubation period depends on the clinical site considered and the fungus to be detected and may vary from 48 hours to 3 to 4 weeks at 30°C. In summary, fungal culture remains an essential investigation for the diagnosis of fungal infections, but because of its poor sensitivity for the diagnosis of invasive infection and the slow growth of certain fungi, other diagnostic tools including molecular tools were developed.

In severely immunocompromised patients, almost every fungal species can cause invasive mycosis (50, 51). Therefore, in this setting correct identification of a fungal isolate to the species level is necessary to appreciate the potential pathogenic role of the fungus, to aid in the interpretation of the *in vitro* antifungal susceptibility testing results, and for epidemiological purposes. Also, for isolates cultured from patients with dermatophytosis, it is helpful to correctly identify the dermatophyte involved in order to apply the best treatment, optimal duration of treatment, and appropriate prophylactic measures to avoid reinfection or transmission of the infection to contacts (52). This applies, for example, to species responsible for tinea capitis such as *Microsporum canis* and *Microsporum audouinii*. These two species are classified in the same teleomorph complex and cannot always be distinguished phenotypically.

However, in the clinical microbiology laboratory, not all fungal isolates should be identified to the species level, since many will not be involved in disease. The clinical context and sample site should always be considered to help to orientate the mycologist in the face of the macroscopical or microscopical aspects of the colony. The identification of complexes to the species level such as for *Aspergillus* of *Fusarium* complexes or within the order *Mucorales* is more of epidemiological relevance than to guide treatment decisions. Since 2009, MALDI-TOF MS has revolutionized fungal speciation in many clinical laboratories because this method can identify yeasts to the species level in only a few minutes and with high reliability, replacing the conventional methods. Nevertheless, when MALDI-TOF MS fails to ascertain a correct identification of a fungal species, the gold standard method is still DNA sequencing targeting fungal ribosomal genes or ITS regions.

The MALDI-TOF MS Technique

MALDI-TOF MS is a soft ionization technique used to analyze the protein composition (ribosomal and membrane-associated proteins) of microorganisms from a positive culture. The process produces ionized molecules which are accelerated in an electric field and directed to an analyzer which classifies them according to the mass-to-charge ratio. The mass spectra are compared to a database containing reference spectra giving identifications associated with a rating score expressed as a log score from 0 to 3 or as percentage of agreement with reference spectra. Currently, three systems are commercialized for microbiology applications: the Microflex LT (Bruker Daltonics system with the Biotyper database) (Bruker, Bremen, Germany), the Vitek MS system (bioMérieux, France), and the LT2-Andromas system (Andromas, France) (54, 55). Numerous publications confirm the performance of the systems, particularly when applied to yeast identification with up to 98.8% accuracy (56–60) and generally good agreement between both MALDI-TOF MS different systems (61). However, and contrary to the algorithm used for bacteria (with the exception of mycobacteria), a lower cutoff score of 1.7 is usually applied to the identification of yeasts to increase the sensitivity of the method (61–63). For yeast identification, there is generally no need to perform protein extraction before analysis, and identification can be performed directly on the colony or even from the blood culture (64). Molds are much more difficult to identify by MALDI-TOF systems than yeasts. Indeed, for filamentous fungi, both lowering the cutoff scores to 1.7 (65–67) and enriching the database are necessary to obtain a correct identification (62, 68–70).

Sequencing Methods

Nucleic Acid Extraction Methods

Extraction methods used for clinical samples can generally also be applied to fungal cultures, but the DNA concentration can be increased by using additional steps in the extraction protocol, for example, freeze-thawing and bead-beating, very powerful methods to break fungal cell walls. Many protocols can be used to extract nucleic acids from fungal cultures, ranging from simple boiling for a rapid preparation of a small amount of impure DNA to more elaborate and time-consuming protocols rendering more high-quality nucleic acid. The latter generally use physical disruption of the fungal wall, for example, bead-beating (with ceramic, zirconium, or glass beads), freeze-thawing (71), high-speed cell disruption (72), sonication (73), or chemical methods with heat-alkali treatment or enzymatic digestion using lyticase and/or proteinase K (51, 73, 74). These methods are often included and/or combined with in-house protocols and are followed by a purification step performed manually (Qiagen columns) or on automates using magnetic beads, for example, MagNAPure (Roche), Easymag (bioMérieux), and Maxwell16 LEV (Promega), or using column-based kits (QiAamp DNA minikit, Qiagen) on a dedicated automate, for example, the Qiacube by

Qiagen (75). Some commercial kits are also available and provide complete protocols, for example, the Ultra Clean DNA Isolation Kit (MoBio Laboratories, United States), which allows extraction of high DNA concentrations when applied to dermatophytes (71). Many other kits are available and applicable to fungal cultures: most of them are preceded by pretreatment including enzymatic digestion or bead-beating. In a recent comparison of five extraction methods (76), the highest DNA yield was obtained by using the ZR/fungal/bacterial DNA kit (Zymo Research, United States), YeastStar genomic DNA (Zymo Research), and QIAamp DNA Mini Kit (Qiagen) with bead-beating pretreatment, a mandatory step to optimize the DNA extraction from fungal cells as demonstrated by White et al (38).

Other approaches have been developed to minimize the duration of the extraction protocols to less than 1 hour (77, 78). One of those is the Whatman FTA filter method developed by Borman et al. (79–81). This method has been used for many years for the sampling, extraction, and storage of many other microorganisms such as protozoa (82) and viruses (83). FTA technology is cheap and fast (about 15 minutes) and uses Whatman FTA filter matrices, which are fibrous cards that are pretreated with chelators and denaturants that lyse and inactivate most of the microorganisms. These cards allow the extraction of as little as 10 ng of genomic DNA depending of the fungal concentration deposited on the cards.

Targets for Molecular Sequencing

Sequencing methods applied to fungi include targets such as genes encoding for structural proteins (actin, β-tubulin, calmodulin) or for enzymes (chitin synthase, enolase, cytochrome b, elongation factor 1-α) or partial sequences of the ribosomal DNA (rDNA) gene complex. The advantages of rDNA over the other targets are their universal representation in fungi, their multicopy nature, and the highly conserved sequences interspaced with highly variable regions, which allows differentiation even between closely related species. But none of the targets provides universal fungal differentiation, and the targeted sequences have to be chosen based on taxonomic background knowledge of the targeted group of fungi. However, overall, the most common target used is the ITS region including ITS1-5,8S-ITS2, which is recognized as the formal metabarcoding sequence (17, 84). The Clinical and Laboratory Standards Institute (CLSI) published a document in 2008 proposing a uniform approach to the molecular identification of fungi by use of broad-range DNA targets using the ITS regions (85). This document provided guidelines for primer design, quality control parameters for amplification and sequencing, measurement of sequence quality, and assessment of databases. Balajee et al. proposed an algorithm for the identification of filamentous fungi without *a priori* knowledge (86). This algorithm classifies a fungus into a complex of species, and the identification can always be completed in more detail using additional molecular targets.

The rDNA gene complex of the fungal genome includes a serial sequence of subunits as follows: 5′-18S/ITS1/5,8S/ITS2/28S/IGS1-5S-IGS2-3′ (Fig. 1) (87). The external transcribed spacers IGS1 and IGS2 are nontranscribed sequences and include the 5S rDNA gene, which has variable positioning and is not transcribed at the same time as the other rDNA genes. The entire rDNA gene complex is repeated many times along the fungal genome.

Regarding the transcribed spacer regions, only the ITS region including the ITS1-5,8S-ITS2 unit is taxonomically interesting, and it is the most widely used for fungal species differentiation because of the variability of the ITS sequences. The majority of the published data describe the use of the entire unit (74, 78, 88). However, ITS2 can also be used alone, particularly if the DNA is potentially degraded. It has been shown recently that ITS2 is more suitable for species differentiation of fungi in comparison with the entire region or ITS1 alone (89).

The length of the entire ITS region, ITS1, and ITS2 is about 600 to 700 bp, 300 bp, and 400 bp, respectively, depending on the fungal group concerned (90). The most commonly used primers have been developed by White et al. (91) for ITS1, ITS2, ITS3, ITS4 and by Gardes et al. (92) for ITSF and ITS4B. The latter are more specific for basidiomycetes than the former. Primer ITS1F is usually combined with ITS2 to amplify the ITS1 region, and ITS3 is generally combined with ITS4 to amplify the ITS2 region (74, 88, 89, 93, 94). These primers have been largely used in mycology, but other primers can also be used, for example, ITS86F (ITS86 forward) combined with ITS4 (95, 96) (Table 1).

The 18S rDNA gene (corresponding to the small subunit rDNA) includes about 1,800 bp with both conserved and variable regions. This region has been regularly suggested for species identification or as a target in PCR-based protocols (97). However, because of high sequence homology among fungal species, it is necessary to choose a long DNA fragment to allow adequate species differentiation (87). The 5.8 S is about 160 bp and is highly conserved but is not appropriate for species identification. This region is often used as a binding site for primers (89, 98, 99). The 28S rDNA gene or large subunit (LSU) rDNA is longer, with about 3,400 bp. It contains very conserved regions but also discrete domains (called "D" regions), ranging from D1

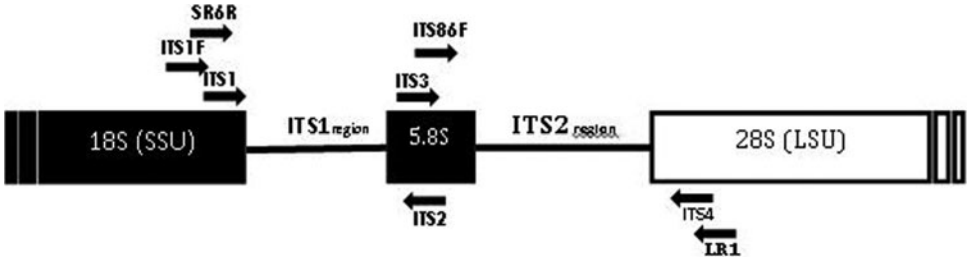

FIGURE 1 Schematic consensus representation of the rDNA gene complex for fungal genomes with the most frequently used internally transcribed spacer (ITS)–targeted primers (arrows). SSU, small subunit; LSU, large subunit.

TABLE 1 The most currently used primers targeting the ITS region of the fungal rDNA genes complex

Primer name[a]	Sequence (5′–3′)	Reference
ITS1 (f)	TCCGTAGGTGAACCTGCGG	91
ITS2 (r)	GCTGCGTTCTTCATCGATGC	
ITS3 (f)	GCATCGATGAAGAACGCAGC	91
ITS4 (r)	TCCTCCGCTTATTGATATGC	
ITS1F (f)	CTTGGTCATTTAGAGGAAGTAA	92
ITS4B (r)	CAGGAGACTTGTACACGGTCCAG	
ITS86 (f)	GTGAATCATCGAATCTTTGAA	96
ITS4 (r)	TCCTCCGCTTATTGATAGC	
SR6R (f)	AAGTAAAAGTCGTAACAAGG	114
LR1 (r)	GGTTGGTTTCTTTTCCT	

[a]f, forward; r, reverse.

to D12, which alternate with more or less conserved regions. The D1 and D2 regions contain the majority of variable sequences and are used for species differentiation (78, 88, 100).

Pyrosequencing

Pyrosequencing is a technique which allows a more detailed characterization of fungal species communities than the Sanger sequencing method. Pyrosequencing, also called sequencing by synthesis, differs from Sanger sequencing in that it relies on the detection of pyrophosphate release on nucleotide incorporation, rather than chain termination with dideoxynucleotides. The limitation of the method is the length of the complementary DNA, which cannot exceed 300 to 500 nucleotides (versus 800 to 1,000 for the Sanger method). This method has been successfully applied to the differentiation of *Candida* spp. (80, 101, 102) and *Aspergillus* spp. (103) and for fungal identification targeting the ITS2 rRNA region (89). Pannanusorn et al. were able to distinguish closely related *Candida* spp. such as those in the *Candida parapsilosis* complex by pyrosequencing of a mere 40 nucleotides in the ITS2 region (102).

Application of Molecular Identification Methods to Different Fungal Groups

Yeasts: *Candida* and *Cryptococcus*

Molecular identification of the majority of the yeast species encountered in clinical microbiology usually targets the full ITS region because even closely related species such as *C. albicans*/*C. dubliniensis*, *Candida inconspicua*/*Candida norvegensis*, *C. parapsilosis*/*Candida metapsilosis*/*Candida orthopsilosis* can be distinguished (88, 104). However, when it comes to differentiating *Cryptococcus neoformans* var. *grubii* from *C. neoformans* var. *neoformans*, the variability of the ITS operon is insufficient, and another target is recommended, for example, the rRNA IGS (105).

Molds

Aspergillus

The genus *Aspergillus* is classified into eight subgenera, and each subgenus is subdivided into several sections (or species complexes) that include many related species (86). Following the recommendations of the European working group (106), ITS sequences can be used for differentiation to the species complex level such as *A. fumigatus* or *A. flavus* complex, while the differentiation to the species level into the sections necessitates the amplification of other targets such as β-tubulin, calmodulin, and rodlet A genes (86).

Fusarium

About 200 species are grouped into the *Fusarium* complexes, and differentiation to the species level requires the use of molecular sequencing tools. The ITS operon (107) and β-tubulin (108) have been successfully used as molecular targets to identify *Fusarium* species. However, the translation elongation factor-1-alpha provides a more detailed differentiation in phylogeny studies (108) and has been used in the most recent epidemiological European survey (109, 110).

Mucormycotina

Most of the isolates of *Mucormycotina* can be identified by using the ITS operon (93, 94). However, it has been demonstrated that some isolates are difficult to identify to the species level. In this case the D1/D2 region, actin gene, and/or elongation factor-1-alpha can be used as complementary target sequences (106).

Dermatophytes

Since 1980, the development of new molecular techniques (restriction fragment length polymorphism, sequencing, fingerprinting) has allowed better comprehension of the genetic diversity of the dermatophytes and has contributed to numerous reclassifications in this group (106, 111). The LSU as well as the full ITS operon have been successfully used as targets in sequencing methods applied to dermatophyte identification, whereas the small subunit (18S rRNA region) is unable to correctly differentiate species. The majority of the primer combinations published in the literature have been reviewed by Cafarchia et al. (111). Briefly, PCR-based techniques have demonstrated that the discriminatory power for dermatophyte identification is identical between the LSU and full ITS region (112, 113). For dermatophyte sequencing many authors use the combination of two primers, LR1 and SR6R (Table 1), with binding sites situated in the flanking 18S and 28S rDNA sequences of the ITS operon (112) as described by Gräser et al. (114). These combinations allow for good discrimination even between closely related species belonging to the same complex, for example, the *Arthroderma benhamiae* or *Arthroderma vanbreuseghemii* complex (114). The same group (111, 115) has proposed a new classification for anthropophilic and zoophilic dermatophytes based on the teleomorphic (perfect or sexual state of the fungus) complexes and anamorphs (asexual state). There is a need to take into account the discriminating power of the technique used to distinguish, for instance, teleomorphic complexes such as *A. vanbreuseghemii* from *A. benhamiae* complexes and whether the name of the anamorph (asexual form of the fungus) can be determined correctly. Currently, ignoring all the classification details or using an incomplete methodology, it is always preferable to refer to the isolate as part of the teleomorphic complex rather than to go to the species level. This is needed to avoid misidentifications (115–117).

For those who prefer to use a commercially available kit, the Microseq D2 LSU rRNA fungal sequencing kit (Applied Biosystems, United States) has been successfully applied to the determination of dermatophyte species using reference strains for sequence comparison (113, 117).

Databases Used for Fungal Molecular Identification

Various databases are used in mycology including GenBank (http://www.ncbi.nlm.nih.gov), CBS (http://www.cbs.knaw.nl/), the European Molecular Biology Laboratory nucleotide sequence database (http://www.ebi.ac.uk/embl/), and the DNA Data Bank of Japan (http://www.ddbj.nig.ac.jp/). The most widely used database is GenBank, which contains a huge number of sequences, but these are combined with unedited and nonvalidated information, which may be updated and corrected only by the original submitter. Errors in fungal sequences within GenBank have been found to be as high as 20% (86). To overcome these problems, specific sequence databases for particular groups of fungi, based on quality-controlled sequences, have been created. Concerning the medically relevant fungi, two ITS databases are available through the CBS Fungal Biodiversity Center (http://www.cbs.knaw.nl) and the Westmead Millennium Institute, University of Sydney (curated database; http://www.mycologylab.org/biolomicsid.aspx) (86). As suggested by the same author, we also recommend creating a unique fungal database with high-quality controlled sequences to gather only reliable mycological sequence data.

CONCLUSIONS

The diagnosis of fungal infections remains challenging due to the nonspecific clinical signs and symptoms of these infections and the limitations of the currently available diagnostic tests. To improve our ability to diagnose these infections and to guide treatment, the value of molecular diagnostic methods in this field is currently being explored extensively.

Numerous protocols for molecular diagnostics in mycology have been published since the 1990s, but their broad implementation was hampered due to the lack of standardization and clinical validation. Important steps forward were made toward the standardization of molecular testing in the fungal field by the publication of recommendations for the development and implementation of home-brew tests, the increasing availability of commercial tests, and external quality control programs. Because these molecular tests generally do not replace conventional tests and are expensive, it is important to demonstrate that they really impact patient management, antifungal use, and outcome and may be cost effective. Therefore, clinical validation studies in carefully selected at-risk patient populations are urgently needed. Based on currently available data, it is most likely that molecular methods will need to be combined with other biomarkers such as antigen detection tests to achieve optimal performance.

Molecular methods as well as MALDI-TOF MS technology play an increasingly important role in the identification of fungi. It is clear that molecular identification strategies render more accurate identification of fungal isolates than strategies based on morphology alone. However, the relevance of identifying an unknown isolate to the narrowest taxonomic unit should be evaluated in the clinical context. Furthermore, it remains essential to maintain morphological expertise to be able to validate the results of molecular tests.

Next-generation sequencing methods have proven to be very powerful in clinical research for the exploration of the human mycobiome and to detect multiple and new markers of antifungal resistance. Whether next-generation sequencing will be introduced in the fungal diagnostic setting in the near future remains unclear.

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Molecular Approaches for Diagnosis of Chagas' Disease and Genotyping of *Trypanosoma cruzi*

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36

Chagas' disease, or American trypanosomiasis, is a vector-borne parasite infection caused by the protozoan *Trypanosoma cruzi*, which affects about 7 to 8 million people, mainly in Latin America. The parasite is primarily transmitted by different species of triatomine bugs (*Insecta*: *Hemiptera*: *Reduviidae*: *Triatominae*). However, *T. cruzi* infection can also be acquired congenitally, by oral transmission (from consumption of food contaminated with infected triatomine bug feces), by blood transfusions, and by organ transplantation (1). *T. cruzi* is a highly diverse species, and six major lineages, or discrete typing units (DTUs) (TcI to TcVI), have been described for this parasite. During its life cycle, *T. cruzi* infects both insect vectors and mammalian hosts and undergoes morphological and functional changes. Three main morphological forms of the parasite are recognized, namely (i) amastigotes (intracellular replicative forms in mammalian tissues), (ii) trypomastigotes (nonreplicative forms: metacyclic trypomastigote in the rectum of triatomine vectors and bloodstream trypomastigotes in mammalian blood), and (iii) epimastigotes (replicative forms found in the triatomine midgut). Intermediate forms are also recognized (2).

Two phases are recognized during the course of *T. cruzi* infection in humans. First, an acute phase develops which is characterized by nonspecific clinical manifestations during a few weeks after infection. However, about 1% of patients in the acute phase present with acute myocarditis, pericardial effusion, and/or meningo-encephalitis. After this first phase, patients enter the chronic phase, which can be categorized into indeterminate, cardiac, and digestive forms (3). Estimates of mortality rates suggest that 10% of infected individuals die annually (4).

During the acute phase of infection by *T. cruzi*, direct observation of circulating parasites in blood by microscopy is possible due to the high-level parasitemia. However, during the chronic phase, the number of circulating parasites decreases below the threshold for microscopic detection.

Presently, vector-borne transmission still occurs in large regions of Latin America. Control strategies mainly involve spraying of the domestic and peridomestic environments with pyrethroids, screening of newborns of infected mothers, and screening of both blood and organ donors. Besides vector-borne transmission, which is almost exclusively confined to Latin America, congenital and transfusion-mediated transmission, as well as infection acquired by organ transplantation, are becoming a global concern due to migration of *T. cruzi*-infected people from endemic countries into nonendemic regions (5).

Two drugs with proven efficacy are currently available to treat *T. cruzi* infection: nifurtimox and benznidazol. Nifurtimox is a nitrofuran derivative which reaches parasitological cure (permanent negative serology) in about 70% of treated patients during acute stages of the disease (6). Benznidazole is a nitroimidazole derivative with an effectiveness similar to nifurtimox (7). Both nifurtimox and benznidazole have an important incidence of side effects which lead to dosage reduction or treatment interruption. A randomized trial (the BENEFIT study) with 3,000 patients is currently evaluating the efficacy of benznidazole to reduce parasite burden in patients with chronic Chagas' cardiomyopathy, as well as assessing the drug's safety and tolerability (8).

METHODS FOR DIAGNOSIS AND FOLLOW-UP OF *T. CRUZI* INFECTION

Serological and Parasitological Methods: Scopes and Limitations

Serology is at present considered the "gold standard" for the diagnosis of *T. cruzi* infection. Two serological techniques with no discordant results should be used to correctly diagnose Chagas' disease. In the framework of public health institutions in Latin America, enzyme-linked immunosorbent assay, indirect hemagglutination, and indirect immunofluorescence are widely accepted serological tests for diagnosis of *T. cruzi* infection. For enzyme-linked immunosorbent assay and indirect hemagglutination, different commercial kits are available. The serological tests currently available are only useful for diagnosis during the chronic phase, while there are no serologic tests for diagnosis during the acute phase. A wide number of native and recombinant antigens have been tested for Chagas' disease diagnosis, and some commercial kits display good levels of sensitivity and specificity: higher than 97% and higher than 96%, respectively (9). However, some serological tests display different levels of resolution among different regions of America, probably due to (clonal) differences between the infecting strains (10). At any rate, during the chronic phase, serological diagnosis is at present the reference approach.

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Direct parasitological methods, such as fresh-blood test or concentration methods (microhematocrit and Strout tests), are only useful during the acute phase, which normally goes unnoticed from a clinical perspective. For suspected congenital cases examined during the first month of life of the newborn, concentration methods can generate an acceptable sensitivity. Indirect parasitological methods, such as xenodiagnosis and hemoculture, are time-consuming. Xenodiagnosis involves having the patient bitten by uninfected triatomine bugs. If the patient is infected, trypanosomes develop in the digestive track of the bug and are found in its feces. These methods are unavailable for routine use in public health institutions, and they have low sensitivity during the chronic phase (20 to 50%).

A particular scenario in which follow-up methods are needed is after specific treatment. Clearly, parasitological methods display low sensitivity, which renders them invalid as a cure criterion. Conventional serology is relatively useful when patients in the acute phase are treated, since a high number of them show seroconversion in the absence of adequate treatment. However, seroconversion only occurs about 2 years after completion of the treatment with either of the two available drugs, benznidazole or nifurtimox (11). Conversely, when chronic patients are submitted to chemotherapy, it takes many years until seroconversion can be observed (12). Moreover, in many cases, seroconversion does not occur.

An alternative approach based on flow cytometry of posttreatment sera for detection of immunoglobulin G antibodies against *T. cruzi* has shown promising results (13–15). However, this method is both expensive and technically complex, which makes its use in public health laboratories difficult.

Molecular Diagnosis of *T. cruzi* Infection

DNA Targets for Molecular Diagnosis

Although aneuploidy has been suspected in *T. cruzi* (16, 17), it is currently assumed that all *T. cruzi* lineages generally exhibit diploidy (18), and it is generally accepted that the mean DNA content of *T. cruzi* is 0.3 pg/parasite (19), varying from 0.12 to 0.33 pg/cell among different strains (17). The genome of this parasite includes nuclear DNA and kinetoplast DNA (kDNA). The kDNA, which is found only in kinetoplastidae parasites, represents 20 to 25% of the total *T. cruzi* DNA (20). It consists of a complex network of concatenated circular DNA molecules located within a big single mitochondrion. This network is constituted by dozens of maxi-circles (which correspond to the mitochondrial genome of other species) and thousands of mini-circles (21). Both nuclear DNA and kDNA sequences have been used as targets for PCR assays. Pioneering work dating back to the late 1980s and early 1990s identified useful targets for PCR detection of *T. cruzi* in blood samples (19, 22, 23). The currently most widely used targets for conventional PCR (cPCR) diagnosis of *T. cruzi* infection are the hyper-variable regions of the mini-circles (mHVR), which are amplified by primers annealing at the conserved region flanking the mHVR (usually referred as the kDNA target), and the repeat tandem sequence of nuclear DNA (satDNA). Mini-circles are organized into four equidistant regions of highly similar sequences (constant regions) and four mHVRs intercalated between the constant regions. The main problem of kDNA-based PCRs is the cross-reactivity with *Trypanosoma rangeli*, a nonpathogenic trypanosomatid parasite distributed from Brazil to Mexico (24). The presence of this parasite may lead to misdiagnosis of Chagas' disease. On the other hand, satDNA is a 195-bp DNA repeat that has around 10^4 to 10^5 copies per nuclear genome in *T. cruzi* (25). The high copy number allows for the detection of low parasitic loads. In addition, satDNA PCR is specific for *T. cruzi* and does not cross-react with *T. rangeli*. Therefore, the misdiagnosis problem of kDNA may be avoided using satDNA-based PCRs.

Other multicopy DNA targets have been used for diagnosis, such as the spliced-leader intergenic region, the 24S ribosomal RNA genes, and the 18S ribosomal RNA genes. However, they exhibit lower analytical sensitivity due to a lower copy number compared to mini-circles and satDNA, but they are useful for strain genotyping when parisitemia is high enough.

Most Efficient Molecular Methods for *T. cruzi* Diagnosis

Different PCR protocols for the detection of *T. cruzi* DNA have been compared in an international collaborative study performed by 26 expert PCR laboratories from 16 countries (26). The participating laboratories used their own PCR strategies for examining three sets of pre-characterized samples: (i) purified DNA from *T. cruzi* stocks belonging to different lineages of the parasites, (ii) seronegative human blood samples mixed with an equivalent volume of a denaturizing guanidine-EDTA buffer and spiked with different dilutions of *T. cruzi* parasites, and (iii) blood samples from naturally infected patients also processed using a guanidine-EDTA buffer. In all cases, appropriate controls were used to rule out possible DNA contamination. The various PCR methods included different DNA extraction methods, different commercial and in-house master mixes, different targets (including both nuclear DNA and kDNA targets), different primer sequences, and both conventional and real-time PCR protocols. Four methods were shown to be the most efficient in all three sets of samples. These four methods were further re-evaluated on a subset of human blood samples from seropositive and seronegative patients, and the results obtained by the participating laboratories were confirmed (26). A synthesis of the four methods showing the best sensitivity and specificity is presented in Table 1. Clearly, according to the results obtained from this multicenter study, the highest sensitivity and specificity were obtained by using the kDNA and satDNA targets. Indeed, both kDNA and satDNA targets showed consistent results when different *T. cruzi* lineages were examined, with slight, nonsignificant differences (see "Analytical Sensitivity in Different DTUs," below). The four PCR methods presented in Table 1 can be considered the most suitable molecular procedures for detecting *T. cruzi* infection to date, taking into account that these tests were rigorously compared with other PCR methods.

An Alternative Simplified Method for Molecular Diagnosis

It is worth noting that, as a rule, both conventional and real-time PCR applied to the diagnosis of *T. cruzi* infection are restricted to specialized research laboratories, and there is a need for simplification of the PCR methods so they can be widely used in public health institutions. To develop simplified PCR methods, PCR-oligochromatography tests for *T. cruzi* detection (*T. cruzi* OligoC-TesT) have

TABLE 1 Best-performing methods for molecular diagnosis of Chagas' disease

Protocol	Extraction method	Target	Primers	PCR method	Master mix	Cycling conditions	LOD[a]	Specificity[b] (%)	Sensitivity[c] (%)
A	Standard phenol chloroform	kDNA	121 5′ AAATAATGTACGGGKGAGATGCATGA 122 5′ GGTTCGATTGGGGTTGGTGTAATATA	cPCR	PCR buffer: 1X dNTPs: 200 μM MgCl: 3 mM Primers: 0.5 μM Taq Platinum (Promega): 1.5 U DNA: 7.5 μl Final volume: 50 μl	94°C 3 min × 1 cycle; 97.5°C 1 min; 64°C 2 min × 2 cycles; 94°C 1 min; 62°C 1 min × 33 cycles; 72°C 10 min × 1 cycle	0.5	100	63
B	Standard phenol chloroform	Sat-DNA	TCZ-F 5′ GCTCTTGCCCACAMGGGTGC TCZ-R 5′ CCAAGCAGCGGATAGTTCAGG	qPCR	QuantiTect Sybr-Green PCR Master Mix (Qiagen) Primers: 0.5 μM DNA: 2 μl Final volume: 20 μl	95°C 15 min × 1 cycle; 95°C 10 s; 55°C 15 s; 72°C 10 s × 50 cycles	0.05	100	69
C	Standard phenol chloroform	Sat-DNA	TCZ-F 5′ GCTCTTGCCCACAMGGGTGC TCZ-R 5′ CCAAGCAGCGGATAGTTCAGG	cPCR	PCR Buffer: 1X dNTPs: 250 μM MgCl: 3 mM Primers: 0.5 μM Taq Platinum (Promega): 1.5 U DNA: 5 μl Final volume: 50 μl	94°C 3 min × 1 cycle; 94°C 45 s; 68°C 1 min; 72°C 1 min × 40 cycles; 72°C 10 min × 1 cycle	0.05	100	63
D	High pure PCR template preparation kit (Roche)	Sat-DNA	Cruzi 1 5′ ASTCGGCTGATCGTTTTCGA Cruzi 2 5′ AATTCCTCCAAGCAGCGGATA Cruzi 3 (probe) 5′ CACACACTGGACACCAA	qPCR	PCR FastStart Universal Probe Master Mix (Roche) Primers: 0.75 μM Probe:0.25 μM DNA: 2 μl Final volume: 20 μl	95°C 15 min × 1 cycle 95°C 10 s; 54°C 60 s × 45 cycles	0.05	100	63

[a]LOD: limit of detection. measured as the minimum number of parasite equivalents per milliliter of blood.
[b]Sensitivity: percentage of PCR-positives in a sample of 32 seropositive patients from endemic regions of Argentina, Bolivia, Brazil, and Paraguay.
[c]Specificity: based on 10 seronegative patients.

been developed, first targeting the satDNA (27), and more recently a new OligoC-TesT targeting the kDNA (mHVR) (28). The OligoC-TesT is based on hybridization of the PCR products with probes conjugated with gold particles, and the hybridization signal is visualized on a dipstick. The kit contains all components needed for performing the PCR, and an internal control DNA is included in the reaction. The *T. cruzi* OligoC-Test showed an overall specificity of 99.1% for the satDNA OligoC-TesT and 97.4% for the kDNA OligoC-TesT when control DNA was tested, while in samples from patients the overall sensitivity was 67.9% for the satDNA OligoC-TesT and 79.1% (95% CI 72.8 to 84.4%) for the kDNA OligoC-Test (28). When the satDNA OligoC-TesT was examined in the previously mentioned multicenter study (26) the sensitivity obtained was 72%, while specificity was 60% in patient samples. The SatDNA OligoC-TesT is commercially available.

Analytical Sensitivity in Different DTUs

Sensitivity obtained with satDNA and kDNA targets can be different for different *T. cruzi* DTUs, mainly due to different copy numbers of the target sequences in different lineages of the parasite. Different limits of detection were observed for satDNA PCRs and kDNA PCRs. The satDNA-based methods detected between 0.05 and 0.5 parasite equivalent per milliliter (par. eq./ml), whereas kDNA-PCR detected 5×10^{-3} par. eq./ml (26). Although the analytical sensitivity of kDNA-PCR is almost homogeneous among different DTUs (29), it is DTU-dependent for satDNA (30). In particular, lower analytical sensitivities were observed for TcI and TcIV (i.e., the analytical sensitivity of the *T. cruzi* OligoC-TesT was 100 to 1,000 times lower for TcI than for the other DTUs). In addition, the satDNA-quantitative PCR (qPCR) was 10-fold less sensitive for TcI and TcIV strains than for TcVI strains. This lower sensitivity is related to a lower number of copies of satDNA in TcI and TcIV (30).

Determining Parasite Load by qPCR

Applications of qPCR

qPCR allows DNA quantification in a sample through the real-time measurement of the DNA amplification curve. Consequently, this method is suitable to determine the number of parasites in blood or tissue samples. The method is as sensitive as cPCR and may be used as a simple diagnostic tool much like cPCR (26). However, determining the parasite load is of particular interest in several cases or presentations of Chagas' disease. First, short-term treatment efficiency in acute and chronic patients cannot be determined using conventional serological methods, as previously mentioned. Second, congenital cases (which can be considered acute cases) are also a suitable target for qPCR-based diagnosis and follow-up. As stated before, congenital cases cannot be diagnosed during the first year using serological methods (due to the persistence of maternal antibodies in the newborn), and parasitological methods have insufficient sensitivity (31). Finally, Chagas' disease reactivation after immune-suppressive treatments or AIDS is another area for implementing qPCR methods because of the presence of circulating parasites. Although diagnosis and follow-up can be also achieved by cPCR, the qPCR strategy may be useful to determine the relative parasite load at the moment of first diagnosis, and it will help to detect a trend toward therapeutic failure or cure during treatment and follow-up.

Several factors affect the sensitivity and specificity of qPCR. Such factors need to be evaluated to establish a highly standardized method. Consequently, different amplification targets, different DNA extraction methods, different internal and external controls, and different qPCR strategies need to be considered to accurately quantify parasite load.

Selection of the qPCR Target

As previously mentioned, the satDNA target provides a good option because of its high copy number and because satDNA-PCR does not cross-react with *T. rangeli* DNA. Only samples containing more than 10 pg of *T. rangeli* DNA were amplified for this target by qPCR (29), an amount which is highly unlikely to occur in a patient sample. However, the main problem of satDNA as a tool to quantify parasite load is related to the variable copy number among different *T. cruzi* lineages (30). A lower copy number leads to a lower analytical sensitivity of qPCR, which is particularly true for DTUs TcI and TcIV, which are of special interest in countries where these DTUs are prevalent. In addition, copy number may vary among strains even within a given DTU. Although several TcI strains have been evaluated, and the variation in copy number is relatively low, few strains of other DTUs have been analyzed. This is important because accuracy in estimating the parasite load depends on our knowledge of the copy number in the genome. Duffy et al. (30) developed a method to determine the DTU based on the melting temperature and characteristics of satDNA amplicons, and this may help in obtaining more accurate estimates of the parasite load. However, the possibility of mixed infections and temporal variations of the circulating *T. cruzi* DTUs should also be accounted for as a potential bias in estimating the parasite load.

kDNA is an interesting alternative diagnostic target to satDNA. qPCR amplification of the constant region of mini-circles provides a slightly lower detection limit (0.234 par. eq./ml) than satDNA (0.698 par. eq./ml) (29). In addition, the analytic sensitivity of kDNA qPCR is almost uniform among different DTUs (29). This uniformity suggests that DTU typing is not required to accurately estimate the parasite load. However, the main problem with this marker is its cross-reactivity with *T. rangeli*. Just 10 fg/μl of *T. rangeli* DNA is enough to be detected by qPCR (29), which generates a serious bias in countries where this parasite is present in the human population.

Diagnostic sensitivity was 100% for both methods when samples of acute patients were tested, whereas qPCR positivity was slightly higher using a kDNA target (84.14% versus 80.69% using satDNA) (29) when chronic patients were considered (Table 2). Another study observed that satDNA has a slightly higher diagnostic sensitivity than kDNA (32). However, different kDNA primers were used in the two aforementioned papers.

Sample Preparation and DNA Extraction Methods

Blood samples for *T. cruzi* DNA amplification are collected and mixed with an equal volume of 6 M guanidine-HCl/0.2 M EDTA solution (GE buffer). GE-blood samples may be boiled for 15 min to disrupt the mini-circle network. Boiling the sample gives a slight improvement in the detection limit for both satDNA (from 0.7 to 0.46 par. eq./ml, $P = 0.044$) and kDNA PCRs (from 0.23 to 0.16 par. eq./ml, $P = 0.013$) (29). Alternatively, some papers

TABLE 2 qPCR methods analyzed by Ramirez et al. (29)

Target	Primers	Master mix	Cycling	Specificity[a] (%)	Sensitivity[b] (%)
SatDNA	Cruzi 1 5-ASTCGGCTGATCGTTTTCGA Cruzi 2 5-AATTCCTCCAAGCAG CGGATA Cruzi 3 (Probe) 5-Fam-CACACACTG GACACCAA-NFQ-MGB	FastStart Universal Probe Master Mix (Roche) Cruzi 1: 0.75 μM Cruzi 2: 0.75 μM Probe: 0.05 μM Final volume: 20 μl	95°C 10 min × 1 cycle; 95°C 15 s; 58°C 1 min × 40 cycles	100	100 in acute phase 80.69 in chronic phase
kDNA	32F 5′-TTTGGGAGGGGCGTTCA 148R 5′-ATATTACACCAACCCCAATCGAA 71P (Probe) 5′-CATCTCACCCGTACATT[c]	FastStart Universal Probe Master Mix (Roche) 32F: 0.4 μM 148R: 0.4 μM Probe: 0.1 μM Final volume: 20 μl	95°C 10 min × 1 cycle; 95°C 15 s; 58°C 1 min × 40 cycles	100	100 in acute phase 84.14 in chronic phase

[a]Specificity based on 50 seronegative patients.
[b]Sensitivity based on 11 chagasic patients in acute phase and 145 seropositive patients in chronic phase.
[c]Underlined bases are the LNA modified nucleotides.

reported a lower limit of detection or diagnostic sensitivity when buffy coats were used instead of whole-blood samples (32–34).

DNA extraction based on solvent methods such as phenol-chloroform may result in a better DNA yield than commercial kits. However, low reproducibility, the toxicity of the solvents, and higher rates of false positives are known drawbacks of this somewhat antique methodology (35). Consequently, commercial kits may provide better alternatives.

Internal and External Controls

Several intrinsic process controls are required for qPCR to be considered a suitable diagnostic tool. As with other methods, nontemplate, negative, and positive controls will help to discard false-negative and false-positive results.

An internal amplification control (IAC) should be used to detect PCR inhibitors in the sample and discard erroneous quantifications of the parasite load, or even false negatives. The human RNase P gene may be used as an IAC (35, 36). However, the host DNA quantity per sample may vary among different clinical specimens, and a high variability in the quantification cycle is observed (29). Consequently, a linearized form of the pZErO-2 recombinant plasmid containing an inserted sequence of *Arabidopsis thaliana* aquaporin has been used as the IAC for *T. cruzi* testing (30, 37). However, it is important to note that any IAC used should be carefully evaluated, because inhibitors copurified with nucleic acids may differentially affect different PCRs (38). In this sense, efficiency variations due to inhibitors may be different for the *T. cruzi* target and the IAC. In addition, if both PCRs are performed in the same tube (i.e., using hydrolysis probes), it is important to verify that accurate quantification (i.e., assay efficiency and the limit of detection) of *T. cruzi* target DNA and/or IAC are not (mutually) impaired. Consequently, a validation comparing simplex and duplex PCR is also required in this case (39).

Calibration Curve and Quality Assays

To quantify the number of parasites in a sample using qPCR, it is necessary to know the efficiency of the PCR. This is achieved by using artificial samples of known and serially diluted parasite numbers as calibrators. The calibration curve is useful to determine PCR efficiency and the dynamic range over which the reaction is linear. In addition, this will help to determine the limit of detection and the limit of quantification. Usually, 10^5 *T. cruzi* parasites are added to a 10-ml GE-blood sample from a healthy patient. The resulting sample is serially diluted (10-fold) with noninfected GE-blood to obtain a concentration range from 10^5 to 0.01 par. eq./ml (30). The curve is reproduced three to five times.

Finally, other quality assays should be performed (39). The limit of detection, the limit of quantification, and the precision (intra-assay repeatability) should be rigorously analyzed in each laboratory.

qPCR Fluorescence Systems: SYBR Green and Hydrolysis Probes

Until now, two fluorescence systems have been used in qPCR for *T. cruzi*: SYBR Green and hydrolysis (or TaqMan) probes. SYBR Green has been used in various studies. Duffy and coworkers used this fluorescence dye in satDNA-qPCR to follow up on 43 pediatric patients after treatment (30). In addition, Bua et al. quantified parasitemia levels during 1-year follow-up after delivery in more than 50 children congenitally infected with *T. cruzi*, showing that SYBR Green-based qPCR has better performance than parasitological methods (22 out of 51 children had a parasite burden below the detection limit of parasitological methods) (31). Finally, Moreira et al. used this dye successfully to detect *T. cruzi* using kDNA-qPCR and satDNA-qPCR in 150 seropositive chronic Chagas' cardiomyopathy patients recruited in the BENEFIT trial (35). The SYBR Green dye has the advantage of being cheaper

than other systems. However, this fluorescent dye does not just label the specifically amplified DNA. Consequently, nonspecifically amplified fragments may bias the quantification (i.e., primer dimers). In addition, only a single PCR per tube is possible, and IAC PCR has to be performed in an independent, second tube (26). Instead, hydrolysis, or TaqMan, probes have the advantage of being specific to the target DNA because they specifically hybridize with an internal region of this target. Consequently, the use of hydrolysis probes allows multiplex PCR in a single tube. Hydrolysis probes were successfully implemented for detection of *T. cruzi* satDNA (37) and kDNA (32) in blood samples for chronic patients. Table 2 shows different hydrolysis probes used for satDNA and kDNA. Finally, it is important to note that SYBR Green and hydrolysis probes are similarly efficient for *T. cruzi* detection (35).

MOLECULAR METHODS FOR *T. CRUZI* GENOTYPING

A Brief Description of the Genetic Diversity of *T. cruzi*

Six main DTUs are distinguishable within *T. cruzi*: TcI to TcVI (40, 41); a seventh DTU has been recently described and was named Tcbat, because it was found infecting bats (42, 43). Population genetics approaches have shown that the DTUs correspond to "near-clades," which are distinct phylogenetic lines that undergo occasional genetic exchange and hybridization in the framework of a predominantly clonal evolution process (44, 45). According to recent molecular analyses (103), *T. cruzi sensu stricto* separated from *T. cruzi marinkellei* (a subspecies found in bats) around 6 million years ago. Approximately 3 million years ago TcII diverged from the ancestor of the lineages TcI, TcIII, and TcIV. Then, TcIV diverged from this last group and, at about the same time, TcI and TcIII split into two well-differentiated groups. Finally, and probably over the last 100,000 years, hybridization events between TcII and TcIII originated TcV and TcVI. This evolutionary time schedule facilitated a wide distribution of the variants across the South American continent. In addition, *T. cruzi* can infect a wide range of mammalian hosts and vectors and circulates in arboreal as well as in terrestrial environments. This great variability of hosts, vectors, environments, and continental distribution clearly favored the strong diversification of the species. Even within given DTUs, significant diversity has been observed.

In particular, TcI has a wide distribution ranging from the south of the United States to Argentina and Chile. TcI is the main cause of Chagas' disease in countries located north of the Amazonian basin (from the southern United States to Venezuela and the Guyanas). Although TcI is often associated with wild cycles in countries located in the south of Latin America, several reports have described it in the domestic cycle as well and even implicated it in human infections (46–48). In addition, a close association of this DTU with the arboreal environment and marsupial hosts (*Didelphis*) has been described. The diversity of TcI is possibly related to its wide distribution, and its genetic diversity appears to be linked to geographical distances. A single group called TcI_{Dom} (also called TcIa) is mainly associated with human infections (49, 50), but other subdivisions have been described within TcI as well. However, the exact genetic boundaries between them are not completely clear (51), and further research is required to determine whether these subdivisions can be validated any further.

TcIII is also a highly diverse DTU that is distributed from Argentina to the northern regions of South America. This lineage is mainly associated with the sylvatic cycle of transmission and the terrestrial ecotope (52). The main hosts are armadillo species (*Dasypodidae*). However, some reports suggest an emergence of this lineage in the domestic cycle in the region of the Gran Chaco Americano (an ecoregion which extends across territories of Argentina, Paraguay, and Bolivia) (48, 53). The genetic diversity of this DTU shows geographical differences, and at least two groups exist, one located in the northern parts of South America and the other one more in the south (52).

TcIV is the DTU about whose genetic diversity we have less information. In addition, it was only rarely associated with human infection (54). At least two groups have been clearly defined within this DTU. Strains from North America are clearly different from those derived from the southern part of the continent. Their separation is almost as ancient as the separation between TcI and TcIII.

TcII is associated with human infections in the southern cone of Latin America. Data about its presence in wild cycles are scarce. Considerable genetic diversity has been noted within TcII. Possible subdivisions within this DTU have not been described yet.

Finally, TcV and TcVI are the main DTUs in the domestic cycles in the southern cone. These DTUs have rarely been described in the sylvatic cycles (55). They exhibit limited genetic diversity, as expected due to their supposedly recent origin.

Methods of Typing I: Isolation-Dependent Methods

In this section, we summarize molecular methods for *T. cruzi* typing which most frequently require prior isolation of the infecting strain and its cultivation in enriched culture media. These methods are usually not suitable for direct typing from biological samples (i.e., blood samples, tissues, etc.) due to their low sensitivity. They are more useful for describing the genetic diversity of the parasite (population genetics and phylogenetic analyses) than for diagnosis. In the following paragraphs we briefly describe classical methods used to define the six DTUs and then more recent methods for *T. cruzi* typing.

Multilocus Enzyme Electrophoresis (MLEE) and Random Amplified Polymorphic DNA

MLEE used to be the gold standard for *T. cruzi* typing. The method was developed before information about the *T. cruzi* genome was available, and it was based on different electrophoretic mobility patterns for various enzymes according to interstrain variation of amino acid sequences. The MLEE method made it possible to show that *T. cruzi* undergoes predominantly clonal evolution with occasional bouts of genetic exchange (44) and to individualize the six major DTUs, or "near-clades" (56, 57). These results have been fully confirmed by modern methods including the nucleic acid-oriented ones (58–60). However, MLEE has been supplanted by DNA-based approaches because they are less time-consuming and more informative.

Random amplified polymorphic DNA was also used as a typing method (57, 61). This method is based on DNA amplification by PCR using a randomly designed short

primer. After amplification, the PCR product is run in an agarose gel to show a pattern of bands. Random amplified polymorphic DNA produces highly polymorphic markers, and it has shown a significant correspondence with MLEE (57). The main advantage of this method is that it allows for obtaining information at a wider genomic level (not just one locus), without previous genome information. However, it is based on the interpretation of banding patterns, which may be subjective. In addition, the method has low interlaboratory reproducibility because of the sensitivity of PCR to even minor variations (i.e., enzyme, reagents, cycler, etc.). Consequently, it is hard to compare results obtained in different laboratories.

A Simple Single-Locus Approach

Quite often, sequences of coding genes allow for differentiation of certain DTUs. However, more than one locus is required to differentiate all DTUs. Cosentino and Aguero (62) identified in the *T. cruzi* genome a gene encoding the putative C-5 sterol desaturase (*TcSC5D*). This gene, located on chromosome 22, is single copy and presents a high density of polymorphic sites. By using specific primers, an 832-bp DNA fragment may be amplified by PCR. Sequencing this fragment allows unequivocal differentiation of the six DTUs. This is due to a predominantly clonal evolution (45, 63), which leads to a considerable linkage disequilibrium (nonrandom association of genotypes at different loci). This shows that the variation at one locus helps to predict the variation at other loci with a high probability (indirect typing). The authors tested between three and seven strains for each DTU, and they observed that the polymorphic sites allowed them to distinguish the different DTUs.

The main drawback of this method is related to differentiation of TcV and TcVI. Just a single polymorphic site (homozygous C in TcVI and heterozygous C/T in TcV) distinguishes the two DTUs. In addition, the method was tested using a limited number of strains of these DTUs only. Apparent loss of heterozygosity (caused by either a real loss of heterozygosis or by mutation in the annealing sites of one or both of the primers) has been described for certain strains in multilocus sequence analyses (59, 60) (see below). If apparent loss of heterozygosity occurred in a TcV or TcVI strain for TcSC5D, this might lead to problems in differentiating the two DTUs. In addition, a risk of homoplasy cannot be ignored, since a TcV strain with a single mutation at the C/T heterozygous locus may be misclassified as TcVI and vice versa. Lastly, the authors proposed a restriction fragment length polymorphism approach as an alternative to sequencing to reduce costs. The restriction enzymes SphI and HpaI may be used in the same PCR tube after PCR in a single incubation step. These enzymes distinguish all lineages with the exception of TcV and TcVI, which require an additional step of PCR-restriction fragment length polymorphism based on an analysis of the Mev-kinase gene (58).

Multilocus Sequence Typing (MLST)

MLST is based on the amplification and sequencing of several housekeeping gene fragments. Usually, between five and seven fragments of different loci are sequenced. Then, the sequences of these loci are analyzed to identify MLST genotypes (also called sequence types or diploid sequence types). Two MLST schemes based on nuclear genes have been developed for *T. cruzi* by different laboratories (59, 60). In addition, a consensus of both schemes selecting the best loci for typing was developed in a collaborative effort (58). This consensus scheme selected 7 out of 19 loci. Each locus is amplified using specific primers, and PCR products are sequenced bidirectionally. MLST has many advantages over other approaches. First, data analysis is based on sequence data and not banding patterns, which reduces the risk of subjective interpretation of data. Second, sequence data can be deposited in specialized online, open-format databases, which facilitates comparison between laboratories. Finally, MLST is a highly discriminatory method, which is useful to detect diversity even at the intra-DTU level.

The MLST consensus scheme is based on sequencing of the seven loci described in Table 3. Once consensus sequences from given gene fragments are obtained, they are aligned using any alignment software such as MEGA or

TABLE 3 Optimized multilocus sequence typing scheme for *T. cruzi*

Gene	Gene ID[a]	Chromosome no.	Primer sequence (5′–3′)	No. of polymorphic sites[b]
GPI[c]	Tc00.1047053506529.508	6	CGCCATGTTGTGAATATTGG (20) GGCGGACCACAATGAGTATC (20)	18
HMCOAR[c]	TC00.1047053506831.40	32	AGGAGGCTTTTGAGTCCACA (20) TCCAACAACACCAACCTCAA (20)	20
RHO1[c]	Tc00.1047053506649.40	8	AGTTGCTGCTTCCCATCAAT (20) CTGCACAGTGTATGCCTGCT (20)	23
Tc MPX[c]	Tc00.1047053509499.14	22	ATGTTTCGTCGTATGGCC (18) TGCGTTTTTCTCAAAATATTC (21)	12
LAP	Tc00.1047053508799.240	27	TGTACATGTTGCTTGGCTGAG (21) GCTGAGGTGATTAGCGACAAA (21)	16
SODB	Tc00.1047053507039.10	35	GCCCCATCTTCAACCTT (17) TAGTACGCATGCTCCCATA (19)	9
RB19	Tc00.1047053507515.60	29	GCCTACACCGAGGAGTACCA (20) TTCTCCAATCCCCAGACTTG (20)	26

[a]Gene ID refers to the access to the gene bank for the complete gene sequence where the primers were designed.
[b]Number of polymorphic sites observed by Diosque et al. (58) in a set of 25 reference strains.
[c]Gene fragments used in reduced four-loci multilocus sequence typing scheme.

SeaView. The procedure is repeated for each strain and for each locus. Once the alignment for all strains is obtained for each locus, they can be analyzed by different software packages. Software specific for MLST data analyses, named MLSTest, was developed with the goal of analyzing diploid sequences of eukaryotic organisms (65). Two approaches were developed to analyze MLST data (Fig. 1 and Fig. 2). In the first approach, different sequences at each locus are considered as distinct alleles. The combination of alleles of several loci generates an allelic profile for each strain (also called a diploid sequence type). Once defined, the identity between allelic profiles can be represented in a distance matrix and analyzed by classical agglomerative methods of clustering, such as the unweighted-pair group method using average linkages or neighbor-joining. This approach has the advantage of buffering the effect of recombination, which is observed in many microorganisms. A single recombination event produces multiple differences between two strains at a given locus. These differences are misleadingly assumed to be a mutational step in sequence-based methods of analysis. Consequently, in such situations, there is no weighting to reflect the number of differences between different alleles. The alternative approach is to concatenate all loci in a super-alignment, which is analyzed by the unweighted-pair group method using average linkages or neighbor-joining. This approach has the advantage of showing a higher resolution at the intra-DTU level (59, 65) and is useful for determining inter-DTU relationships (58). Fortunately, MLSTest has several tools to discard these biases caused by genetic exchange.

An interesting advantage of MLST is that reference sequences of different DTUs are now available in GenBank, and they can be used in the analyses without *de novo* amplification and sequencing.

The consensus scheme is highly discriminative. It was able to distinguish 25 out of 25 reference strains (58). In addition, it is able to assign strains to their appropriate DTU with high-level confidence. However, the main disadvantage of MLST is the relatively high cost of sequencing. Consequently, a reduced scheme of MLST has been proposed to correctly identify strains to the appropriate DTU, although it loses some of the discriminative power of the seven-loci scheme at the intra-DTU level. The scheme relies on only four loci and is able to differentiate the DTUs with high confidence. Since sequencing is becoming cheaper and of higher throughput, these problems will cease to exist in the near future, when even whole-genome sequencing will become affordable.

The MLST scheme described above is based on nuclear loci, and it does not include any locus from kDNA. Another MLST scheme was developed based on kDNA loci (kMLST) (66). Although this last scheme is highly discriminatory, it should be carefully applied for DTU assignment given the evidence of mitochondrial introgression that could lead to a misclassification (66–68).

Multilocus Microsatellite Typing (MLMT)

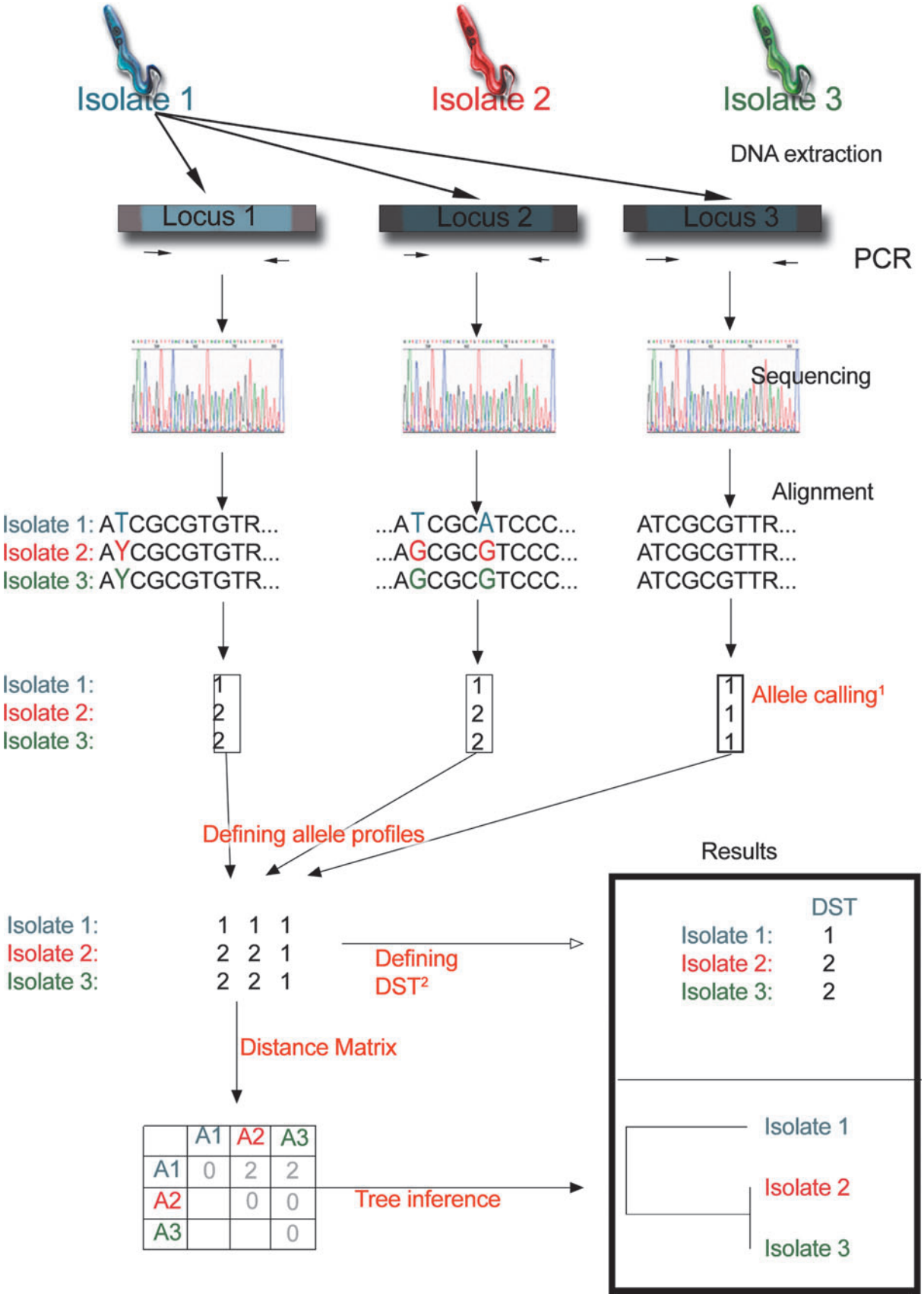
MLMT is a highly discriminatory method. It is based on the amplification of DNA fragments containing short tandem repeats. These microsatellites are length-variable among different *T. cruzi* strains. Amplified PCR products containing the microsatellite are evaluated using capillary electrophoresis to detect the potentially small length variations. This marker is based on single-copy loci and is considered to be unaffected by selective pressures. Consequently, it is a well-appreciated tool for population genetics studies.

Oliveira et al. (69) developed the first MLMT scheme for *T. cruzi*. This scheme was based on 8 polymorphic $(CA)_n$ microsatellites, and the analysis of 54 strains using this scheme showed correspondence with other markers (70). Further, different MLMT schemes have been used for population studies in *T. cruzi*. However, no consensus scheme has been designed yet. Llewellyn and coworkers used 48 and 49 microsatellite loci to analyze internal diversity within TcI (49) and TcIII (52), respectively. In addition, Baptista et al. (71) used seven polymorphic microsatellites to analyze genetic exchange in TcII populations from Brazil. These studies have shown high degrees of polymorphism in these three DTUs. Moreover, Lewis and coworkers (72) analyzed 28 microsatellite polymorphic loci for 16 strains of TcV and TcVI, showing reduced genetic diversity within both DTUs. In spite of its strong resolution, MLMT markers have the disadvantage of high levels of homoplasy. Consequently, DTU assignment may fail if not enough loci are used for typing.

In addition, microsatellites have been proposed for genotyping directly on biological samples. Macedo and coworkers (73) reported a limit of detection ranging from 10 to 100 fg of DNA for microsatellite PCR, which is unexpectedly sensitive for single-copy genes. They proposed this tool as a marker in biological samples. However, it is important to consider that this analytical sensitivity is far lower than that of the molecular diagnostic methods described above. As an example, the most efficient PCR methods for *T. cruzi* DNA detection have analytical sensitivities ranging from 1 to 0.1 fg of DNA. Consequently, microsatellite PCRs would have a 100 to 1,000 times lower analytical sensitivity than optimized molecular diagnostic methods. In addition, considering that the most efficient methods have sensitivity levels around 60% in chronic patients (Table 1), a very low sensitivity for blood samples of chronic patients is expected with microsatellite markers.

In spite of this expected low sensitivity, Venegas and coworkers (74) used three out of the eight microsatellite loci described by Oliveira et al. (69) to analyze blood samples of 42 noncardiopathic and 32 cardiopathic individuals who had positive cPCR for kDNA. They implemented double rounded PCRs using the same primers. This enhances the number of cycles during PCR and hence enhances sensitivity. A result unwished for in this case may be the amplification of nonspecific by-products. The researchers observed amplification for 21.6%, 75.7%, and 87.8% of samples for the three loci examined. In addition, Valadares et al. (75) analyzed different microsatellite markers using a nested PCR to increase sensitivity. However, reported analytical sensitivity for these nested PCRs was around 200 fg, which is lower than the previous analytical sensitivity reported by Macedo and coworkers for cPCR of

FIGURE 1 Simplified scheme for multilocus sequence typing (MLST) data analysis based on allelic profiles. (1) Allele numbers are arbitrarily assigned considering the rule that two identical sequences always have the same allele number. (2) DSTs are arbitrarily assigned considering that two isolates with the same allelic profile must have the same DST number.


Isolate 1
Isolate 2
Isolate 3
DNA extraction
Locus 1
Locus 2
Locus 3
PCR
Sequencing
Alignment
Isolate 1: ATCGCGTGTR...
Isolate 2: AYCGCGTGTR...
Isolate 3: AYCGCGTGTR...
...ATCGCATCCC...
...AGCGCGTCCC...
...AGCGCGTCCC...
ATCGCGTTR...
ATCGCGTTR...
ATCGCGTTR...
Isolate 1:
Isolate 2:
Isolate 3:
1
2
2
1
2
2
1
1
1
Allele calling[1]
Defining allele profiles
Results
Isolate 1: 1 1 1
Isolate 2: 2 2 1
Isolate 3: 2 2 1
Defining DST[2]
DST
Isolate 1: 1
Isolate 2: 2
Isolate 3: 2
Distance Matrix
A1 A2 A3
A1 0 2 2
A2 0 0
A3 0
Tree inference
Isolate 1
Isolate 2
Isolate 3

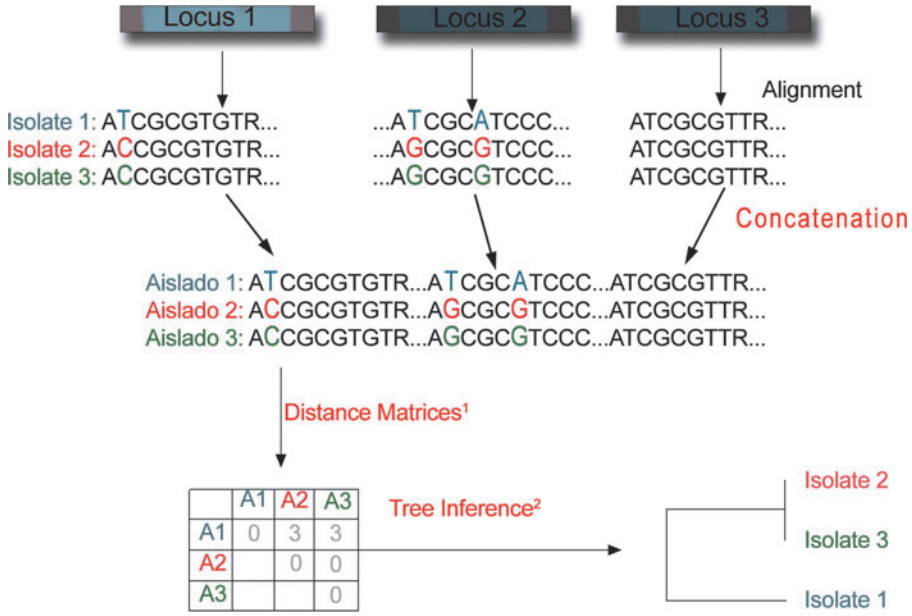


FIGURE 2 Simplified scheme for multilocus sequence typing data analysis based on concatenation of fragments. (1) Distance matrix is calculated by counting the number of nucleotide differences between isolates. (2) Tree inference is made by neighbor-joining or unweighted-pair group method using average linkages methods.

microsatellites. Consequently, further validation is required to implement this marker as a diagnostic tool for direct use on biological samples.

The discussion of MLMT in this section (isolation-dependent methods) was included to differentiate it from methods which are successfully implemented for direct genotyping on biological samples.

Typing Methods II: Culture-Independent Methods

In this section, we describe molecular methods that can be used for direct *T. cruzi* genotyping in blood or tissue samples without the necessity of isolation and cultivation of the parasite. These methods are also applicable to cultured parasites.

Multicopy Sequences for Typing

Multicopy DNA fragments are usually implemented for typing due to some advantages in relation to single-copy genes. First, multiple copies of a certain DNA target are easier to amplify than single-copy targets, particularly in samples such as blood, tissues, or triatomine feces. In addition, several multicopy genes are organized in tandem. Usually, tandemly repeated genes are subjected to homogenization processes (i.e., concerted evolution or birth-and-death evolution) that lead to limited intragenomic variation (76). However, in spite of this low intercopy variability, some multicopy genes that do not code for proteins have high inter- and intrastrain variability. Consequently, these multicopy genes may be useful to describe genetic diversity across different strains and may also be suitable for direct typing in biological samples.

Two multicopy targets have been used for *T. cruzi* genotyping: rRNA genes and spliced-leader genes. rRNA genes are organized into cistrons, which are present in around 100 copies per genome and are located on at least two different chromosomes (77). These rRNA cistrons are separated by sequences larger than 20 kb (77). Each cistron encodes the sequences of several rRNA types. These rRNAs include 24S-alpha, 24S-beta, and five low-molecular-weight RNAs which are components of the large ribosomal subunit (78). In addition, an 18S fragment is embedded in the small ribosomal subunit (78). Lastly, different rRNAs are separated by internal transcribed spacers, which are eliminated during the ribosome biogenesis (79). Other small rRNA genes are located outside the cistron (79). Particularly useful polymorphisms were detected at the D7 domain of ~100 bp located in the 24S-alpha locus (80). PCR amplification of the D7 domain revealed three groups simply according to amplicon size (81). The amplicon sizes were 110 bp in TcI and TcIII strains, 125 bp in TcII and TcVI, 110 bp and 125 bp in TcV, and variable sizes for TcIV. In addition, variable DNA fragment sizes were observed by amplification of a region of the 18S locus, which were 175 bp for TcI (with a few exceptions), 155 bp for TcIV, and 165 bp for TcII, TcIII, and TcV; no fragment was observed for TcVI (82, 83).

The other multicopy target used for *T. cruzi* genotyping is the intergenic region of the spliced leader. The spliced leader is a short RNA sequence added to the 5′-end of every mRNA through a process called trans-splicing, which is only observed in kinetoplastids. The spliced leader (SL) is organized in clusters of tandem repeats with around 200 gene copies per genome (84). Each SL copy is flanked by intergenic regions (SL-IR) of around 500 bp. The SL is relatively well conserved among different species (85). However, the SL-IR is highly polymorphic among *T. cruzi* strains (47, 86–89). Interestingly, the SL-IR is also highly similar between different intragenomic copies, which suggests concerted evolution (89). The interstrain variability of the SL-IR was used for *T. cruzi* genotyping. Using two primer combinations, Souto and coworkers (81) were able

to amplify different fragment sizes for different *T. cruzi* groups. With the primers Tc2 (5′-CCTGCAGGCACA CGTGTGTGTG) and Tcc (5′-CCCCCCTCCCAGGCC ACACTG), a 350-bp fragment was amplified in TcI only. The Tc1 and Tcc primers allowed the amplification of 300 bp in TcII, TcV, and TcVI. No amplicon was observed for TcIII or TcIV with any primers in most isolates. In addition, sequencing of the SL-IR made it possible to detect genetic structuration (the presence of subclustering resulting in a smaller number of near clades) within TcI (48, 88, 90).

Based on the amplified fragments for 24S-alpha, 18S, and SL-IR, Brisse and coworkers proposed a sequential algorithm to identify the six DTUs. However, some DTUs were characterized by the absence of amplification, which is distressing. Consequently, Brisse and coworkers proposed confirmatory specific PCRs designed to amplify sequences that are DTU-specific (82). Later, Burgos et al. (90) modified this algorithm to avoid typing based on the absence of amplification. Using three different SL-IR PCRs, Burgos and coworkers were able to distinguish different groups: TcI, TcIII-TcIV, and TcII-TcV-TcVI. In addition, a 24S-alpha hemi-nested PCR was used to differentiate TcIII from TcIV and TcV from TcII/TcVI. Finally, instead of 18S PCR, they implemented another hemi-nested PCR directed at the A10 fragment previously used by Brisse et al. as a confirmatory test, which made it possible to discriminate between TcVI (525 bp) and TcII (585 bp).

However, it is important to note that the 24S-alpha plus A10 fragments cannot show certain mixed infections or cultures (TcV/TcVI mix and several TcV have both 125/140-bp fragments for 24S-alpha and 525-bp fragments for A10). Burgos and coworkers (90) evaluated the limits of detection for the various PCR protocols in their algorithm. They observed detection limits ranging from 100 fg to 10 pg of DNA. Burgos et al. used this algorithm for genotyping congenitally infected children, transmitting and nontransmitting mothers, and unrelated Chagas' disease patients from Argentina. DTU assignment could be made on 38 (80.4%) out of 47 infected children. However, sensitivity was lower in infected mothers (ranging from 40.6 to 77.3%). The same algorithm was implemented in heart tissue of end-stage chronic Chagas' disease patients (46). The authors successfully amplified spliced leader fragments in 8 out of 15 heart tissues, but they could not clearly discriminate TcII, TcV, and TcVI because they failed to amplify 24S-alpha and/or A10 fragments.

The mHVR as a Target for Strain Typing

The mHVR is a very good target for the sensitive molecular detection of *T. cruzi* due to its high copy number. In addition, the mHVR has been used as a target for typing via different approaches. The main advantage of using the mHVR for genotyping is sensitivity: it can be amplified from blood samples containing less than 1 par. eq./ml.

Below, we briefly describe the three main *T. cruzi* typing approaches that use the mHVR as a target.

PCR-Restriction Fragment Length Polymorphism of the mHVR

The pioneering studies of Morel and coworkers showed that digestion of the kDNA by restriction endonucleases could be used for *T. cruzi* strain typing (91). A simpler approach, based on PCR of the mHVR and the posterior digestion of the PCR products using restriction endonucleases, can also be used with a good resolution power. Burgos and coworkers implemented this method using MspI and RsaI restriction enzymes to compare parasite populations in mothers and their congenitally infected siblings (90). In addition, Burgos and coworkers used this method to compare *T. cruzi* populations of different tissues of transplantation patients infected by *T. cruzi* (46).

mHVR Probes

The hypervariable region of the mini-circles used for *T. cruzi* diagnosis has also been used for typing in reciprocal hybridization assays. These assays showed that positive hybridization occurs among amplicons belonging to genetically closely related strains (92, 93). This approach is based on the Southern blot technique. The sequences of the primers used for the construction of specific probes are similar to those used for diagnosis but include restriction sites which allow elimination of the mini-circle constant region of these PCR fragments by digestion, using restriction endonucleases. This technique has been widely used in the molecular epidemiology of Chagas' disease (94–99).

It should be taken into account that most of these hybridization patterns should be considered "black-box hybridization patterns," because the mHVR is composed of a diverse number of sequences. However, different results suggest that for TcV and TcVI, the hybridization patterns could be explained by the presence of DTU-specific predominant sequences (100), while in TcI, the absence of predominant sequences suggests that the patterns of hybridization could be due to the global similarities of the total set of sequences (101). A clear weakness of this approach is the reproducibility: constant experimental conditions are required for obtaining reproducible results.

Low-Stringency Single Specific Primer PCR

This technique consists of submitting the 330-bp mHVR amplicon (obtained by cPCR) to multiple cycles of PCR amplification under conditions of very low stringency and using a single oligonucleotide primer (driver) specific for one of the extremities of the fragment. In this way, the driver hybridizes with high specificity to its complementary extremity and with low specificity to multiple sites within the fragment in a sequence-dependent manner. The obtained amplicons are observed as complex banding patterns when submitted to polyacrylamide gel electrophoresis. This approach has been successfully used for *T. cruzi* typing, allowing identification of strains with good discrimination and showing adequate correlations with other genetic markers (102). Taking into account that this technique is based on a low-stringency PCR, reproducibility may be its main weakness.

FINAL REMARKS

Presently, specific, sensitive, and standardized molecular approaches for diagnosis of *T. cruzi* infection and genetic characterization of the causative agent are available. Advances in the field of qPCR approaches for the quantification of parasitic load are promising; however, some difficulties regarding the quantification of different lineages of the parasite should still be resolved.

Regarding genotyping, it is clear that the MLST approach is emerging as the new gold standard for typing of *T. cruzi* isolates; however, more sensitive approaches, which allow typing directly from biological samples (avoiding isolation and growth in culture media), are needed. The mHVR target is a good candidate for direct parasite typing from biological samples, but standardized methods are not yet available. The mHVR is built from a highly

diverse set of sequences, and the molecular bases of some of the typing patterns are not fully understood, which in part blocks the development of even more standardized methods.

Finally, it is worth noting that serological approaches for typing could be complementary to molecular approaches and that next-generation sequencing approaches will soon invade the field of both the diagnosis of Chagas' disease and the characterization of the parasites themselves.

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Molecular Approaches for Diagnosis of Malaria and the Characterization of Genetic Markers for Drug Resistance

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37

INTRODUCTION TO MALARIA

Human malaria is caused by infection with protozoan parasites of the genus *Plasmodium*. Four species of *Plasmodium* are well known to cause malaria in humans: *P. falciparum*, *P. vivax*, *P. malariae*, and *P. ovale* (with two subspecies, *P. ovale curtisi* and *P. ovale wallikeri* [1]). Recently, a fifth species has been added to the list: the simian malaria species *Plasmodium knowlesi* has been discovered to be a major cause of zoonotic human malaria in parts of Southeast Asia (2). The parasite species vary in the severity of disease they cause, as a result of differences in their biology, with the highest morbidity and mortality resulting from infection with *P. falciparum*. Correct identification of the infecting species is required to select appropriate and effective antimalarial drugs for treatment.

The disease is transmitted through the bite of an *Anopheles* mosquito, which injects parasites into the skin and bloodstream while taking a blood meal. After an initial, asymptomatic multiplication period in the liver, parasites emerge to infect erythrocytes. Thereafter, there is a cycle of intraerythrocytic growth and parasite replication, followed by lysis of the red blood cell and emergence of daughter parasites that reinvade new erythrocytes. This intraerythrocytic cycle is associated with the major pathology of malaria disease. The periodicity of the cycle is characteristic of the infecting parasite species, varying from ~24 hours for *P. knowlesi* to ~48 hours for *P. falciparum*, *P. vivax*, and *P. ovale* and ~72 hours for *P. malariae*.

P. falciparum presents an additional complication for diagnosis, because the parasite circulates in the peripheral blood for only the first 18 to 24 hours after invasion (ring-stage parasites) and is then sequestered (as the trophozoite and schizont stages) for a further 24 to 30 hours in the capillary beds of the deep tissues, as a result of cytoadherence to the capillary endothelium. Following reinvasion, the ring forms again circulate; the larger stages of the parasite (trophozoites and schizonts) are rarely seen in the peripheral blood.

Infected individuals can present with symptoms including fever, headache, and vomiting, but the timing of onset of symptoms and their severity vary greatly between individuals and the infecting parasite species. Diagnosis of malaria from the symptoms alone is not recommended, because not all febrile illness is due to malaria, and presumptive treatment leads to drug waste and undertreatment of other febrile illnesses (3). If not diagnosed and treated promptly with an effective antimalarial drug, the infection can progress to life-threatening severe disease very quickly.

Early and accurate diagnosis of malaria is considered to be essential for effective management of the disease; misdiagnosis can result in significantly increased morbidity and mortality (4). The WHO recommends malaria diagnosis (evidence of parasite infection) before antimalarial drugs are given, both to improve the treatment of patients with any febrile illnesses and also to help to reduce the emergence and spread of drug resistance by reserving antimalarials for those who actually have the disease (4).

Diagnosis of malaria is most commonly undertaken through microscopic examination of Giemsa-stained blood smears, which allows confirmation of parasite infection, determination of the levels of parasite present in the blood (parasitemia), and differentiation of the parasite species by differences in the morphology of the parasite and the infected erythrocyte.

Following a positive diagnosis of malaria, a patient should be treated with an appropriate antimalarial drug; the choice of chemotherapeutic agent is dictated by the species of parasite present, the severity of disease in the patient, and any particular risk group to which the patient belongs (3, 4). In many parts of the world, parasites have developed resistance to a number of antimalarial drugs, which restricts the drug options; testing for drug resistance before treatment is not currently practiced. Because of widespread resistance in *P. falciparum* to antimalarials such as chloroquine and sulfadoxine-pyrimethamine (Fansidar), WHO currently recommends that uncomplicated malaria cases caused by *P. falciparum* be treated with artemisinin combination therapy (3). *P. vivax* malaria is usually treated with chloroquine, but in areas where chloroquine-resistant *P. vivax* has been reported, WHO recommends an appropriate artemisinin combination therapy (3). However, drug resistance is rarely at 100% in an area, so cheaper and safer drugs can be used if resistance can be tested before treatment. Options for the diagnosis of antimalarial drug resistance will be discussed later in this chapter.

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Several methods for the diagnosis of malaria infection have been developed and are currently used in the diagnostic laboratory and in field (malaria-endemic) settings. They can be divided into three groups: microscopy-based tests, immune-chromatographic tests, and molecular methods. The sensitivity of all diagnostic tests—the likelihood of detecting at least one parasitized erythrocyte in a blood sample—is influenced by the volume of blood that can be screened in the test and by the density of parasites in the blood sample.

MICROSCOPY METHODS

Light Microscopy: Thin and Thick Blood Smears

The detection of *Plasmodium* species by light microscopic examination of blood smears stained with Giemsa stain is the most widely used technique for the detection of malaria parasites, and it is still the routine method for the diagnosis of malaria in many disease-endemic countries (4). In 2012, there were an estimated 188 million microscopic examinations for malaria worldwide (5). Microscopy is widely used because of low cost and the relatively unsophisticated equipment needed, but it is slow, and accurate diagnosis requires experienced microscopists and technicians. Microscopic diagnosis can be inadequate because of poor-quality equipment, insufficient training, or a lack of quality assurance (6). Microscopy has been shown to perform well in the diagnosis of clinical cases in areas where malaria is endemic and in nonendemic settings but was found to be less sensitive for diagnosis of asymptomatic individuals in malaria-endemic regions (7). These differences in performance can be explained by the higher parasite densities found in clinical cases and in individuals with no previous exposure to the disease, compared to the low parasite densities common in asymptomatic infections, which occur only in those with some degree of acquired immunity to malaria, gained as a result of continuous exposure to the parasite in the field.

The blood sample used for both thick and thin films is usually obtained by pricking a finger. The thick film (Fig. 1) consists of 6 to 20 layers of red and white blood cells which are stained as an unfixed preparation; dehemoglobinization occurs during the staining process so that large amounts of blood can be examined quickly and easily (8). Thick films therefore allow more rapid identification of parasites in the blood because of the larger volume of blood examined, compared with the thin blood film. Parasitemia can be estimated relative to the white blood cell count, using a standard value for the white blood cell count (8,000 white blood cells/μl of blood) (8). Examination of thick blood films can detect a parasite density as low as 5 to 20 parasites/μl of blood (approximately 0.0001 to 0.0004% of erythrocytes infected), but a more realistic estimate in field settings is a threshold of 50 to 100 parasites/μl of blood (9).

The thin film (Fig. 1) consists of a single layer of red and white blood cells, which is fixed with methanol before staining with Giemsa stain, thus preserving the red cells and providing an enhanced capacity to identify the species of parasite present. A combination of size, shape, and appearance of the infected erythrocyte, and the morphology of the parasite, allows a differential diagnosis of the species of parasite (8). Errors in differentiation of species are common, especially between morphologically similar species such as *P. vivax* and *P. ovale* or *P. malariae* and *P. knowlesi* (2). Parasitemia (the percentage of red blood cells infected with parasites) can also be estimated directly from red blood cell counts.

Fluorescence Microscopy

Fluorescent dyes such as acridine orange (AO) (10) can be used to stain parasite nuclei, either as a direct stain followed by fluorescence microscopy or in combination with a concentration method such as the centrifugal quantitative buffy coat (QBC) (11). The dyes bind to nucleic acid and fluoresce under UV excitation. The QBC method combines a capillary tube, precoated with AO, containing a float; following centrifugation, the white blood cells (which also take up AO) are separated from the parasitized red blood cells, and the latter can be viewed in a thin layer around the float, through the capillary tube wall, using a fluorescent microscope. The use of fluorescence speeds up diagnosis, and the test is simpler to interpret, but neither AO nor QBC tests allow the identification of the species of parasite present (12). The QBC test was reported as having low sensitivity for parasitemias below 100 parasites/μl of blood (13). Because of the relatively high cost of both the test capillaries and equipment needed, coupled with safety concerns over breakage of the glass capillaries, the QBC test is not widely used.

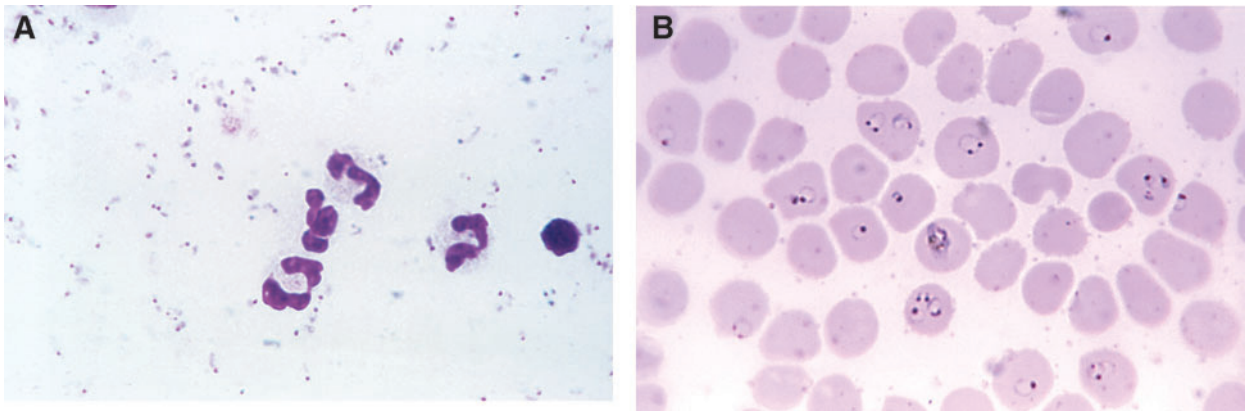

FIGURE 1 (A) Thick and (B) thin blood films showing *P. falciparum* ring forms. (Images taken from the Public Health Image Library, Centers for Disease Control and Prevention, courtesy CDC/Dr. Mae Melvin [public domain image]).

IMMUNE-CHROMATOGRAPHIC METHODS

These tests, also known as rapid diagnostic tests (RDTs), are usually available as dipstick or plastic cassette format kits (14). Diagnosis is based on the capture of parasite antigens present in a drop of blood, using monoclonal antibodies. The migration of the antigen-antibody complex in a strip of nitrocellulose, on which a second antibody has been immobilized, produces a visible colored line, which represents the detection of the parasites in the blood. Most of the commercially available RDTs target one or more of three malaria antigens: the parasite lactate dehydrogenase enzyme (pLDH), the parasite histidine-rich protein 2 (HRP-2), and the parasite enzyme aldolase.

pLDH is an enzyme found in the glycolytic pathway of the malaria parasite and is produced by both sexual and asexual stages of four human malaria species (not yet confirmed in *P. knowlesi*) (15, 16). The level of pLDH in the blood is strictly correlated with the level of viable parasites; therefore this assay can be used to monitor the progress of the patient during therapy and as an indication of recrudescence and possible drug-resistance infections (15). Antibodies to pLDH used in the RDTs can be species-specific for *P. falciparum* or *P. vivax* or can be pan-specific and detect all human species except for *P. knowlesi* (17, 18).

HRP-2 is a highly abundant protein, specific to *P. falciparum*, secreted by the asexual and gametocyte (transmission) stages of the parasite (19). HRP-2 antigen has been shown to persist after the clinical symptoms of malaria (and circulating parasites) have disappeared (20); thus, this antigen cannot be used for monitoring the patient to determine if treatment has successfully cleared parasites from the blood. Furthermore, false-negative results have been reported due to a deletion or mutation of the HRP-2 gene in some parasites (21–23), and false positive results have been attributed to cross-reaction with immunological factors such as rheumatoid factor (24) and with other infections including schistosomes (25) and human African trypanosomes (26), which are sympatric with malaria in many parts of the world.

Aldolase is another key enzyme of the glycolytic pathway, and it is produced by all human malaria species (27). The detection of aldolase antigen identifies live parasites only, and it is highly conserved within a species. Different monoclonal antibodies used in RDTs can detect aldolase from all human malaria species (pan-specific), or antibodies specific for *P. vivax* or for *P. falciparum* can be used in species-specific assays. Aldolase-only RDTs are not commonly used and have shown poorer sensitivity for the detection of malaria (18). Many RDTs combine the detection of HRP-2 or pLDH with aldolase.

Performance and Choice of RDTs

The performance of different RDTs has been evaluated in laboratory tests by WHO (28). RDTs perform well with samples with high parasite density (2,000 to 5,000 parasites/μl of blood) for both *P. falciparum* and *P. vivax*, but performance is more variable at low parasite densities (200 parasites/μl of blood). Detection of low-density *P. vivax* infection (200 parasites/μl of blood) in patient samples was better with tests targeting pLDH than those targeting aldolase, but generally, performance was lower than for detection of *P. falciparum* (28).

Published data on the performance of pLDH- and HRP-2-based tests (including combination assays with aldolase) in field and laboratory settings have been the subject of a recent Cochrane Review (29). This meta-analysis of published trials of RDTs showed that HRP-based tests had higher sensitivity than those based on pLDH. However, specificity was lower for HRP-2-based tests.

RDTs are increasingly used in malaria-endemic countries, and for *P. falciparum*, RDTs have been shown to have detection thresholds similar to standard microscopy and to perform as well as microscopy in field settings (7). Unlike microscopy, RDTs do not allow an estimate of parasite density.

RDTs which target only HRP-2 are likely to miss *P. falciparum* infections in areas where there is a high frequency of parasites with no expression of HRP-2 (21, 30). Because they do not detect non-*falciparum* infections, HRP-2-based RDTs are of limited use in areas where *P. falciparum* is not the main malaria parasite species. Tests detecting pLDH are likely to be of more use in these areas and avoid false positives due to HRP-2 antigen persistence. Existing RDTs lack sensitivity and specificity for *P. knowlesi*. Antibodies recognizing HRP-2 do not detect *P. knowlesi*, and antibodies specific to pLDH of *P. falciparum* and of *P. vivax* can cross-react with *P. knowlesi* (17, 18). Current RDTs are therefore inadequate to distinguish *P. knowlesi* infections from *P. falciparum* and *P. vivax*, and RDTs are not currently recommended in settings where *P. knowlesi* is the predominant species (17). The RDTs that are currently available do not perform as well as microscopy for non-*falciparum* infections, and microscopy outperforms RDTs in nonendemic settings for the detection of both *P. falciparum* and non-*falciparum* infections (7, 28).

RDTs give quick results (in 5 to 20 minutes) and are useful in low-resource settings because they do not require electricity, are inexpensive, and are simple to perform and easy to interpret. However, they have a limited shelf life and need to be kept dry and away from extremes of temperature (>30°C), or their performance can be affected (31).

Low-level parasitemia (≤1 parasite/μl of blood) is common in asymptomatic infections of people in regions of malaria endemicity (32). These individuals may contribute to ongoing transmission (33); active case detection with highly sensitive diagnostics will be necessary to identify and treat these people in areas seeking to eliminate malaria (6, 34). This requires alternative molecular assays, particularly PCR (and quantitative PCR), nucleic acid sequence-based amplification (NASBA), and loop-mediated isothermal amplification (LAMP).

SEROLOGICAL METHODS

Serologic assays, which detect the presence of antibodies against *Plasmodium* species, are rarely used for acute disease diagnosis and management. In regions where malaria is endemic, it is likely that every individual has antibodies that recognize parasites, so this is not a useful criterion in determining an ongoing parasite infection. Serological assays such as enzyme-linked immunosorbent assays (ELISAs) are occasionally used in patients from nonendemic countries with acquired infection, to detect seroconversion and thus to confirm an initially doubtful diagnosis of malaria (35).

MOLECULAR DIAGNOSIS WITH PCR

PCR, and particularly nested PCR, has increased the sensitivity and specificity of malaria diagnosis since it was first introduced in the 1990s. The majority of the molecular

assays target the small subunit (SSU) rRNA genes of the parasite. The highly conserved nature of these genes has favored their use in numerous phylogenetic studies of microorganisms, including *Plasmodium* (36, 37). The loci have also been widely used for the detection and differentiation of human malaria parasites (38–40). Other loci that have been used for diagnosis and differentiation of *Plasmodium* species include the parasite mitochondrial genes cytochrome oxidase (*cox*) (41) and cytochrome *b* (*cytb*) (42) and the nuclear genes encoding the enzyme dihydrofolate reductase (*dhfr*) (43), the circumsporozoite protein (*csp*) (44, 45), and multicopy loci such as Stevor (46).

Malaria Diagnosis Based on PCR Amplification of the SSU rRNA Locus

In several *Plasmodium* species, the SSU rRNA genes exist as multiple copies (usually between four and eight) consisting of two or three differentially expressed sets of genes (47). *P. falciparum* has two types of SSU rRNA genes, denoted the A- and S- types, which are transcribed during the asexual (blood stage) and sexual/sporozoite (mosquito) stages, respectively (48). *P. vivax* has an additional type, denoted the O-type, that is transcribed only in a specific stage in the mosquito (the oocyst) (49). Only a single SSU rRNA gene type has been reported for *P. malariae* and *P. ovale*, but significant sequence variation has been reported for *P. ovale* (50–52). The variation in the sequence of *P. ovale* genes has been suggested to cause the failure of PCR-based assays to detect this parasite species in some parts of Southeast Asia (51, 52). Some PCR assays target only one type of SSU rRNA in those species that have more than one type. Therefore, the assays usually detect a multicopy gene, but the number of copies present is different in the different species, which can lead to differences in the sensitivity of detection of infections with low parasite density.

Amplification of the SSU rRNA locus in a nested PCR assay allows for the discrimination of distinct malaria species based on different-sized PCR products (38, 53). This assay has been considered the molecular "gold standard" for malaria diagnosis, but it is time-consuming and labor-intensive because it requires at least six separate reactions to test for the five human malaria species. Modifications include seminested PCR (54) and multiplex PCR (55–57), allowing diagnosis of several species in a one-tube reaction. A hexaplex, one-step, one-tube system has recently been developed that allows the simultaneous amplification of all five human malaria species (58). Size differences in the PCR products visualized by gel electrophoresis allow the different species to be identified.

The threshold of parasite detection for nested PCR is usually reported to be around 5 to 10 parasites/μl of blood, but the method is not quantitative. However, the hexaplex PCR system has been shown to have thresholds below 0.5 parasites/μl of blood for all five human *Plasmodium* parasites (58). Two further advantages of the multiplex approach are the savings in time (3 hours compared to ~6 hours for seminested PCR and ~20 hours for nested PCR) and the lower risk of carryover and possible external contamination during transfer between primary and secondary reactions compared to a nested or seminested approach.

PCR Diagnosis Using Alternative Loci to SSU rRNA

The cytochrome *b* and cytochrome oxidase genes are present as single copies on the 6-kb circular mitochondrial genome of the parasite (59). Each asexual parasite contains a single mitochondrion with around 20 copies of the mitochondrial genome, and the transmission stages (gametocytes) typically have 4 to 8 mitochondria (60, 61). PCR assays based on amplification of mitochondrial genes therefore should show higher sensitivity compared to the SSU rRNA locus assay (42, 62), especially for detection of gametocytes, where the copy number of the mitochondrial genome is approximately 10 to 32 times higher than the copy number of the SSU rRNA locus. Initial results show that single-amplification mitochondrial gene assays have excellent specificity and sensitivity, that is, statistically noninferior to that of the reference SSU rRNA nested PCR. Further testing will be necessary with a larger number of field samples to determine if the mitochondrial assays have a lower threshold for detection than those based on amplification of the SSU rRNA loci (62). These assays may be of particular value to detect gametocytes, which are typically at very low densities in human infections (63). A further advantage of amplifying the mitochondrial genes *cytb* and *cox* is the lack of sequence polymorphism (at the primer sites) observed in parasites from diverse geographical locations (64).

Advantages of PCR Diagnosis

The application of molecular methods for diagnosis of infection in many studies revealed a reservoir of asymptomatic individuals with low parasitemia, especially in areas of low transmission. On average, the prevalence of infection measured by microscopy was around half of that identified by PCR, but in areas of low transmission (defined as a low [<10%] prevalence of infection detected by PCR), almost 90% of infections were not identified by microscopy (65). This is probably explained by higher parasite densities in individuals in areas of high transmission intensity, which are more likely to be identified by microscopy (66). Mixed infections with different species were also found at a much higher rate than previously suspected (67–69).

The basic PCR diagnostic method is used in research and in diagnostic laboratories in non-malaria-endemic countries that have the required resources, but the considerable time lag between sample collection, transportation and processing, and dissemination of the results back to the physician limits the usefulness of this technique in routine clinical practice. In areas with high malaria transmission, factors such as limited financial resources and inadequate laboratory equipment preclude the use of PCR as a diagnostic method in field clinics (70).

Quantitative PCR for Malaria Diagnosis

The development of quantitative real-time PCR (qPCR) techniques, usually targeting the SSU rRNA locus, allows quantification of parasite levels at the same time as the identification of the species of parasite in the infection (71–73). The method has the advantage of speed over conventional PCR, with results typically being available within 1 hour, and similar or slightly lower thresholds for detection (quoted thresholds range from 3 to 30 [73] to 0.02 [72] parasites/μl of blood). The greater sensitivity offered by qPCR methods allows detection of parasites in the blood 1 to 4 days earlier than microscopy in human challenge vaccine trials; this has been useful in allowing vaccinees to be monitored and treated earlier, often before the onset of symptoms (74). The ability to measure parasite density afforded by qPCR also allows the monitoring of

parasite clearance rates during chemotherapy, which revealed the early signs of resistance to artemisinin drugs in Cambodia (75).

ISOTHERMAL AMPLIFICATION DIAGNOSTIC METHODS

Two isothermal molecular methods, NASBA and LAMP, have been applied to the diagnosis of malaria. Isothermal amplification methods do not require a thermocycler and can be performed using simple and portable heating systems.

Nucleic acid sequence-based amplification (NASBA)

NASBA is an alternative technique to PCR and RT-PCR. RNA is amplified in an isothermal process involving cycles of production of cDNA from the RNA templates and T7 RNA polymerase for subsequent amplification (Fig. 2) (76). Because NASBA is capable of generating very high numbers of RNA copies per cycle in a short time frame, it has been used for diagnosis of many infectious diseases (77). For malaria, a quantitative NASBA was developed that targets the SSU rRNA locus for both *P. falciparum* and *P. vivax* and was found to have a threshold of detection of 0.01 to 0.1 parasites/μl of blood, via serial dilutions of clinical samples (78). The assay was further developed to provide quantitative identification of the four human *Plasmodium* species (excluding *P. knowlesi*) with a similar sensitivity (detection limit of 0.1 *Plasmodium* parasites/diagnostic sample of 50 μl of blood) (79).

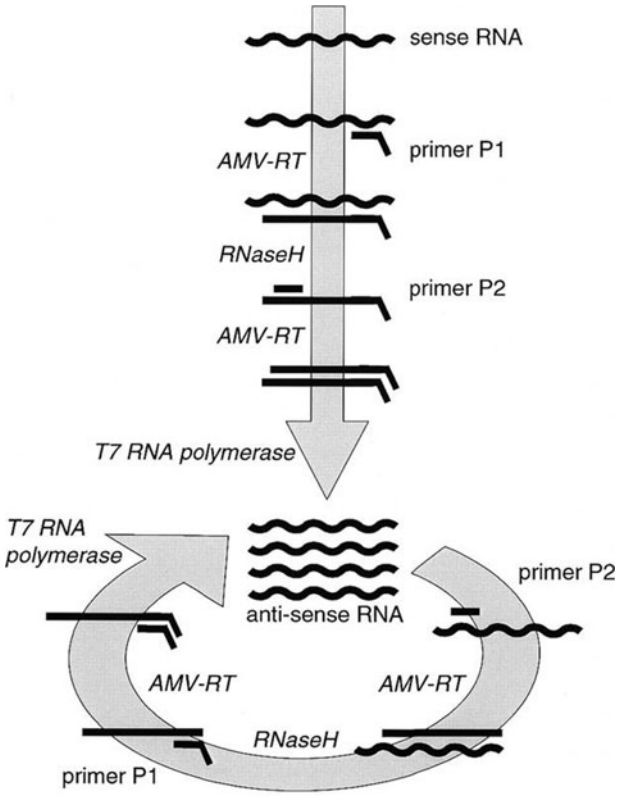

FIGURE 2 NASBA. Reprinted from *Nucleic Acids Research* (146) with permission of the publisher.

Loop-mediated isothermal amplification (LAMP)

LAMP is an isothermal amplification method in which DNA is amplified in isothermal conditions using a polymerase (*Bst* polymerase) with strand displacement properties (80). Amplification proceeds via a complex set of stem-loop structures; most LAMP assays use four to six different primers recognizing distinct regions on the target gene (Fig. 3), giving a high specificity (81). An added advantage is that *Bst* polymerase tolerates low concentrations of PCR inhibitors, including heme, so DNA extraction methods from blood can be rapid and simple (e.g., boil and spin [82]). Reaction times are very short (DNA is amplified 10^9 to 10^{10} times in 15 to 60 minutes). Detection of product can be achieved in two ways, both dependent on the production of magnesium pyrophosphate as a by-product of the amplification reaction, in proportion to amplified products. Magnesium pyrophosphate forms a white precipitate which can be observed by the eye or measured using a real-time turbidimeter (83). Second, calcein and manganese ions can be added to the reaction mix: the green fluorescence of calcein is blocked when bound by manganese ions, but the pyrophosphate ions generated in the amplification reaction displace the manganese ions, allowing calcein to fluoresce under UV excitation. A cheap UV-emitting LED, such as those found in counterfeit note detectors, can be used for detection (84). Quantitative LAMP can be achieved through the use of real-time turbidimeters or fluorimeters.

LAMP assays have been developed for four species of human-infecting *Plasmodium*, targeting either the SSU rRNA locus (85, 86) or a mitochondrial DNA target (64). One SSU rRNA–based LAMP assay was found to have a threshold of six parasites/μl of blood for *P. falciparum*, based on serial dilution of patient samples, with a high sensitivity (95%) and specificity (98%) when compared to nested PCR (85). An assay using primers able to distinguish four human malaria species was found to have a similar sensitivity and specificity as PCR (86); thresholds for detection were determined using a plasmid copy of the gene and were found to be 10 copies for *P. malariae* and *P. ovale*, and 100 for *P. falciparum* and *P. vivax* (86). Performance in the field with patient samples was not as good, with a reported sensitivity of 76% and specificity of 89% compared to a nested PCR (87).

An alternative LAMP assay with primers targeting the mitochondrial genome was developed to distinguish *P. falciparum* as a single-species infection, with non-*falciparum* species not resolved to the species level (64). The primers target the noncoding intergenic regions between the *cox1* and *cox3* genes for the *P. falciparum*–specific LAMP and between *cytb* and *cox1* for the pan-genus LAMP. This LAMP assay performed as well as nested PCR in a United Kingdom reference laboratory, with a high sensitivity compared to nested PCR of 98.4% (*P. falciparum* only) and 97.0% (*Plasmodium* genus). Specificity for the *P. falciparum* assay was 98.1% and 99.2% for all *Plasmodium* species (64). The LAMP tests were superior to expert microscopy for detection of *P. falciparum* and all other human *Plasmodium* species and gave positive results with sample parasite densities as low as five parasites/μl of blood (64). In field testing in a remote setting in Uganda, the same assay, with a fluorescence readout, was much more sensitive than expert microscopy and was similar to a three-well nested PCR (82). The assay had a very high sensitivity for infections of ≥2 parasites/μl of blood (~98% sensitivity to

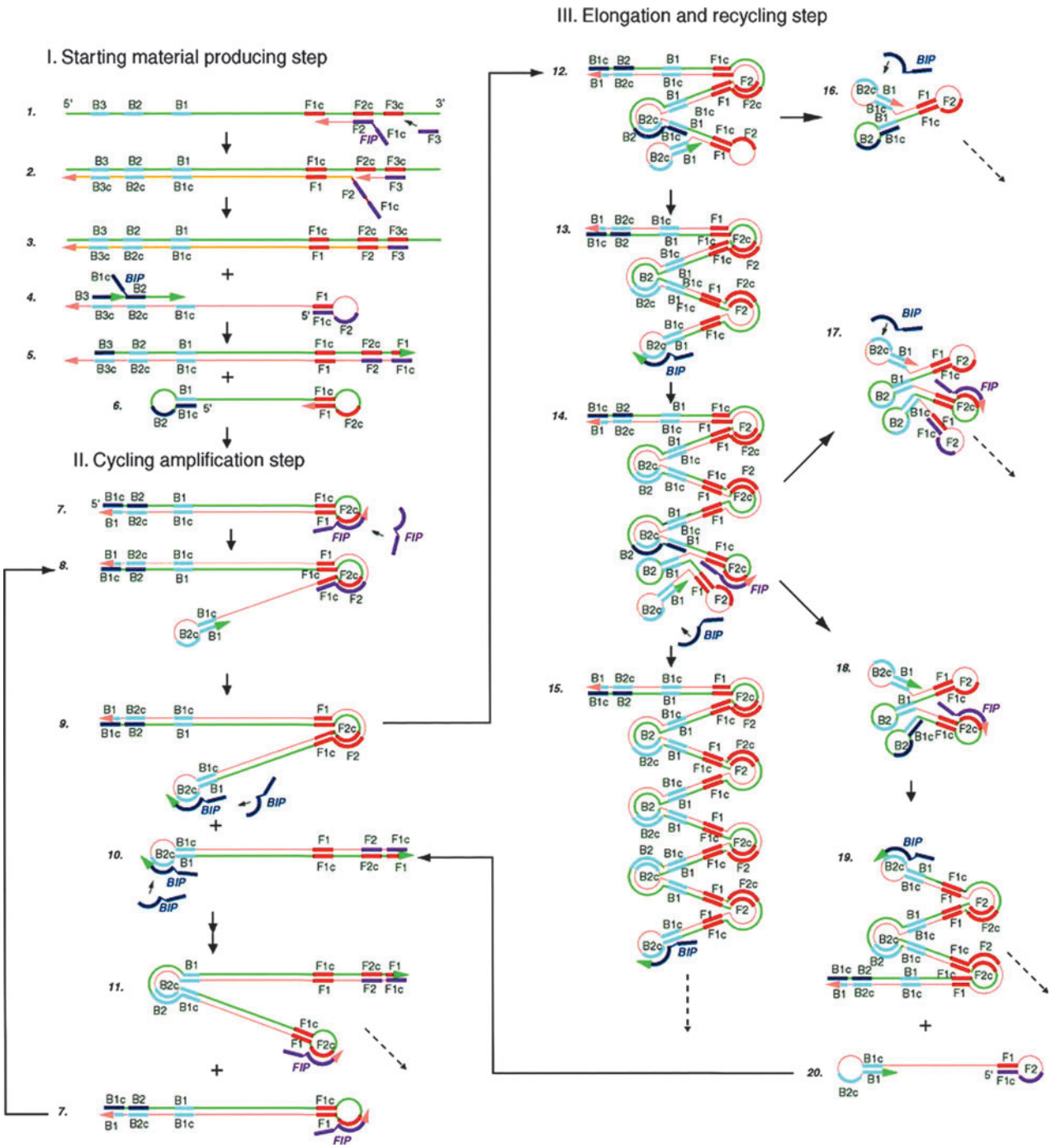

FIGURE 3 Schematic representation of the mechanism of LAMP. Reprinted from *Nucleic Acids Research* (80) with permission of the publisher.

detect parasite densities of 1 to 2 parasites/μl of blood in patient samples), but below that parasite level, both LAMP and nested PCR declined in sensitivity (82). LAMP occasionally failed to detect parasites in samples that had very low parasite densities which were still detected by qPCR. The mitochondrial-target-based assay forms the basis for a CE-marked product commercially released in July 2012 as the Loopamp Malaria Pan/Pf Detection Kit, list number LMC562 (Eiken Chemical).

LAMP assays have been reported recently for the detection of *P. knowlesi* infection through amplification of the species-specific beta-tubulin gene (88) and the apical membrane antigen-1 (AMA-1) gene (89). In addition, assays specific for gametocytes of *P. falciparum* have been developed using reverse-transcription LAMP, targeting two genes, *Pfs16* and *Pfs25*, that are known to be expressed only in these sexual transmission stages (90). *Pfs16* mRNA is expressed throughout gametocyte development, whereas

Pfs25 mRNA is limited to mature gametocytes (91, 92). A detection limit of only 1 gametocyte in 500 μl of blood was obtained for both *Pfs16* and *Pfs25* transcripts using reverse-transcription LAMP (90). Sensitivity and specificity compared to a nested RT-PCR assay were very high (100% and 98%, respectively, for the *Pfs16* assay and 100% for both for the *Pfs25* assay). The LAMP assays were much more sensitive than microscopy at detecting low-level gametocytemia; for mature gametocytes the *Pfs25* LAMP assay gave a positive result in 29% of microscopy-negative samples (90). Detection of gametocytes is necessary to identify infected individuals that are able to transmit the infection to mosquitoes, and the new reverse-transcription LAMP technique thus provides a simple and reliable tool for epidemiological studies of malaria transmission and in gametocyte-targeted control programs.

The major strengths of the LAMP assays are their high sensitivity and specificity, especially at parasite loads below five parasites/μl of blood, the very fast time from blood sampling to results (60 to 90 minutes) as a result of simplified DNA extraction methods, the minimum training required to perform the assay, and the simple equipment required. The disadvantages, as with any molecular system, are the relative costs of the assays and the risk of workspace amplicon contamination (and therefore false positives); this risk is reduced by closed amplification and reading systems. Developing new LAMP assays is also difficult because of the complexity of the primer design.

Both NASBA and LAMP are promising diagnostic methods for point-of-care settings compared to other molecular diagnostics because of lower equipment and set-up costs, speed of results, and capacity to detect infections at low parasite densities. NASBA has better performance than LAMP below one parasite/μl of blood but is slower and much more expensive, with more sophisticated equipment and reagents that require cold storage. LAMP has a higher risk of contamination but has the major advantages of easy and rapid sample preparation and monitoring of the outcomes of the test, limited and relatively cheap specialist equipment, and assay reagents that do not require cold storage.

Novel Detection Methods for PCR-Based Assays

PCR has been coupled with an ELISA-based readout, through the use of biotinylated primers for amplification followed by capture oligonucleotides instead of the antibodies usual in ELISA (93). The PCR-ELISA was found to have a detection limit of 30 parasites/μl of blood (the lowest parasitemia, identified by microscopy, detected in the patient samples), with a sensitivity of 91.4% and a specificity of 95.8% against microscopy as the gold standard (93).

PCR-ligase detection reaction coupled with a fluorescence microsphere assay has also been tested. In a multiplex PCR assay, DNA probes for each species are resolved by length difference by gel electrophoresis (94). The electrophoresis step in the PCR-ligase detection reaction is replaced by binding of fluorescent microspheres tagged with a specific sequence (Luminex Flexmap probes), which recognize and bind to species-specific sequences in the PCR product (94). Limited testing using cultured parasites revealed a detection threshold of 20 parasites/μl of blood.

A similar approach uses fluorescent resonance energy transfer–based DNA probes and a commercial quantitative PCR thermocycler that incorporates a fluorimeter (Lightcycler system). Probes recognizing regions of the SSU rRNA PCR-product specific for each parasite species were designed to have different melting temperatures, and the binding of the different probes could then be distinguished in melting curve analysis (95). Compared to a gold standard of microscopy, the technique had very high sensitivity (97%) and specificity (100%), with a limit of detection (as determined by spiking blood samples with a positive control plasmid) of one parasite/μl of blood.

All of these methods currently require sophisticated and expensive detection equipment and therefore are probably not suitable for widespread use in the field. A detection method with greater promise in this regard is nucleic acid lateral flow immunoassay (NALFIA); the principle of detection is similar to the antigen capture used in RDTs, but in this case, DNA is captured by sequence hybridization, using specific probes, antibodies, and reporter molecules bound to nitrocellulose. Instead of blood being applied to the nitrocellulose, the amplified product from LAMP or PCR is applied. Proof-of-principle field testing of a NALFIA system for diagnosis of malaria has been carried out in Kenya, using PCR products (96). The detection of amplified product using NALFIA strips was very similar to that obtained on standard gel electrophoresis, with a sensitivity of 98% and specificity of 99% compared to PCR. The threshold for detection, using diluted patient samples, was estimated to be 0.3 to 3 parasites/μl of blood. The major advantage of NALFIA is the simplicity of use and the speed with which the amplified product is detected (10 minutes); the disadvantage is reagent instability at high temperature and humidity (as for RDTs). NALFIA is most likely to be used as an alternative method to detect products from isothermal amplification, especially LAMP.

FUTURE PERSPECTIVES: MICROFLUIDIC APPROACHES

Microfluidic systems are emerging as a powerful method which could address the challenges of conventional diagnostic devices. Sample handling and detection are integrated within a single device, which can be mass-produced at low cost, and can be used by individuals with only basic training (reviewed in reference 97). There is strong potential to integrate molecular tests into a lab-on-chip format, where the processing of the sample to isolate parasite DNA is integrated onto a single device alongside a suitable molecular detection method.

One limitation of current diagnostic methods is the volume of blood that is analyzed (usually in the low-microliter range) in a diagnostic test. As parasite density decreases, the probability of having even one parasite within the sample decreases, so to detect very low parasite densities (<0.1 parasite/μl of blood), which are commonly found in asymptomatic infections of *P. falciparum*, a larger volume of blood needs to be analyzed. Microfluidic techniques could be used to enrich for parasite-infected erythrocytes in a larger blood sample (ml) that could then be analyzed using conventional diagnostics. The detection of asymptomatic malaria will be a priority as countries and regions move toward malaria elimination (6).

Microfluidic Parasite Enrichment Methods

Several physico-mechanical methods to isolate/purify, or to enrich, parasitized erythrocytes from the majority of uninfected red blood cells have been proposed. These rely on changes in the physical properties of the red blood cell as a result of infection with *Plasmodium*. As the parasite

matures, the erythrocyte becomes progressively less deformable (98–101). This is partly a result of the rigidity of the more mature parasite stages themselves (trophozoites and schizonts) (100) but is also due to the cross-linking of spectrin in the red cell membrane through interactions with certain *Plasmodium* proteins (102, 103). These changes in deformability of the infected erythrocyte can be exploited in various microfluidic systems to separate infected and uninfected red blood cells (104). For example, noninertial hydrodynamic lift has been used to enrich ring-stage parasites of *P. falciparum* by a factor of around 4 (105).

The electrical conductivity of erythrocytes has been shown to increase markedly when infected with *Plasmodium* (106, 107). This change in conductivity can be used to separate infected red blood cells using dielectrophoresis. Cells are polarized, according to their conductivity, within a nonuniform electric field and move differentially according to the degree of polarization (108). Proof-of-principle studies have demonstrated that *Plasmodium*-infected red blood cells can be separated from uninfected cells in a dielectrophoresis system using small electrode arrays (109), and this has then been coupled with field-flow fractionation to collect the infected cell fraction for further analysis (97, 110).

Ultrasonic Manipulations

Acoustic waves, generated by ultrasonic transducers on microfluidic chips, can also be used to manipulate cells (111). Surface acoustic waves can generate circular flow patterns, which coupled with a density gradient allow the separation of parasite-infected erythrocytes from uninfected cells (112) as a result of small differences in their density (100). The method allowed 100-fold enrichment of low-density (25 parasites/μl) ring-stage parasites, enabling subsequent microscopic detection at parasitemia levels as low as 0.0005%. Acoustic waves can also be used to perform PCR amplification in microfluidic devices. Surface acoustic waves were used to induce mechanical lysis of erythrocytes and malaria parasites to release parasite DNA. Further adjustment of surface acoustic wave frequency was used to generate heating within the sample, which coupled with passive cooling allowed PCR amplification and detection of low parasite densities (113).

Several proven technologies thus exist to allow the development of a low-cost microfluidic-based system, both to enrich parasites from low-parasite-density infections and to detect and differentiate the species of parasites, using existing technology such as LAMP or PCR, on the same microfluidic device.

DIAGNOSIS OF DRUG-RESISTANT MALARIA

Drug resistance is a particular problem with infections caused by *P. falciparum* but is becoming an increasing issue with infections of *P. vivax*. *P. falciparum* has developed clinically significant resistance to all classes of antimalarial drugs, with the latest being reduced sensitivity to artemisinin and its derivatives (75). For *P. vivax*, chloroquine resistance has been reported from most countries where it is endemic and is a particular problem in New Guinea and Indonesia (reviewed in reference 114). Resistance to primaquine, a drug used to treat the hypnozoite form of the parasite and thus to prevent relapse, has also been reported (reviewed in reference 115).

Currently, there is no diagnosis of drug susceptibility at the point of care. Once diagnosed with malaria, patients need to be treated immediately; there is no time to wait for an additional diagnostic test to inform the chemotherapeutic choice. For *P. falciparum*, most countries use an artemisinin combination as the first-line drug, in accordance with WHO recommendations. For *P. vivax*, chloroquine is the usual first-line drug, although in areas where chloroquine resistance is considered a problem, an artemisinin combination therapy can be used (3).

Surveillance of Antimalarial Drug Susceptibility

Since the emergence of *P. falciparum* resistant to chloroquine in the 1960s, the subsequent global spread of parasites resistant to commonly used antimalarials has been monitored nationally and globally (116), both by *in vitro* drug susceptibility testing (117) and by *in vivo* therapeutic efficacy trials (118).

The *in vitro* tests require the incubation of parasites (sampled from infected people) with varying concentrations of antimalarial drugs for defined periods of time (usually between 24 and 72 hours, depending on the drug and test). Various assays are available with different measurements of parasite growth including the use of microscopy, radioisotope incorporation, ELISA, and fluorescent DNA-binding dyes (reviewed in reference 119). The results are recorded as inhibitory concentration of drug that kills 50% (IC_{50}) or 90% (IC_{90}) of parasites, or the MIC. The tests allow relative monitoring of changes in drug susceptibility over time in a region but do not directly predict treatment failure rates, because the correlation of *in vitro* response and *in vivo* treatment outcome is not well defined. The *in vitro* sensitivity assays provide an accurate measurement of parasite sensitivity to drugs that is not confounded by host factors such as immunity. They are, however, slow, technically demanding, and require specialist equipment and training. They are not used to make clinical decisions on patient treatment at the point of care, but rather are used for surveillance by national malaria control programs. Because the tests require *in vitro* cultivation, they are restricted currently to *P. falciparum*, since none of the other human malaria species are yet amenable to routine *in vitro* culture.

The gold standard for measurement of drug resistance is the *in vivo* therapeutic efficacy test (118). This is a field-based trial consisting of the administration of antimalarials and follow-up of individuals using active and passive case detection for 14 to 42 days (depending on the drug and the level of endemicity/transmission). The outcome of treatment is measured both clinically (presence or absence of symptoms) and parasitologically (presence or absence of parasites). Later developments of the therapeutic efficacy test incorporate PCR analysis of any recrudescent parasites appearing in the follow-up period, to discriminate reinfections from genuinely resistant parasites.

Molecular Markers of Drug Resistance in *P. falciparum*

Resistance to most antimalarial drugs has arisen as a result of spontaneous mutations within the coding regions of the targets of the drugs in the parasite (reviewed in reference 120). Treatment failure *in vivo* is strongly associated with the presence of one or more of the key mutations (Table 1), although other factors such as immunity (121) and host genetics (122) can promote clearance of drug-resistant parasites. The mutations are necessary but not sufficient to cause treatment failure.

TABLE 1 Molecular markers of antimalarial drug resistance in *P. falciparum*[a]

Drug	Candidate gene	Plasmodb gene ID	Specific amino acid changes/regions associated with resistance	Reference(s)
Chloroquine	*crt*	*PF3D7_0709000*	K76T	147–149
	mdr1	*PF3D7_0523000*	Y184F/S1034C/N1042D/D1246Y	150
Proguanil	*dhfr*	*PF3D7_0417200*	A16V/S108T	151
Pyrimethamine	*dhfr*	*PF3D7_0417200*	S108N/C59R/N51I/I164L	152–154
Sulfadoxine	*dhps*	*PF3D7_0810800*	S436F/A437G/K540E	155, 156
Atovaquone	*cytb*	*mal_mito_3*	Y286S/C/N	157–160
Mefloquine	*mdr1*	*PF3D7_0523000*	S1034C/N1042D/D1246Y	161
			Gene copy number	162, 163
Artemisinin	Kelch propeller domain (*K13-propeller*)	*PF3D7_1343700*	C580Y/R539T/Y493H	164, 165

[a]Amino acid changes are shown as single-letter codes.

The molecular markers for chloroquine (*Pfcrt*) and for sulfadoxine/pyrimethamine (*dhfr* and *dhps*) (Table 1) have been used extensively in countries where malaria is endemic to monitor the spread of resistant alleles as a cheaper and simpler alternative to *in vitro* and *in vivo* drug efficacy screening. Their use has been instrumental in decisions to change the first-line antimalarial drug policy in some countries (123–125). Molecular tests permit the levels of resistance to be monitored when that drug has been withdrawn due to high resistance, when it would be unethical to conduct *in vivo* efficacy trials, so that the drug could be reintroduced if resistance levels have dropped sufficiently (126). The assays have also been used retrospectively in archived samples, which would not otherwise have been typed for drug resistance, to allow tracking of the initial emergence and spread of drug-resistant parasites (127–130).

Molecular Markers of Drug Resistance in *P. vivax*

P. vivax is not yet amenable to routine *in vitro* culture, so the evidence for association of genetic polymorphism and drug resistance is obtained from *in vivo* efficacy studies. The mechanism of resistance to chloroquine in *P. vivax* is not well understood but does not appear to involve mutations in the *P. vivax* homologues of the *Pfcrt* and *Pfmdr1* genes associated with chloroquine resistance in *P. falciparum* (131, 132). Although sulfadoxine-pyrimethamine is not usually used for treatment of *P. vivax* infections, mixed infections with *P. falciparum* are common in some geographical regions, and *P. vivax* is therefore exposed to sulfadoxine-pyrimethamine, and resistance can be selected. As for *P. falciparum*, single nucleotide polymorphisms in the genes encoding dihydrofolate reductase (*dhfr*) and dihydropteroate synthetase (*dhps*) are associated with sulfadoxine-pyrimethamine resistance (133–135).

Methods for Typing Drug Resistance Genes

Most of the mutations associated with antimalarial drug resistance are single nucleotide polymorphisms. Early typing methods were all based on DNA sequencing, which was too expensive for most large-scale epidemiological studies. Alternative assays more suited to larger-scale studies may involve standard nested PCR (124, 136) followed by restriction digestion of the PCR products with enzymes that differentially cleave resistant and sensitive alleles (PCR–restriction fragment length polymorphism) (137, 138), allele-specific PCR (mutation-specific PCR) (139, 140), and dot-blotting of PCR products and hybridization with labeled probes (141). These assays are sensitive (down to 10 parasites in each PCR). While the PCR–restriction fragment length polymorphism and dot blot techniques have good specificity, false positives can occur in mutation-specific PCR, especially when the infection has an excess of the "wrong" allele; mixed infections of sensitive and resistant parasites are common (142). The nested-PCR-based methods are labor-intensive and suffer from low throughput. The advent of real-time PCR allowed novel methods to be applied (reviewed in reference 143). Real-time PCR also allowed the development of assays to measure gene copy number, which is necessary for the analysis of mefloquine resistance associated with amplification of the *Pfmdr1* gene (Table 1) (144).

The new methodology being developed for the molecular diagnosis of parasite species is likely to be adapted for analysis of drug resistance, although discrimination of sequences differing by one single nucleotide polymorphism presents technical challenges.

SUMMARY

There has been remarkable progress and improvement in the diagnosis of malaria over the last 20 years, led by the advent of molecular techniques. The development of rapid diagnostic tests has been invaluable for the control of epidemics of malaria in resource-poor environments and in the absence of more advanced methods of diagnosis. The challenge for the future is to improve the sensitivity of diagnostics to begin to address the large number of asymptomatic infections that contribute to transmission of the disease but are difficult to identify and treat with the current methodology. Any new diagnostics for use in resource-limited settings should be affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and deliverable to end users (ASSURED) (145). Accurate and sensitive diagnostic tools to identify and treat all individuals carrying the parasite are required for areas where malaria transmission is in decline and there is the possibility of elimination (reduction of transmission to zero) (6).

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Molecular Detection of Gastrointestinal Parasites

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38

In parasitology, the importance of DNA-based testing methods became apparent in the 1990s following the redescription of the potentially invasive *Entamoeba histolytica* and the nonpathogenic *Entamoeba dispar* (1). Since these two species are morphologically indistinguishable, nucleic acid-based detection methods were introduced in the clinical laboratory for their differentiation in cases where microscopy lacked specificity. In a similar way, molecular applications gradually replaced tests in which microscopy was time-consuming or inconclusive, for example, in the detection of microsporidia (2). When real-time PCR was introduced, the idea of using multiplex PCR assays for the simultaneous detection of various pathogens as first-line diagnosis gradually developed (3).

The application of (multiplex) real-time PCR for the detection of intestinal parasites has been validated against microscopy in a variety of settings (Table 1) (4–14). These studies have clearly shown that molecular diagnostics outperforms microscopy in multiple areas, including, for instance, diagnostic sensitivity and standardizability. However, in many studies, microscopy of only one sample is compared to PCR performed on one sample. Since the "gold standard" for microscopy involves repeated examination of consecutive stool samples, only this may explain the better sensitivities of the molecular tests (6, 15). Interestingly, PCR performed on a single sample appears to be at least as sensitive as microscopy performed on three samples (16).

The lower DNA load found in microscopy-negative samples compared to the higher load found by microscopy in positive samples shows that the superior results obtained by PCR approaches are caused by higher sensitivity rather than lower specificity (4, 6, 14). Also, the same PCR used on different populations may give high positivity rates in one study group while being almost negative in another group. For example, high rates of *Cryptosporidium* spp. infections were found in a study performed in late summer, and low detection rates were correctly documented in winter (4, 6). Similar results can be found for different age groups, where a similar difference in the age distribution of *Giardia lamblia* infections was found by both PCR and microscopy (4).

One of the key questions in molecular parasitology is how long DNA can be detected in stool after successful treatment. *Enterocytozoon bieneusi* DNA became undetectable in stool in two patients at day 7 and day 4, respectively, after treatment (17). *Ascaris lumbricoides* DNA could not be detected 21 days after albendazole/ivermectin treatment in children who were previously PCR positive (11). In a study in the Netherlands, G. *lamblia* DNA became undetectable in all patients tested (n = 75) 1 week after antibiotic treatment (18). Rapid disappearance of DNA is expected in most other gut infections that are limited to the lumen. In contrast, it has been postulated that, for example, remaining *Schistosoma* eggs in the gut wall could cause "leakage" of parasitic DNA for extended periods.

In microscopy, a range of species-specific staining, culture, and concentration techniques is used to achieve higher sensitivity and specificity. Such techniques will not be applied to all samples that are submitted; rather, choices will be made based on travel history, eosinophilia, increased IgE level, urticaria, immune status, etc. In rare cases, microscopy can help discover an unexpected parasite, but this expertise in such a situation is waning. Therefore, the same algorithm used to decide between the conventional techniques including microscopy can be used for selecting the optimal PCR panel, aiming at the most common and relevant intestinal parasites.

DNA-based methods provide sensitive and specific detection of intestinal parasitic infections. Quality assessment schemes are essential to ensure such high diagnostic accuracy since there is a variety of protocols that may be used in different laboratories. Alternative platforms capable of multiplexing have been developed and are in the process of being commercialized. The application of broad-range PCRs in combination with classical sequencing or next-generation sequencing offers an interesting broad-target approach. The positioning of such tests in future molecular parasitology laboratories has not been defined at this stage.

This chapter will provide guidance on overcoming the hurdles that complicate the introduction and adoption of molecular diagnostic methods in the clinical parasitology laboratory. We will limit the discussion primarily to (multiplex) PCR testing.

DNA ISOLATION

Without an appropriate nucleic acid extraction method, DNA amplification techniques will not be reliable. The method must be able to release all nucleic acids from all parasitic stages present in the clinical sample. The nucleic acids need to be free of inhibitory substances that may interfere with DNA amplification. This is especially important

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TABLE 1 Examples of real-time PCRs for the detection of intestinal parasitic infections[a]

Protozoa	Targets[b]	Reference(s)
Blastocystis	SSU rDNA	90–92
Cryptosporidium	DNAJ-like protein, SSU rDNA, COWP	3, 4, 6, 11, 13, 23, 41, 64, 106, 139
Cyclospora	SSU rDNA, HSP70	74, 75,140–143
Dientamoeba fragilis	SSU rDNA, 5.8S rDNA	6, 50, 139,144–147
Entamoeba histolytica	SSU rDNA	3, 6, 28, 30, 41, 106, 148, 149
Entamoeba dispar		13, 28,150–159
Giardia lamblia	SSU rDNA	3, 15
Microsporidium	SSU rDNA, ITS	42, 147, 160–163
Nematodes		
Ascaris lumbricoides	ITS-1	11, 104, 105,164–167
Hookworm	ITS	11, 105,164–166168–171
Strongyloides stercoralis	SSU rDNA	11, 41, 105, 117, 169, 172
Trichuris trichiura	SSU rDNA, ITS-1	11, 106
Trematodes		
Clonorchis sinensis	ITS-1, ITS-2, NAD-2	122, 123, 173
Opisthorchis viverrini	*O. viverrini*-specific repetitive DNA fragment, NAD-2	173–175
Paragonimus	ITS-2	176
Schistosoma	SSU rDNA, ITS-2, NADH-1, Cox1, *S. mansoni* tandem repeat	125, 128, 130, 131, 166,177–182
Cestodes		
Taenia solium	Tsol9 repeat sequence, ITS-1	183, 184
Taenia saginata	ITS-1	184

[a]Reprinted with permission from reference 185.
[b]SSU, small subunit; COWP, *Cryptosporidium* oocyst wall protein; ITS, internal transcribed spacer.

with complex matrices such as feces (19–22). DNA isolation and PCR should be repeated using a fresh or diluted sample once inhibition is apparent from lack of amplification of an internal control molecule. It appears that efficient release of nucleic acids depends on a combination of features of the DNA isolation procedure. Preserving clinical specimens such as feces in formalin or other fixatives may also prevent successful amplification of parasite DNA; this effect increases with the duration of fixation (19–21). Species-specific DNA extraction using magnetic beads with capture oligonucleotides specific for *Cryptosporidium* and *Giardia* improved the PCR significantly (23).

ASSAY DEVELOPMENT AND VALIDATION

Detailed knowledge of genetic variation across isolates, species, and genera is paramount in efforts to develop and tailor DNA-based assays. It is important to know that assays used to define one species may also detect closely related species. This can be overcome by optimizing the assay and targeting other diagnostic sequences. In the design of genotype-specific detection assays, target sequences that show variation between different isolates belonging to the same species need to be used (24). While *in silico* evaluation of primer/probe specificity and sensitivity is a key component in the development, integration, and validation of diagnostic assays, extensive laboratory testing using panels of control DNA samples is mandatory. Of note, the small subunit (SSU) rRNA gene is highly conserved among some apicomplexan genera, so it has limited species-specific diagnostic value, which shows that diagnostic target sequences need to be selected with care (24).

An extensive body of literature exists on the optimization of nucleic acid extraction and assay development, and the reader is referred to the many excellent reviews on these topics. In addition, other chapters in this book provide information on the methodology of test development and validation from a practical but also from a regulatory perspective.

Below, we will focus on some practical examples of molecular diagnosis of gastrointestinal pathogens of the clinically most important and most prevalent species.

AMOEBOZOA

E. histolytica is the agent of amoebic dysentery and is able to penetrate through the gut wall and reach the liver, where it can cause severe damage (25). In 1997, the existence of *E. histolytica* and *E. dispar* as two distinct but morphologically identical species was officially acknowledged (25, 26). Clinical studies have shown a 1:10 *E. histolytica*: *E. dispar* prevalence ratio, which is currently assumed to be the worldwide ratio (26). Simple stool antigen assays appear to be specific and sensitive for the detection of *E. histolytica* infections in an endemic setting (27) but lack sensitivity in nonendemic settings (28). The genetic universe of *Entamoeba* is currently rapidly expanding, which is mainly due to the application of sequencing of PCR products obtained by amplification of *Entamoeba*-specific DNA directly from genomic, fecal DNA. This has resulted in the discovery of a large number of novel *Entamoeba* lineages

and additional subtypes from human and nonhuman hosts (29). Such new lineages warrant additional sampling to identify their clinical significance and to enable further intrageneric analysis of *Entamoeba*.

A range of single- and multicopy DNA targets, including the SSU rRNA gene, have been used in single, nested, and real-time PCRs (30, 31). PCR technology also facilitates detection of *E. histolytica* in samples other than stools such as abscess aspirates and tissue biopsies (32). A Luminex method for detection and differentiation of five species of *Entamoeba*, including *E. histolytica*, was recently developed (33).

Ali et al. (34) reviewed genetic variation within *E. histolytica* in protein coding genes, noncoding regions, and short tandem repeat loci facilitating epidemiological investigations and differences in clinical outcome. A multilocus sequence typing system was developed based on 16 polymorphic loci identified by genomic next-generation sequencing of *E. histolytica* (35).

METAMONADS

The genus *Giardia* comprises multiple species, with *G. lamblia* (syn. *Giardia duodenalis*, *Giardia intestinalis*) being one of the most common intestinal parasites of humans (36). Transmission is fecal-oral, and the parasite has a simple life cycle comprising excystation in the duodenum, colonization by rapidly multiplying, noninvasive trophozoites in the small intestine, and eventually the production of cysts that are shed with feces. In developing countries, this parasite adds yet another burden to already disadvantaged populations (37).

Conventional and real-time PCRs for the diagnosis of giardiasis have been published targeting the SSU rDNA, β-giardin, triosephosphate isomerase, and intergenic spacer regions (38). Other targets are being used for further genotyping. SSU rDNA, glutamate dehydrogenase, triosephosphate isomerase, β-giardin, intergenic spacer region, and elongation factor 1-alpha (ef1-alpha) gene amplification products are used for direct sequencing or analysis by restriction fragment length polymorphism (RFLP) (13, 39, 40).

As for other parasites, the introduction of (multiplex) PCR-based diagnostic assays for *Giardia* took place about a decade ago (3). State-of-the-art diagnosis included microscopy, staining of fixed fecal smears, and antigen detection using enzyme-linked immunosorbent assay or direct fluorescent antibody tests. The newer DNA amplification techniques have excellent sensitivity and specificity compared to microscopy and antigen detection (14, 41, 42).

Dientamoeba fragilis was first described in 1918 as an amoeba of the human intestinal tract (43). Later, by means of antigen and ultrastructural studies and analysis of the ribosomal RNA, the organism was reclassified as a trichomonad flagellate (44, 45). *D. fragilis* appears to be common and may have a cosmopolitan distribution with variation in prevalence. Notably, *D. fragilis* has been linked to intestinal symptoms, especially in children (46); however, recent comprehensive studies have not been able to support a general clinical role for *Dientamoeba* (47, 48). The sensitivity of a single microscopic examination is low because the variation of *D. fragilis* trophozoites in feces seems to be more irregular than that observed for other intestinal protozoa (49). Conventional PCRs have been used mainly for confirmation of microscopy and subsequent characterization of the *Dientamoeba* ribosomal genes. A real-time PCR targeting 77 bp of the SSU rRNA gene was the first molecular assay developed as a screening tool (50). In a study comparing microscopy and real-time PCR Bruijnesteijn et al. showed a *D. fragilis* prevalence of 17% and 31%, respectively (6). RFLP analysis has been used to distinguish between the two currently known genotypes (51). Genotyping has also been performed by single nucleotide polymorphism (SNP) analysis via PCR and pyrosequencing (52). A profiling method using the variability within the internal transcribed spacer (ITS)-1 of *D. fragilis* was developed (53), but so far little is known of its epidemiological relevance. The fact that *D. fragilis* detection can now be easily integrated in multiplex nucleic acid-based detection techniques means that it is relatively inexpensive and straightforward. This can provide accurate data on possible differences in prevalence and infection intensity between symptomatic and asymptomatic carriers, which can be exploited in randomized controlled treatment studies.

APICOMPLEXA

Cryptosporidium has emerged as a cause of diarrheal illness worldwide (54). Infections in immunocompetent individuals are self-limiting and last for 1 to 2 weeks. Asymptomatic shedding of oocysts may be common (55), although cryptosporidiosis may be chronic in certain patient groups (56). Transmission is fecal-oral by ingestion of oocysts containing infectious sporozoites. Infection can result from contact with mammalian feces, food, or water contaminated by oocysts. In several countries, surveillance systems, including genotyping, have been implemented (57). The genus comprises over 20 species, of which the morphologically indistinguishable *Cryptosporidium parvum* and *Cryptosporidium hominis* account for most human cases. Around 10% of all human cryptosporidiosis may be due to species other than *C. parvum*, *C. hominis*, and *Cryptosporidium meleagridis* (58). *C. parvum* may be seen more commonly in mixed infections than *C. hominis* (59). Clinical samples include biopsies, bile, sputum, and antral washouts, in addition to stool (56). In many laboratories, *Cryptosporidium* is not routinely included in test panels for gastroenteritis. Conventional diagnosis relies on microscopy of modified acid-fast-stained fecal concentrates or auramine-phenol staining, and/or antigen detection by direct immunofluorescence or immunochromatographic assays. The main targets for diagnostic PCRs typically include the SSU rRNA gene, *Cryptosporidium* oocyst wall protein, or the DnaJ-like protein gene (60). SSU rDNA sequences are available for all species of *Cryptosporidium* known to infect humans. Such data are not available for most other species, which may go undetected using this assay (12, 61). Because there is little genetic variation across the *Cryptosporidium* SSU rRNA gene, the design of primers/probes targeting the entire genus is relatively straightforward, but there are limitations for designing species-specific primers/probes. Therefore, other loci have been targeted (59, 62).

It has been argued that SSU rDNA PCR is compromised in its ability to detect mixed species due to preferential amplification of the predominant species in a sample (63–65). Detection and differentiation of *C. hominis*, *C. parvum*, and *C. meleagridis* can be performed using high-resolution melting curve analysis of ITS2 amplicons (66, 67). Fluorescence resonance energy transfer (FRET) probes have also been used to distinguish between *C. parvum* and *C. hominis* (68). For phylogenetic analysis, molecular epidemiological, and outbreak investigations, PCR-RFLP or

PCR-sequencing analysis of various loci have been useful (69, 70). PCR resulted in a 22-fold increase in the detection of *Cryptosporidium* and *Giardia* compared to conventional microscopy (71). An increased detection rate was found using a DnaJ-like gene-based TaqMan assay (60) compared to commonly used commercial kits such as Merifluor (Meridian Bioscience, Inc.). High sensitivities were reported for auramine-phenol microscopy and commercial kits based on immunofluorescence microscopy and enzyme immunoassays (72). The validation study was carried out using *Cryptosporidium* samples, of which 97% were either *C. parvum* or *C. hominis*, and the sensitivity reported must be interpreted carefully.

Species of *Cyclospora* are obligate intracellular apicomplexan parasites infecting primates and other mammals, reptiles, and arthropods. Sporozoites released from ingested oocysts infect the duodenum and jejunum. Asymptomatic presentation is not uncommon in endemic areas, but symptoms related to *Cyclospora* infection, seen primarily in children and HIV/AIDS patients, may include low-grade fever, anorexia, nausea, diarrhea, and weight loss (73). Traditional diagnostic methods include autofluorescence and modified Ziehl-Neelsen staining of fecal concentrates in which oocysts can be detected based on morphological characteristics. In 2003, the first TaqMan assays were published (74), enabling incorporation of *Cyclospora* into multiplex screening assays (75). A multiplex PCR method to detect *Cyclospora*, *Cystoisospora*, and microsporidia in stool samples based on Luminex technology was recently published (76). Unfortunately, little has been published on nucleic acid-based testing for *Cyclospora* infections in a clinical setting (77). There is currently still no method available for genotyping.

MICROSPORIDIA

Microsporidia are obligate, intracellular, eukaryotic, single-celled fungi that infect a wide variety of vertebrate and invertebrate hosts, comprising more than 160 genera and representing over 1,500 species, of which at least 14 are known to infect humans. Intestinal microsporidiosis in humans is due mainly to *E. bieneusi* and species of *Encephalitozoon*, mainly *E. intestinalis* and, probably to a lesser extent, *E. cuniculi* and *E. hellem* (78). In humans, opportunistic microsporidial infections associated with self-limiting but sometimes persistent diarrhea and weight loss may be seen (79). Both food- and waterborne outbreaks have been reported (80). The traditional gold standard for detection of microsporidia relies on demonstration of 1- to 2-μm spores by electron microscopy. Detection of microsporidia in stool often includes identification of spores in fecal smears by nonspecific histochemical chemofluorescent or trichrome stain as well as monoclonal antibody immunofluorescence assays (80). PCR has been shown to be a very sensitive and specific diagnostic alternative for the labor-intensive and expertise-dependent microscopy (2, 81, 82).

While microsporidia may not currently qualify as part of a routine test panel for intestinal pathogens, PCR-based detection appears relevant in cases of HIV-related diarrhea, diarrhea in patients undergoing organ transplantation, and in cases of unexplained diarrhea in otherwise immunocompromised patients, for instance in outbreaks of diarrhea (83). Few PCRs have been developed for species causing systemic infections, and the lack of validation studies on DNA extracted from clinical samples other than stool remains a limiting factor.

STRAMENOPHILES

Blastocystis is a strictly anaerobic, unicellular intestinal parasitic protist of human and nonhuman hosts (84). It is one of the most common microbial eukaryotes to colonize the human intestine and has a largely unknown clinical significance, but still it remains a poorly studied parasite. It is not known how to eradicate the parasite, and *Blastocystis* is not even remotely related to the other eukaryotes that colonize or infect the human intestine. Not all stages of the life cycle of *Blastocystis* have been clarified, and transmission is fecal-oral (85). While asymptomatic carriage is common (86), *Blastocystis* has often been linked to disease (87). The genus comprises numerous ribosomal lineages, at least nine of which have been found in humans. It is possible that differences in clinical outcome reflect differences in the subtypes' relative ability to cause disease (88). Microscopy of fecal concentrates has low diagnostic sensitivity, which has most likely led to substantial underreporting (89), whereas use of stained smears of stool specimens or culture has the potential to increase the detection rate of *Blastocystis*.

The first publication of a diagnostic PCR for *Blastocystis* was partly inspired by the tendency toward screening fecal DNAs for other intestinal protozoa as a supplement to microscopy (89). A real-time FRET-based PCR based on an uncharacterized gene was validated against subtypes (ST) ST1, ST3, and ST4 (90). A SYBR Green real-time SSU rRNA PCR for the detection of *Blastocystis*-specific DNA and subtyping by melting curve analysis was designed (91). Low sensitivity can be expected due to the large PCR product (320 to 342 bp), and the specificity of the assay was only 95%. Another real-time assay, using a hydrolysis probe based on the SSU rRNA gene, displayed 100% specificity (92). Two methods have been well employed for the genetic subtyping of *Blastocystis*: barcoding and sequence-tagged-site PCR (93). Subtyping has revealed significant differences in *Blastocystis* epidemiology: ST4 appears common in Europe. ST6 and ST7, usually occurring in birds, account for about 20% of the cases in Africa, while only sporadic cases are seen elsewhere. Data from Denmark and Spain show associations between ST4 and diarrhea, and ST4 is common in United Kingdom patients suffering from irritable bowel syndrome (94–96). Geographical differences in the distribution of subtypes may hamper attempts to identify subtypes linked to disease. Multilocus sequence typing of *Blastocystis* is currently based on loci in the mitochondrion-like genome (97); however, information obtained by the SSU rDNA analysis is a cost-effective alternative (98). Due to the limited knowledge of the clinical significance of *Blastocystis*, implementation of molecular diagnostics in the routine clinical setting may be premature (95). If differences in clinical outcome of *Blastocystis* infections reflect differences in subtypes or strains, nucleic acid-based methods will most likely be implemented as first-line diagnostics.

SOIL-TRANSMITTED HELMINTHS

Soil-transmitted helminths are a group of intestinal worm infections—*Trichuris trichiura*, *A. lumbricoides*, *Necator americanus*, and *Ancylostoma duodenale*—of which the eggs develop into infective eggs or larvae when excreted onto soil. Ingestion of infective eggs or penetration of larvae through the skin leads to new infections. Infections with soil-transmitted helminths are the most common infections in the world, with more than a billion people infected (99). To date, few papers have been published on

PCR for detection of *T. trichiura*-specific DNA. This might be due to difficulties in the isolation of parasite DNA from its robust eggs (7, 100). While testing analytical performance using spiked stool samples, the assay showed 100% sensitivity and specificity. A *T. trichiura*-specific singleplex real-time PCR designed from the ITS1 sequence was performed as part of a screen of stool samples from school children (11). Phylogenetic analysis of *Trichuris* SSU, ITS1, 5.8S, ITS2, and mitochondrial genes derived from worms and eggs has been performed. Although the closely related *T. trichiura* and *Trichuris suis* cannot be differentiated using morphological and biometrical measurements, their ITS1 and ITS2 sequences revealed clear differences (101). Pyrosequencing assays have been developed for the detection of SNPs in the β-tubulin gene that are associated with benzimidazole resistance. The finding of a benzimidazole resistance-associated SNP in *T. trichiura* might explain the reported ineffectiveness of this drug (102).

Conventional PCR assays targeting the *A. lumbricoides* ITS1 region and cytochrome *b* gene were used to detect and genotype *Ascaris* DNA from coprolites found in pre-Columbian South American archaeological sites and in fecal samples from Brazilian patients (103). Another PCR for the detection of *A. lumbricoides* DNA was designed from the ITS1 sequence (104). A multiplex real-time PCR using hydrolysis (TaqMan) probes for the detection and quantification of *A. lumbricoides*, *N. americanus*, *A. duodenale*, and *Strongyloides stercoralis* was validated against a large panel of Malaysian and Indonesian samples (104, 105). Microscopy-negative samples showed higher median cycle-threshold values compared to microscopy-positive samples. This ITS1-based *Ascaris* PCR was also validated in a TaqMan Array Card for the detection of 19 enteropathogens (106). The same primer and probe design for the detection of *A. lumbricoides* DNA was incorporated into a multiplex PCR for the detection of three intestinal protozoa and four helminth infections using Luminex beads (107, 106). TaqMan probe chemistry with another ITS1 primer and probe was used when an *Ascaris* PCR was performed in parallel with singleplex PCRs for seven additional parasites (11). DNA loads showed an excellent correlation with the egg counts. Whole-genome fingerprinting by amplified fragment length polymorphism, RFLP, and sequencing of the ITS1 and mitochondrial genes and microsatellites have been used to study the epidemiology of *Ascaris* (108–110).

Species-specific markers defined in the ribosomal and mitochondrial genes were validated for species identification of adult hookworms and later employed in epidemiological studies as well (111). There was a good correlation between egg counts and DNA load. The same ITS2 primers and probe were used in multiplex TaqMan assays for different helminths. Singleplex assays were tested in parallel and in a multiplex PCR combined with probe-based detection with Luminex beads. Real-time PCR targeting the ITS2 sequence, followed by high-resolution melting (HRM), differentiated *N. americanus*, *A. duodenale*, *Ancylostoma ceylanicum*, and *Ancylostoma canium*. Although hookworm-specific amplification was detected in an additional 35 of 166 microscopy-negative fecal samples, both PCRs remained negative in 11 of 52 microscopy-positive samples. The possibility of a less optimal DNA isolation procedure or the misidentification of hookworm-like eggs (e.g., *Trichostrongylus*) cannot be excluded. Infections with a moderate and high *A. duodenale* DNA load were associated with severe anemia, whereas iron deficiency measured in bone marrow was positively associated with an increasing load of DNA. Using adult worms from Africa, Asia, and South America, multiple genetically distinct groups of *N. americanus* were found by amplified fragment length polymorphism (112), confirming previous findings from ribosomal and mitochondrial genetics (111). Pyrosequencing tests were designed to identify benzimidazole resistance SNPs in the β-tubulin gene of *N. americanus*, *A. lumbricoides*, and *T. trichiura* (113). Recently, next-generation sequencing and analysis of the *N. americanus* transcriptome revealed 18 drug targets without human homologues (114).

S. stercoralis is endemic in many tropical and subtropical regions; it is estimated that 30 to 100 million people worldwide are infected, with a prevalence in some cohorts as high as 50% (115). Chronic strongyloidiasis may result in hyperinfection or systemic strongyloidiasis, often seen in immunocompromised hosts (116). The laboratory diagnosis is based on serology and the detection of larvae by microscopy of fecal samples. Recently, an *S. stercoralis* real-time PCR with primers and FRET probes designed from the 28S rDNA was described showing high specificity and sensitivity (117). Another real-time PCR with primers and an SSU rRNA hydrolysis probe showed a 10 to 100 times higher sensitivity compared to assays designed from the cytochrome *c* oxidase subunit I gene and an *S. stercoralis*-specific sequence (118). The SSU rDNA primers and probe were multiplexed as well (107). A conventional 5.8S rRNA PCR amplified *S. stercoralis*-specific DNA in all 16 positive samples and 5 of 30 microscopy-negative samples from 782 Iranians (119). Considering the time-consuming concentration and stool culture methods that have to be applied on multiple fresh stool samples to achieve good sensitivity in the diagnosis of *S. stercoralis* infections, molecular diagnostics appears to be a worthwhile alternative.

FOODBORNE TREMATODES

Foodborne trematode infections are zoonotic and can be caused by more than 80 species (120). The species considered to be of public health importance are the liver flukes *Clonorchis sinensis*, *Opisthorchis* spp., and *Fasciola* spp.; the lung flukes *Paragonimus* spp.; and a number of intestinal flukes. Although *Fasciola* spp., *Paragonimus* spp., and intestinal flukes are found worldwide, higher transmission is observed in certain areas (121). DNA-based methods for the detection and differentiation of these trematodes include PCR, nested PCR, real-time PCR, and loop-mediated isothermal amplification (LAMP), all designed from the *Opisthorchis viverrini*-specific repetitive DNA fragment, mitochondrial sequences, ITS1-2 sequences, and the SSU rRNA gene. The ITS2 sequence of *C. sinensis* was used to develop a TaqMan real-time PCR for *C. sinensis*-specific DNA in feces. The cycle-threshold values were strongly correlated with the intensity of infections as determined by egg counts (122). A similar approach involving the *C. sinensis* ITS1 provided specific and sensitive detection of *C. sinensis* in fecal and fish tissue samples (123). A multiplex real-time PCR based on DNA sequences of *C. sinensis* and *O. viverrini* was developed using FRET probes and HRM. Molecular tools for the detection, differentiation, and genetic characterization of *Fasciola* spp. have been reviewed recently (124). Molecular approaches based on ITS1, ITS2, *nad1*, and *cox1* have been used for the differentiation of adult flukes of *Fasciola hepatica*, *Fasciola gigantica*, and the novel "intermediate *Fasciola*" and to detect genetic variation among *Fasciola* spp.

SCHISTOSOMA

Although adult schistosome worms mostly reside in the blood vessels around the intestines or urinary bladder and are therefore blood-borne parasites, the eggs of *Schistosoma mansoni* are excreted in the feces, and the eggs of *Schistosoma haematobium* pass through the bladder wall into the urine. The detection of *Schistosoma*-specific DNA can achieve a higher sensitivity in the diagnosis of acute schistosomiasis compared to serology (125). The *S. mansoni* and *S. haematobium* tandem repeat sequences (126) have been used in a number of studies using conventional and real-time PCR in stool, urine, and serum samples. The ribosomal SSU rDNA, 28S, and ITS sequences and mitochondrial genes were used for the same purpose. In areas of endemicity, *Schistosoma*-specific PCR performed on DNA isolated from urine or feces was more sensitive than microscopy (127). The higher sensitivity of the real-time PCR resulted in much higher negative predictive values as well (128). Latent class analysis of hematuria, microscopy, and PCR targeting the *S. haematobium*-specific *DraI* repeat sequence using DNA isolated from a quarter of a filter paper after filtration of 50 ml of urine showed sensitivities of 87%, 70%, and 100%, respectively (129). *S. haematobium* DNA in vaginal lavage samples was highly associated with mucosal manifestations typical for female genital schistosomiasis (130). A high sensitivity of the detection of *Schistosoma* DNA using a real-time PCR targeting the *S. mansoni* tandem repeat sequence has been reported using DNA isolated from 10 ml of plasma of patients with chronic disease and in patients with Katayama syndrome (131).

CESTODES

The impressive length of tapeworms has placed them among the best-known parasites. Although the pathology of intestinal cestodes is minor, if metacestode stages occur in human tissues, morbidity can be severe. Several species are known to infect humans including *Taenia solium*, the pork tapeworm. Frequently, the diagnosis is made when patients notice the presence of proglottids in their feces. Microscopic detection of eggs in feces is known to be insensitive because eggs are not homogeneously distributed. Immunodiagnostic tests for the detection of *Taenia* antigens in stool have been used in epidemiological studies, and DNA-based methods are used mainly for species discrimination (132). Primarily, conventional (multiplex) PCR and PCR-RFLP designed from the HDP1 repeat, HDP2, mitochondrial 12S rDNA, *cox1*, pTsol9 repeat, and Tso31 sequence have been used for species identification. In a study comparing LAMP and PCR using 43 known positive fecal samples, LAMP was more sensitive than a conventional multiplex PCR (133). The lower sensitivity of the PCR might be explained by the relatively large amplicon sizes that have to be generated for the different *Taenia* species. Molecular diagnostics have proven to be extremely valuable in the diagnosis of neurocysticercosis (134). PCR and sequencing are used to identify and confirm the species identity of tapeworms as well as interspecies variation needed to construct phylogenies (135).

CONCLUDING REMARKS

A large number of studies using a variety of nucleic acid-based techniques have contributed to our understanding of the genetic diversity, epidemiology, and clinical relevance of intestinal parasites. In a routine setting, standardization and harmonization of protocols are essential for cost-effective implementation of these techniques. Multiplex testing offers a highly sensitive and specific alternative to labor-intensive microscopy. Because molecular diagnostic facilities in microbiology laboratories are already available, an increasing number of laboratories have implemented real-time PCR for the detection of diarrhea-causing pathogens including parasitic targets. In comparison with microscopic examination, the detection rate of parasitic infections when the targets are included in a multiplex PCR is higher (4, 6, 12, 13). Use of additional diagnostic methods for the detection of those parasitic infections that are not included in a standard feces PCR panel can be limited to a selected group of patients. The same algorithms that are used to decide on additional methods when PCR is not a method of choice for the first-line diagnosis of parasitic infections can be used. For example, in immunocompromised patients with diarrhea an additional method for the detection of opportunistic parasitoses may be appropriate. Molecular techniques are ideally suited for automation of the nucleic acid isolation, PCR set-up, and PCR itself when integrated into the laboratory information management system. In this process, primary screening through a general algorithm can be followed by additional techniques decided upon during the medical authorization based on the individual clinical records and patient history.

Obviously, just as quality assessment schemes are common practice for microscopy-based diagnosis, the application of molecular diagnostics requires quality assurance as well. Initiatives to this end have been taken (136). Since 2012, Quality Control for Molecular Diagnostics (http://www.qcmd.org) has provided a yearly quality assessment scheme for gastrointestinal diseases including a panel for protozoa.

FUTURE DIRECTIONS

Real-time PCR is available in an increasing number of research centers, and molecular diagnosis of parasitic infections can be applied to large-scale epidemiological and fundamental research. Samples can be stored and used later for testing additional targets when new research questions arise. Although PCR seems more expensive than conventional microscopy, this must be weighed against, for instance, the complexities of organizing the prolonged stay of a large team of technicians and support staff in field laboratories. Commercial multiplex PCRs for the detection of a range of enteropathogens that are already optimized and validated for a variety of instruments, as well as kits to be used on more extended multiplex platforms and variations on micro-arrays, are all available (137). This trend will make it easier for laboratories that are just starting to use molecular diagnostics to catch up. The first FDA approval of a multiplex gastroenteric panel and others that will undoubtedly follow will give rise to more common use of nucleic acid-based tests in the United States as well. While there is no doubt that amplification-based molecular diagnostic methods will continue to be used, new-generation sequencing platforms will become more user friendly and integrated into diagnostic settings. This will enable screening of fecal DNAs using broad-specificity primers for in-depth analysis of the microbial diversity present in such samples (138). In addition to targeting microorganisms, the host factors potentially involved in the pathogenesis can also be explored.

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section VIII

Point-of-Care/Near-Care Diagnostics

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Molecular Diagnostics and the Changing Face of Point-of-Care

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39

In vitro diagnostics (IVDs) are undergoing an evolution that is being driven by nucleic acid–based molecular detection technologies, from fully automated, multistep miniaturized platforms that integrate sample prep, detection, and signal amplification into single-step cassettes processed on handheld or portable instrumentation (1, 2), to novel real-time detection chemistries that are platform-agnostic and allow high target multiplexing (3). With simplified laboratory requirements, portable equipment and wireless links to remote health care services, these sophisticated technology platforms have the potential to revolutionize patient testing, putting actionable information rapidly into the hands of the treating clinician during the patient encounter, regardless of where the interaction occurs.

The implications of these technologies are disruptive and paradigm-shifting for point-of-care (POC) capabilities: effective, timely treatment for symptomatic patients, disease control through low-cost screening with immediate referral or treatment of asymptomatic individuals, ease of monitoring during therapeutic treatment to improve compliance and patient outcome, and cost-effective clinical trial tools to substantially decrease the time and cost of patient selection and monitoring during experimental drug assessment (4). Less than 10 years after the publication of the multinational research endeavor, the Human Genome Project (5), next-generation sequencing companies are envisioning implementing POC nucleic acid sequencing technology near or at the patient bedside, particularly in the therapeutic area of cancer diagnosis and treatment monitoring.

Multiple reviews on new diagnostic platforms and technologies have catalogued recent technological and commercial advances that have the potential to enable molecular diagnostics at the POC, with example breakthrough advances reported for nanotechnology (2, 6–8), biomaterial engineering (6, 8–11), microfluidics (11–14), and novel enzymology (15, 16) advancing toward product development and commercialization. In many respects, the rapid generation of new solutions to diagnostic needs through molecular technologies makes review of this changing technology landscape obsolete almost as soon as it is published. Therefore, this review will focus more on the requirements for POC testing, particularly around infectious diseases, and how these are being addressed through new innovations. The gaps and shortcomings that still need critical consideration and innovation to deliver highly functional, quality-assured, and practically affordable diagnostic products will also be examined as essential ingredients for effective adoption in the developed world as well as low-resource areas. With regard to any particular products or technologies highlighted in this review, these examples are provided and discussed solely for illustrative and informational purposes and are not meant as endorsements of any particular product or company. Similarly, this review is not meant to be a comprehensive survey of the burgeoning literature and information available on technologies with the potential to assume roles as POC diagnostic platforms, but hopefully will stimulate discussion of the requirements that need to be addressed for the successful deployment and adoption of these platforms to enhance health care services.

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RECONSIDERING THE CURRENT POC PARADIGM

Defining POC

When reviewing the literature, one finds varied definitions of what constitutes a POC diagnostic device, regardless of the platform. The U.S. National Library of Medicine defines a "point-of-care system" as "laboratory and other services provided to a patient at the bedside. These include diagnostic and laboratory testing using automated information entry" (17). A broader definition, found in *Stedman's Medical Dictionary for the Health Professions and Nursing* (18), indicates that a POC device should allow for "performance of clinical laboratory testing at the site of patient care rather than in a laboratory, and often by nonlaboratorians." Unfortunately, neither of these definitions, nor most of the descriptions currently circulated in the literature, is universally accepted (19). Therefore, it may be more instructive to understand the principal requirements for POC diagnostics and its relationship to "near-patient" testing.

The essential performance requirements for a POC diagnostic system have been defined as a "rapid turn-around and communication of results to guide clinical decisions with completion of testing and follow-up action in the *same clinical encounter*" (20; italics added for emphasis), delivering actionable results that can then be linked to impactful treatment decisions and treatment outcome. The key to understanding the challenges of a POC device is

the phrase "same clinical encounter." A standard doctor office visit for disease diagnosis presents many difficulties and challenges for the POC product requirements or specifications, including specimen acquisition and, in the case of a molecular device, nucleic acid extraction integrated into a simplified diagnostic platform that requires minimum manipulation to achieve the optimal shortened time-to-results constraints. These constraints, though they vary in definition, can be viewed as no more than a 2-h office visit, with less than 20 min being optimal. The more practical approach is envisioned in "near-patient testing," where the specimen is sent to a local clinical laboratory for frequently ordered testing, with results available between 12 and 48 h. Near-patient testing can be viewed as an intermediate step in the testing schema. But the concepts can blur at times: near-patient testing becomes "POC" in an emergency room, hospital, or clinical setting if single-use diagnostics technologies with a rapid turnaround time from patient sample collection are available to the clinician and admitted or emergency room patient, allowing for a complete diagnostic work-up during the clinical encounter. In any case, the key to the success of a POC or near-patient test is a rapid turnaround time to interpretable and actionable results, and ready access to the patient, for best clinical practices to be fully realized from the results.

POC rapid diagnosis of infectious disease can be most readily visualized in clinical settings such as a teaching hospital with an appropriately staffed and outfitted laboratory that can process specimens for routine testing in-house, interpret results, and expedite decision-making for effective patient care. Unfortunately, most patients are not tested in hospitals, so additional requirements of sample transport, infrastructure, highly trained staff, facilitated communication, and time available for follow-up between laboratory, clinician, and patient are essential to effectively link diagnosis to treatment. This requires centralized laboratory facilities and highly trained personnel, creating a significant and costly barrier to implementing molecular diagnostics as effective POC tools (21). While the developed world takes these conditions for granted, these additional factors are often cited as the challenge of bringing near-patient diagnostics to the developing world; the basic need for a continuous and reliable electrical power supply to run the instrumentation alone poses a major barrier to molecular diagnostics at local clinics, resulting in critical delays in treatment and loss to follow-up (22).

However, overhead costs and infrastructure concerns are no longer confined to health care delivery in the developing world: recent economic drivers of the health care systems of the developed world (i.e., decentralized health care services, more home-based patient-controlled testing and monitoring, trained technical staff, universally available personalized medicine, cost containment and antibiotic stewardship) are converging with the absolute necessity in low-resource emerging markets for accessible, low-cost diagnostics tools that can be adapted to the local health care environment. These constraints are beginning to place significant demands on product developers to offer sophisticated and high-quality diagnostic tools that are easy to use, require minimal training, and provide rapid and affordable diagnostic solutions, delivering definitive answers to expedite therapeutic decision-making, whether at the field hospital or at the local pharmacy (21, 23).

Cancer clinical trials offer another example of the shift toward rapid and sophisticated molecular POC diagnostics tools that can aid in appropriate patient selection, stratification, and monitoring, as well as contribute to patient compliance during the trial. These tests are becoming standard operating practice in the enrollment and monitoring of patients. For example, investigators conducting clinical trials with novel cancer therapies are looking for sensitive diagnostics that can rapidly assess the complex genetic profiles of these diseases, to monitor a patient in remission who may be reverting to a cancerous genotype and re-initiate treatment long before any signs or symptoms are displayed (24–26). Across diverse clinical sites, decentralized testing with rapid turnaround is now viewed as the best means to decrease the cost and shorten the time of these trials, as well as ensuring improved patient compliance and interpretable outcomes, accelerating clinical decision-making, regulatory submission processes, and ultimately, market access to new therapeutics (24).

Current POC Testing

Unfortunately, the current repertoire of POC diagnostics generally falls short of addressing the expanding need for rapid and reliable diagnosis. Rapid diagnostic tests (RDTs), which use a single drop of blood, dip into urine, or cheek swab applied to a single-use cassette for antigen- or antibody-based detection, are the most widely available devices meeting the criteria of readily accessible, easy-to-use, and cost-effective POC diagnosis (27). However, few are sensitive enough to serve as stand-alone, definitive diagnostic tools and are generally considered a first-line diagnostic strategy for screening. Positive results, in most cases, need to be verified with more definitive testing, particularly if the patient's symptoms persist. For many infectious diseases, more definitive testing is usually mandated if the results are negative, especially if signs and symptoms of illness are at odds with a test that has known low sensitivity (28). While secondary, confirmative testing has been the norm in well-developed health care systems, easy-to-use and low-cost RDTs may be the stand-alone decision-making test, an imperfect solution to initiating medical treatment in low-resource and remote areas.

RDT performance has generally not been proven to be sufficient to address expanding and emerging diagnostic needs, including direct measurement of infectious pathogens (as opposed to humoral immune responses to pathogenic antigens), high sensitivity required for screening assays and detection of early infections, enhanced multi-analyte profiling from a single specimen source to better understand the course of a disease in an individual, and rapid discrimination of mutations conferring drug resistance. Even in situations where antigen- or antibody-based detection has been shown to be safe, effective, and convenient, as is the case with the recently FDA-approved over-the-counter OraSure HIV1/2 RDT (27), these tests have limited utility (28), while other rapid HIV tests have failed altogether (29). Follow-up confirmatory testing in a clinical setting, as well as counseling, is strongly urged regardless of the results of the test, because of individual variability of the immune response and potential false-negative results due to low sensitivity in acute infection (31, 32), as well as capitalizing on the opportunity for effective and timely patient treatment (30). Quantitative viral load assessment to facilitate treatment monitoring (33), mutant detection and analysis (34), direct viral detection in the case of vaccinated individuals, and early infant detection (where maternal antibodies confound the detection of viral infection) (35) have all necessitated nucleic acid–based

molecular diagnostics to correctly identify HIV or mutant variants for optimized treatment and treatment monitoring. For tuberculosis (TB), the low sensitivity and variable manufacturing lot quality documented for lateral flow serological RDTs prompted the WHO to issue strong recommendations to discontinue use of these devices (36). Similarly, RDTs targeting sexually transmitted *Chlamydia trachomatis* (37) have produced marginal results with a poor performance record and have been less reliable than syndromic management in some studies, compounding the dilemma caused by the fact that in-home testing kits are widely available without medical supervision through Internet retail sites (www.stdrapidtestkits.com/chlamydia-rapid-test-kit-for-home-use.html).

Obviously, performance validations, quality control of manufacturing, and post-market surveillance have not been universally embraced, a situation that begs for action from the global health community. In certain cases, this has led to successful and collaborative monitoring programs. In response to the high rate of new device and lot failures of malaria RDTs, WHO and FIND (Foundation for Innovative New Diagnostics), with support from concerned collaborators, instituted an external quality assurance program in 2004, requiring manufacturers to submit new devices, or lots of marketed devices, for separate quality-control testing at independent laboratories (38, 39). This labor-intensive program has been very successful in upgrading the overall quality of malaria IVD products made available through ministries of health in many countries where the disease is endemic. These successes come at a cost in time and resources and continued vigilance. Even with this extensive quality-control program, lack of standardized positive controls or continuous training often makes interpretation of test results difficult and inconsistent among the health care workers surveyed (40).

Antibody- and antigen-based detection RDTs have their place and are certainly not inherently flawed. Given strong system regulation that incorporates internal controls and training standards for these devices, RDTs with good performance characteristics will be "fit-for-purpose," particularly to guide treatment of symptomatic patients (39). These RDTs can uniquely fill the niche of home-based testing, fulfilling privacy needs when appropriate while bringing into the clinic patients who would not normally have sought care. But studies have demonstrated that RDTs do not substitute for IVDs with the sensitivity and specificity required for detection of low-level infection or asymptomatic patient populations (41), and they do not fill the need for rapid tests that can provide a fast definitive diagnosis, discriminate closely related pathogens, identify emerging drug resistance, and can be readily adopted as POC tests.

Strategic Direction for POC Testing

The issues and inadequacies evidenced in current POC testing, emerging primarily from the need for low-cost, high-performance diagnostics in regions with high disease burden but little access to advanced health care and quality advocacy, are driving the strategic change in the development requirements for molecular diagnostics, particularly around diagnosis of infectious diseases and antibiotic stewardship. ASSURED (Table 1), a roadmap proposed for the development of new high-performance POC diagnostics in the developing world (42, 43), mandates that developers and manufacturers address the physical environments and limited resources while focusing on ease of use and low cost from concept to product launch. Standing for Affordable, Sensitive, Specific, User-friendly, Robust, Equipment-free, and Deliverable, the ASSURED requirements could equally apply to cost-containment of companion diagnostics for clinical trials and thus provide a practical guide for next-generation molecular diagnostics innovation in general (44).

ASSURED is an idealized standard that imposes challenging constraints and standards on molecular POC diagnostics developers. Affordability is typically at price points far below what has been deemed desirable for commercialization and market entry, even though commitments from numerous governmental and nongovernmental donors have helped lower this barrier. Sensitivity and Specificity are hallmarks of molecular diagnostics in general. However, while most molecular diagnostics have excellent analytical and clinical sensitivity/specificity profiles for detection of active infection, higher levels of performance are mandated for screening asymptomatic patients in endemic populations to aid in disease control and eradication. Robust and Rapid are often at odds with the requirements for adequate nucleic acid enrichment, reagent stability, or instrumentation environmental requirements. The requirements for User-friendly operations and Deliverability, while always assumed goals of manufacturing, are amplified in certain geographic regions where transportation infrastructure, local laboratory facilities, electric power, and air conditioning are typically inadequate, and experienced personnel are stretched thin (44, 45).

The qualification for "Equipment-free test or detection," at first glance, would seem difficult or unreasonable to conceive for nucleic acid-based testing without instrumentation. In a broader, more practical sense, this criterion aims to minimize reliability on a steady electric power supply and encourage instrument mobility while requiring ease of use and flexibility of the testing in general. However, recent innovations in molecular test development, either targeted directly at ASSURED for the developing world or crossing over from innovations designed to improve efficiencies and turnaround times and cost-effectiveness in centralized laboratories, demonstrate the potential to address the ideals of ASSURED in all laboratory settings.

MEETING THE ASSURED CHALLENGE: TECHNOLOGICAL INNOVATIONS FOR POC MOLECULAR DIAGNOSTICS

Several recent reviews have highlighted the advances in molecular biochemistry, nanotechnology, and microfluidics that are driving diagnostic development to meet the challenges posed by the nucleic acid–based POC platforms in the developing world (16, 46, 47).

PCR-Based Amplification

PCR-based nucleic acid testing is a highly effective and selective process for sensitive and specific detection of given analytes. Molecular diagnostics technologies were originally envisioned for the centralized laboratory because of complex assay assembly, complicated sample preparation, elaborate and energy-consuming thermocycling instrumentation, and detection systems. Due to the high cost of platform development, diagnostics companies have focused primarily on high-volume centralized clinical laboratories with the capability to perform a menu of different assays

TABLE 1 Breakdown of the ASSURED principles for diagnostics development

ASSURED principle	Analytical performance versus clinical requirements	Risk-benefit considerations
Affordable	To whom and at what level of the health care system? What if it is affordable but provides no actionable information?	Dependent on outcome-based study looking at the assay readout versus economic benefits versus patient outcome.
Sensitive	What level of sensitivity is required? In certain cases an assay which can be deployed at the POC can be too sensitive. Or do we mean "limit of detection," which defines analytical performance but may not be necessary for clinical outcome? "Fit for purpose" must be understood.	Based upon the analyte and a risk-benefit analysis. Sensitivity = TP/(TP + FN)[a] From a risk standpoint, what is the outcome of a false-negative answer in light of the intended use of the test? Sensitivity and specificity are inversely related to each other for most technologies. But this is all a trade-off depending on the intent of the assay and what is required for a negative predictive value
Specific	All assays should be specific for the analyte which is to be detected, but what about the potential change in the analyte, e.g., influenza and rapid lateral flow tests or mass-spectrometry-assisted enzyme-screening (MES) and molecular tests?	Specificity = TN/(TN + FP)[b] From a risk standpoint, what is the outcome of a false-positive answer in light of the intended use of the test? Sensitivity and specificity are inversely related to each other for most technologies. But this is all a trade-off depending on the intent of the assay and what is required for a positive predictive value.
User-friendly	Based on what criteria, is the situation and level at which the test will be employed critical to understand?	This is dependent on the level of the health care system where the test will be deployed, but it is also dependent on the output of the assay. If the output is actionable information, then action needs to be the ultimate outcome of the assay.
Rapid/**R**obust	How fast is fast, and does it depend on the location and the analyte which is being tested? Robustness is dependent on the user and the setting where the assay will be deployed.	Rapidity is again dependent on the analyte and the actionable information that is the output of the test. Robustness for a test can be defined by multiple parameters. What is more important is the validity and acceptance of the test output.
Equipment-free	Why, if a simple device improves sensitivity, reproducibility, and/or accuracy, should the device not be deemed to be a POC device?	This should not be a limitation if the instrumentation increases the performance of the test. Even with currently available RDTs, a reliable reader increases reproducibility and accuracy of the test. But the equipment needs to be robust, reliable, accurate, and reproducible from run-to-run, user-to-user, and day-to-day.
Deliverable	This is a reflection of the infrastructure of the health care system and the manufacturer.	This should reflect the usability of the test at its intended location, the stability of the test, and transportation stability

[a]TP, total positive; FN, false negative.
[b]TN, true negative; FP, false positive.

designed specifically for the instruments. However, innovations in diagnostics platforms have the potential to simplify the sample-in to results-out process while still maintaining the high sensitivity and specificity associated with specific target identification through molecular detection. This again leads to reimbursement issues because platform development is costly, market sizes are not a given, assay uptake is not a given, and the product life cycle may be shorter than 5 years. All of these factors and more must be fully investigated and understood prior to a developer becoming involved in a POC market (48).

Microfluidics and miniaturization have allowed full assay integration, from target enrichment of raw specimens to PCR-based nucleic acid amplification and multiplex detection, in a single-use closed cassette–based system (49). The instrumentation currently in use or evaluation typically have small footprints, from the size of a small computer tower (such as the Cepheid GeneXpert single-cassette or four-cassette instrument) (50–52) to battery-powered, handheld devices (such as the Epistem GeneDrive) (53).

The GeneXpert system has received much scrutiny from the TB community over the past few years as a benchmark for performance features that attempts to address ASSURED for POC testing in developing countries: a single-assay, closed-cartridge system that integrates (i) onboard sample preparation from liquefied-decontaminated specimen for nucleic acid target enrichment, (ii) target-specific detection of single or multiple pathogens through real-time PCR, (iii) potential differentiation of multiple mutations of the same pathogen through use of sequence-discriminating DNA hybridization signal beacons, (iv) specimen sampling control, and (v) cartridge quality control (50, 51). These features are demonstrated in the performance of the Cepheid Xpert MTB/RIF Cartridge Assay, which enables detection of both *Mycobacterium tuberculosis*

complex and M. *tuberculosis* strains associated with rifampin resistance from sputum after a single decontamination and liquidation step followed by addition of the prepared specimen to the sample portal and placement into the GeneXpert cartridge port. This minimizes health care worker exposure to harmful contagious pathogens and decreases the risk of cross-contamination that often plagues high-throughput open-tube sample preparation and detection platforms. Multiple clinical trials conducted in low- and middle-income countries (52) demonstrated the robustness of the assay under less than optimal conditions and led to WHO endorsement of the platform for TB and multidrug-resistant TB detection in the developing world (51).

The GeneXpert system addresses several of the ASSURED targets, but not all. This is an instrument-focused platform. The system still requires a controlled environment (temperature, humidity, and dust) and a steady electrical source when operating, although the shorter run times (2 h for MTB/RIF assay) have been accomplished on battery power (54). The sensitivity of the assay drops significantly for sputum samples that are acid fast staining negative but culture positive (55). GeneXpert requires an external computer/laptop, which presents issues of theft in resource-stretched laboratories with marginal security. And the cost, even at prices supplemented by nongovernmental organizations, is still beyond the reach of many health care systems in areas where TB is endemic (56). Still, GeneXpert MTB/RIF demonstrates the potential for routine molecular diagnostic near-patient testing in resource-constrained settings.

Other amplification-based analytical systems are being introduced to developing markets to specifically address the need for accessibility with low-cost, multiplex technology. Several commercialized systems are being introduced. The Epistem Genedrive is a portable system that is initially being developed for TB testing (M. *tuberculosis* complex and rifampin-resistant M. *tuberculosis*) from sputum. Sample preparation is minimal but does involve extensive manipulation separate from the PCR-based amplification and detection. Sample preparation involves spotting a volume of sputum onto proprietary, functionalized filter paper that purportedly renders the pathogen nonviable. The dried filter paper spot is punched and transferred to the closed GeneDrive Cassette, a miniaturized assay platform containing lyophilized components required for target nucleic acid amplification. Amplification and endpoint detection require approximately 25 min. Although separate sample preparation is a drawback, the system could provide rapid and quantitative multiplex results with minimal specimen input, enabling detection from patients providing suboptimal specimens for initial testing or further mutational/biomarker analyses (e.g., HIV-positive patients and juvenile patients) (53).

Similarly, MolBio Diagnostics has developed a semi-automated real-time PCR instrument: the Truelab integrated chip-based amplification and detection process claiming "specimen-to-results" turnaround times of under 1 h, with the analytical hardware integrated directly into a handheld device (47). MolBio Diagnostics has developed an instrument that can operate on battery power for up to 8 h. Both Truelab and Genedrive, while offering speed, convenience, and flexibility, require separate nucleic acid extraction and sample preparation steps, which add a level of complexity that can impact their adoption as a POC device in the absence of trained laboratory personnel. These two devices are currently undergoing clinical assessment to determine operation feasibility and failure rate for detection of M. *tuberculosis* complex and rifampin-resistant M. *tuberculosis* in a noninferiority trial (57) with a cost analysis that will evaluate various aspects of assay operations (including transportation, equipment, reagents, sample prep, and hands-on personnel time) compared to the performance of the GeneXpert MTB/RIF assay.

An all-in-one platform, cobas Liat System developed by IQuum (a division of Roche Molecular Diagnostics), seeks to integrate sample preparation and rapid, multiplex PCR capabilities in a compact microfluidics chamber run in a small desktop device. The current FDA-cleared assay for detection of influenza A/B (including H1N1) is conducted from a nasopharyngeal swab, detecting and discriminating influenza A from influenza B in as little as 20 min, making it an invaluable tool for an emergency clinical setting. However, the assay is considered "moderately complicated" by the FDA and therefore is restricted to use in CLIA-qualified labs (58).

It is important to note that these near-patient technologies are still operationally "moderately complex technologies" (i.e., would not qualify for CLIA waiver in the United States and would require qualified laboratory facilities and personnel for operations), particularly in the handling of highly infectious specimens, such as sputum from suspected TB patients (58). While each of these PCR amplification platforms has specific strengths and weaknesses, their performance in clinical trials in low-resource areas suggests that next-generation technologies can potentially integrate all steps from sample preparation to amplification to signal detection, with minimum requirements for specimen manipulation, in a portable platform designed to be user-friendly, flexible, and cost-effective. But unfortunately, the "holy grail" of a true single-use, low-cost molecular POC is still a vision of the future (2, 59).

Isothermal Amplification

Several thorough and extensive reviews of isothermal amplification (involving enzymatic DNA strand separation rather than temperature-based denaturation-annealing cycles) highlight several assay systems that can be run at a single temperature (as low as body temperature) using a simplified heating block to perform the assay (although most systems rely on an initial high-temperature denaturation step). Versatile detection chemistries, as simple as precipitation-based turbidity for loop-mediated isothermal amplification (or LAMP) reactions for single amplicon detection to multiple target detection with helicase-dependent amplication (helicase-dependent amplication–based colorimetric lateral flow cassette or fluorometric-based real-time detection) can provide interpretable results in less than 2 h (16, 60); an assay using a helicase-dependent amplication cassette for detection and discrimination of herpes simplex virus 1/2 has been cleared for use by the FDA (61) and can potentially serve as a near-patient device. Highly portable, battery-operated detection devices for real-time detection of isothermal reactions, such as the OptiGene Genie II fluorescent platform and the TwistDX fluorometric instrument for multiplex recombinase polymerase amplification–based detection, further demonstrate the potential of isothermal systems as POC platforms (16). Again, based on the moderate complexity of these platforms (as defined by CLIA waiver criteria), these IVDs would still be considered near-patient in developed markets, requiring some level of laboratory-based oversight.

Future Developments

The new wave of nucleic acid–based technologies currently in development takes a radically different approach. One intriguing technology is based on gold-based nanoparticles cross-linked with highly specific complementary sequences that can be induced to aggregate through salt concentration gradient and be visualized by a simple colorimetric shift. Control of aggregation, through binding of target DNA sequences, allows for sequence-specific detection that can discriminate between pathogens or determine the presence of mutations conferring resistance (62). The strength of this technology and its application in POC testing lies in the ability to detect low levels of target pathogen DNA in the absence of extensive sample preparation or target sequence amplification, using a handheld, paper-based optical or electrochemical microplate reader for detection. Proof-of-concept studies with a variety of clinical samples have demonstrated the potential of this sophisticated chemistry to deliver a simple and cost-effective POC detection system. Other nanoparticle technologies are being explored that can differentiate specific target binding by size (through formation of defined-size X- and Y-DNA nanoarchitecture) or in microfluidic platforms that integrate sample enrichment, binding, and detection through functionalized magnetic nanoparticles (1, 63–65). Other microfluidics platforms include the uBAR system, which can potentially be agnostic to nucleic acid detection chemistry achieved on an economical, robust handheld platform (65).

Probably the most anticipated revolution in POC technology is advances in next-generation sequencing. Already, next-generation sequencing is being sought for companion diagnostic development to capitalize on the sensitivity and ability of the technology to evaluate sequence information from single strands of DNA, to assess low-abundance mutations corresponding to druggable targets for anticancer therapies, or as early indicators for disease in patients classified as "in remission." The complexity and expense of the instrumentation and the software requirements for storage and manipulation of "big data" associated with this technology preclude current use as a POC system. However, the ever-evolving technology, which can rapidly identify sequences of infectious agents in a low-level infection or mutations arising in a majority wild-type population, is decreasing the cost and time of sequencing while generating data that will identify mutational "hot-spots" for disease profiling and development of secondary targeted detection assays (66, 67). It is anticipated that this technology will become a standard in near-patient testing and clinical trials within the next 10 years.

CHALLENGES FOR POC MOLECULAR DIAGNOSTICS

Sample Preparation

Sample preparation is often underestimated in the early development of these new technologies, where proof-of-concept studies are performed with purified nucleic acids or concentrated biological targets (i.e., defined pathogenic strains or isolated genomic DNA). This step becomes particularly confounding later in development when attempts to migrate to complex and heterogeneous specimen types, such as sputum, whole blood, and fixed tissue samples, become a major impediment to implementation of innovative technologies. Enzymatic amplification typically comes with the requirement of purified, or at minimum, significantly enriched, nucleic acid substrate on the front end of the assay. As can be seen in the technologies discussed here, sample preparation is usually done as a separate step. These additional manipulations could require centrifugation steps or isolation apparatus, increasing the potential for contamination and sample loss and for variable assay performance through contamination interference and decreased sensitivity, while potentially increasing assay complexity, turnaround time, and cost.

Biofire (bioMérieux) has developed a highly multiplex, PCR-based FilmArray device in a single-cassette format that integrates and simplifies (i.e., minimizes) the interface between sample processing and sample input to assay. Three 20+-plex diverse infectious pathogen panels (for respiratory disease, blood culture identification, and gastrointestinal tract infections) all utilize the same strategy and interface with the cassette, demonstrating how sample preparation for diverse specimen types (i.e., blood, oral swab, stool) can be integrated into a common platform (68). Other approaches are being researched to mitigate issues and streamline specimen processing, including low-cost paper-based filtration devices that can concentrate bacteria from viscous materials, a necessary component for evaluating sputum or homogenized tissue (69), or the use of specific bacteriophage tail-spike proteins, which bind specifically to cognate bacteria. However, to avoid limiting the application of the technology to only a single target or specimen type, the hurdle of specimen preparation must be addressed early in development, and innovations to overcome this step will need to become the focus of intense research in the near future. Of course, molecular diagnostics–based chemistries that are robust and can tolerate specimen impurities (e.g., loop-mediated isothermal amplification–based gene amplification) may offer the ideal solution through the simplest requirements, as long as sensitivity is not compromised (16, 64).

Adoption Strategy and Implementation Planning

POC technology, like home-based detection technology, is geared toward end users with little formal training or expertise in testing. Unfortunately, no IVD technology is foolproof or obviously straightforward. The issues and difficulties inherent in any detection technology have the potential to be magnified in inexperienced hands, derailing the cost savings by requiring laboratory infrastructure for implementation, or worse, losing the momentum to place the new product in emerging markets where it will have a positive impact on health care. This will be particularly true of breakthrough technologies such as molecular-based detection systems; in their current configurations, most molecular-based diagnostics still have multiple steps which may seem trivial to experienced users but are challenging to untrained personnel.

In emerging markets, the following concerns compound the issues faced by all medical device manufacturers and distributors: limited infrastructure and distribution channels and unclear regulatory requirements. So the roll-out strategy, incorporated into the planning phase of the device early in development, must envision and understand the needs of the end users in a challenging health care environment, the training that will be required to effectively introduce the POC IVD, and the support that will continue as the device gains acceptance and filters down to local health providers. A carefully crafted strategy continually incorporates

lessons learned from the early adopters. The WHO Prequalification Program, which does the assessment, recommendation, and ultimate endorsement (when appropriate) of high-risk/high-impact medical diagnostics (currently limited to HIV and malaria tests, as well as an endorsement process for TB), includes as its criteria clinical trials at the point of use, to demonstrate that the device can function and impact therapeutic outcome in limited-resource environments (70).

These activities can significantly impact the cost of IVD uptake. As noted above, some of the issues can be mitigated through intense and ongoing education, as well as a "boots on the ground" presence to ease the transition of the new technology into the clinical setting. Device manufacturers need to develop strategies for best practices in communication through social media, remote technologies, and early adopter/end user virtual forums to gain insight and alleviate issues as they arise while helping to build confidence in the product and the technology (70).

Facilitating Communication of Results

The emphasis in design and development of POC devices is the turnaround time from sample-in to results-out for the device, typically requiring instrumentation and software (either included with device or separate, requiring additional steps for input of raw data) to provide the final result. However, the goal of ASSURED emphasizes assays that require no or minimal instrumentation, for ease-of-use, cost-effectiveness, and infrastructure requirements.

Certainly, the trend of instruments with smaller footprints moving toward handheld devices is a step in the right direction. However, it still requires a separate dedicated instrument, with all the inherent requirements for validation, care, and downtime cost.

At least one alternative strategy that can greatly simplify the requirement for instrumentation would use optical imaging through cell phone–based telemedicine, and these capabilities are under consideration for RDTs in the developing world. Mobile phones are certainly ubiquitous worldwide, and the familiar "mobile app" can be developed and validated that could greatly enhance the adoption of visual or colorimetric cassette-based molecular diagnostics. Integrating this approach with cassette-based lateral flow molecular devices, such as the Biohelix/Quidel Amplivue cassettes or UStar nucleic acid detection strip, would be an affordable, readily accessible way to enhance the sensitivity of the visible readout, particularly for the scannable faint band that might be read as negative or indeterminate by an inexperienced user. A mobile phone application developed specifically for these types of platforms would aid in standardizing result reporting and training less-experienced personnel, through remote interactions with a central laboratory, with raw-image files that can be stored and linked to encrypted patient data for remote analysis (71). Similarly, cell phone–based applications could enhance the experience, compliance, and follow-up for the use of home-based RDT testing, by encouraging submission of data for more sensitive screening, and feedback on next steps based on results. Prototypes that provide easy attachment to a mobile device are currently being tested at the research level (72). Other remote applications, providing system monitoring and automated instrument calibration (as is available for the GeneXpert platform), reduce the cost of instrument maintenance and downtime for technologies requiring frequent calibration and quality control review.

One important consideration with the use of mobile technologies is standardizing image acquisition and application performance across different brands, makes, and models of phone, similar to requirements for IVD instrumentation. Quality control assessments and specifications around minimal pixel requirements and image reproducibility should aid in defining minimal performance standards. Another concern is protection of patient data and confidentiality, and software and encryption validation must be planned into the validation of data security of these systems to ensure patients' rights and privacy. Back-up systems for storage of patient data will be an important consideration for the successful adoption of telemedicine, but the value of integrating low-cost, familiar, and easy-to-use consumer electronics into the diagnostic process has great potential that should be exploited.

A PRESSING NEED: QUALITY ASSURANCE FOR POC MOLECULAR DIAGNOSTICS

A common theme resonating through all of the requirements outlined in this review is the need for quality assurance at all stages of design and development, from documenting the end users' needs and requirements through supporting end users during the lifetime of the marketed IVD. While the ultimate test of any candidate diagnostic is its outcome-based performance in well-controlled and unbiased clinical studies demonstrating intended use (73), the basis for this performance starts back at the laboratory bench of the developer.

"The planned and systematic activities implemented in a quality system so that quality requirements for a product or service will be fulfilled" is a broad, somewhat circular definition of quality assurance. At the root is quality, the concept that dictates processes and procedures that ensure that design, validation, and manufacturing of a product will be "fit for purpose" consistently, lot after lot, so that the performance of the device will meet the objectives of the intended use. For IVD design and development, the guiding principles for quality are exemplified by various standards, such as International Organization for Standardization standard ISO13485, various Global Harmonization Task Force nonbinding guidances supported by the IMDRF (International Medical Device Regulatory Forum), and the regulations imposed by U.S. law on manufacturing entities commercializing IVDs in the United States and embodied in the Code of Federal Regulations 21 (i.e., 21CFR Part 820), also known as Quality Systems Regulations.

These guidances and regulations detail the requirements for design control (74) and good manufacturing practices that govern all aspects of product realization, continued improvements, corrective/preventative actions, and post-market surveillance under controlled and documented standard operating policies and procedures and are embedded within an organization's quality management system. A good quality management system makes the process of validating performance of an IVD, as well as determining and mitigating the risks of device failure in the marketplace, transparent to national regulatory agencies or their representatives (i.e., notified bodies in the European Union) as well as to the manufacturer itself. The importance of these guiding practices to IVD development cannot be overstated, because the risk of supplying POC devices that fail in areas of endemic disease is life-threatening and disease-promoting and results in diverting and

draining scarce resources for procurement of effective diagnostics or therapeutic treatments.

A key element within a quality management system is design control, the process of defining and validating the intended use of an IVD, the functional performance and molecular target of the IVD, the patient population targeted (and excluded), specimen types appropriate for use, and the clinical setting appropriate for use. For POC IVDs, these criteria become critical, since the device performance must be carefully validated for use outside of highly controlled laboratory settings with personnel that may have little familiarity or training in the use of diagnostic devices or interpretation of results. As discussed previously, the increased requirements for robust performance of these products in developing countries can impose extreme challenges to even a well-conceived and experienced IVD development team.

To assist manufacturers in conceptualizing molecular POC diagnostics that can fulfill the criteria of "fit for purpose" in challenging and resource-limited clinical settings, advocates for POC IVDs have launched several initiatives:

- Target product profiles have been vetted by expert panels consisting of governmental and nongovernmental organizations, regulators, researchers, and clinicians knowledgeable in product performance to provide developers and manufacturers with essential and specific requirements for product performance in resource-limited areas with challenging medical infrastructures and unfavorable environments (47, 75).
- Various organizations have partnered with manufacturers in protocol design and clinical evaluations in diverse geographic and resource-limited settings to accumulate the evidence needed to demonstrate the feasibility and performance of novel technologies to address underserved communities where neglected diseases are endemic. The data collected have been used to support the approval process and implementation of novel technologies in the developing world (51).
- External quality assurance programs have been developed to independently assess the performance of new devices and the lot-to-lot quality of existing devices (75). As previously mentioned, the Malaria Rapid Diagnostic Test Performance, a program currently sponsored through WHO and FIND, with participation from the CDC and other collaborators, evaluates the performance of malaria RDTs submitted from manufacturers to determine and distinguish properly performing tests from poorly performing POC devices. The program is now in its sixth round of testing, and the evaluation results, including the identity of specific devices, manufacturers, and manufacturing lots, are published on the WHO website and are used to guide the procurement of high-quality RDTs by ministries of health in malaria-endemic countries, as well as funding agencies. The program has been highly successful in encouraging the quality of manufacturing for these devices (39). A similar scheme for evaluating the plethora of malaria molecular diagnostics coming onto the market has recently been beta-tested and is now advocated to ensure the quality and utility of these devices slated for use in screening of both symptomatic and asymptomatic patients, toward the goal of eradicating malaria (77).
- Access to well-characterized, biologically relevant, and geographically diverse pathogen strains and patient specimens collected in controlled clinical evaluations is a necessary component for analytical and preclinical validation of molecular IVD performance. However, access to these specimens is difficult to attain, and the collection at relevant clinical sites can be prohibitively expensive, which can discourage developers from properly validating IVDs such as TB detection molecular diagnostics. In response to this need, the WHO Tropical Disease Research unit, FIND, the Institute of Tropical Medicine, and other organizations and national laboratories have derived and characterized pathogen strains or archived specimen collections from multiple clinical trials. FIND makes its TB specimen archive, complete with clinical data, available to assay developers worldwide, to encourage product development and validation on a wide range of specimen types, as well as to foster biomarker discovery to expand the range of testing possibilities to novel targets (76; http://www.finddiagnostics.org/programs/tb/find_activities/tb_specimen_bank.html). The Institute of Tropical Medicine has a similar policy for providing well-characterized TB strains for research and product development (http://www.tbonline.info/posts/2013/12/13/worlds-largest-collection-tb-strains-antwerp). Commercial entities, such as Zeptometrix Corporation, also provide innovative and validated surrogate solutions to enable safe validation activities and provide realistic controls, critical for replicating device detection capabilities for highly contagious and life-threatening pathogens. With the rise in drug resistance threatening to eclipse the availability of effective antibiotics, these resources will be invaluable for the validation of IVDs targeting the detection of drug-resistant TB to ensure that proper therapeutic treatment is initiated as soon as possible.

These initiatives and resources are at the disposal of IVD developers and manufacturers, particularly POC developers, to encourage the development and manufacturing of high-quality and fit-to-purpose devices. While the programs focus on delivering POC technology to the developing world, the concepts and guiding principles are the same for quality-assured manufacturing practices in any setting. However, the ultimate responsibility for high-quality manufacturing and supply of much-needed, high-performance molecular diagnostics, whether slated for use in well-resourced clinical laboratories or at the POC in a field hospital, rests with the manufacturers and the agencies responsible for their oversight.

Another key element that is often overlooked, particularly in the development of POC diagnostics, is external and whole-process controls and standards, which can help ensure effective training and consistent use and interpretation of results (77). Particularly as novel and untested technologies find their way into the POC market, it will be incumbent on manufacturers and regulators alike to evaluate controls and standards that mimic the clinical reality of device deployment, providing tools to build confidence in device performance and the validity of results.

UNDERSTANDING THE CONTEXT FOR POC TESTING

Cost-Benefit Analysis of POC Testing

The transition to POC molecular diagnostics, with the potential to address a wide range of diseases with sensitive, rapid testing, has game-changing potential in the clinical

testing arena. However, in the final analysis, it is also important to take a broader view of the actual requirements for disease diagnosis, so that innovative POC testing can truly meet the needs and be cost-effective at the POC. A recent cost analysis of GeneXpert MTB/RIF implementation in diverse laboratory settings in South Africa concluded that implementation in decentralized POC clinical settings was significantly more costly than use in more traditional clinical laboratories, without significantly increasing the overall outcome for affected TB patients (78). This is just one modeling analysis, and it does not question the innovation, performance, or value of the test itself, which can rapidly detect both infection and potential drug-resistant infection in patients. It does, however, bring into consideration the costs and benefits of decentralized, POC testing, unless the cost of the diagnostic test is radically offset by benefits to patients.

Evaluating Judicious Use of Rapid POC Diagnosis

It is tempting to speculate about deployment of rapid POC testing in all clinical settings, but there may be clinical situations when POC technology is not so effective, exemplified by POC detection and discrimination of human papilloma viruses (HPV), particularly high-risk forms (HR-HPV testing) associated with high-grade cervical cancer. Given the generally low prevalence of cervical cancer, the medical community urges caution in rapid follow-up to any form of cervical cancer screening because of the likelihood of unnecessary treatment. A meta-analysis of randomized clinical trials looked at the screening outcome of approximately 235,000 women tested with conventional cytology (i.e., Pap smear) compared to screening with any HPV test (alone or in conjunction with cytology) (79). The results showed that high-grade precancerous lesions (at or above cervical intraepithelial neoplasia grade 3) were detected earlier with HPV testing (alone and in combination in this analysis) compared to Pap smear, but the study did not note any long-term outcome benefit (decreased mortality from cervical cancer or higher quality of life). Unfortunately, the study was unable to determine how many unnecessary treatments were performed due to false positives or true-positive HPV infections that would have naturally resolved without any intervention. Once again, the value of a sensitive, rapid assay that can alert a physician to a potential precancerous clinical condition for a disease that is typically asymptomatic is outstanding, but the need to have that information immediately during a routine office visit is questionable, particularly if performing the test at the POC is not cost-effective and leads to a higher rate of unnecessary, and at times invasive, treatment (79). In resource-constrained health care settings, other factors need to be considered, such as loss of the patient to follow-up and the impact of a patient's condition on the community at large. But in a time of scarce medical resources, these factors must be evaluated in the larger context of the ability to generate a diagnosis rapidly from a POC test (regardless of the cost) when the need for a thoughtful diagnosis and "wait and see" monitoring of disease progress may have a more positive impact on clinical outcome to the patient.

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Point-of-Care Technologies for the Diagnosis of Active Tuberculosis

GRANT THERON

40

INTRODUCTION

The Tuberculosis Epidemic

Tuberculosis (TB) is a preventable and curable disease, yet it is responsible for over 1.5 million deaths every year (1). In 2012, 6 million new cases of TB were diagnosed, yet an estimated two-thirds cases were missed. Almost half of the TB cases in the world are in Brazil, Russia, India, China, and South Africa, with the highest incidence in sub-Saharan Africa. Although TB is readily curable, the failure to diagnose more cases rapidly means that patients have poorer outcomes and prolonged infectiousness (2). TB therefore remains a global threat to public health.

TB is the biggest killer of people infected with HIV, and in 2014, 400,000 people living with HIV died from TB (1). The need for better diagnostics is particularly dire for these patients because specimen collection is challenging, pulmonary fluids have few bacilli (paucibacillary disease), and extrapulmonary TB is more frequent (3, 4). TB is underdiagnosed in children too, who have atypical clinical signs and symptoms and paucibacillary disease, and in whom currently available diagnostics perform poorly (5–7). Globally, only 35% of cases of pediatric TB are detected (8).

Approximately 480,000 patients were diagnosed with multidrug-resistant TB (MDR-TB) in 2014. MDR-TB is defined as resistance to rifampin and isoniazid, which are two of the key drugs used to treat drug-susceptible TB. MDR-TB is extremely costly to treat. For example, in South Africa, MDR-TB comprises 2% of the case burden yet consumes 32% of the national TB budget (9). Globally, it is estimated that less than one in four cases of MDR-TB are correctly diagnosed (1). Furthermore, only 5% of patients with a new diagnosis of TB ever undergo drug susceptibility testing (DST), despite evidence from China (10) and South Africa (11) suggesting that ~80% of MDR-TB is in patients who have not had TB before. Most MDR-TB therefore appears to be caused by person-to-person transmission, rather than the acquisition of resistance.

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The Importance of Rapid, Decentralized TB Diagnostics

The most important feature of point-of-care (POC) or near-care diagnostic tests is their ability to inform clinical decision making during a single encounter. In Asia and Africa, between 1 in 10 and 1 in 5 patients who have a positive test result never start treatment (12). Smear microscopy is routinely used in these settings, and most patients who test positive but do not start treatment have high bacillary load, heightened infectiousness (13, 14), and a greater risk of death (15–17). POC or near-care diagnostics can allow these "pre-treatment dropouts" to be reduced. In a single day, a patient could present to a health facility, be screened, provide a specimen that can be tested on-site or nearby, and, once the result has been reported, start treatment. This can reduce the number of visits patients and their companions make to facilities prior to treatment initiation, which can reduce nosocomial transmission. Furthermore, even when diagnostics and treatment are available for free, TB patients and their households can incur dramatic and catastrophic economic loss while seeking care (18).

Because the burden of TB is highest in countries that have weak health systems, POC or near-care tests for TB should be simple to use and not require sophisticated infrastructure. They must be able to rapidly impact clinical decision-making at the primary care level, which is where the bulk of TB patients (~65%) are seen (19). While not the focus of this chapter, the parallel strengthening of the health care system, including aspects of clinical training, supply chain management, and health information services, is key to ensuring that the benefits offered by new POC or near-care tests for TB are not undermined (20, 21). Indeed, patients who test positive by rapid, potentially same-day tests can still take months to start treatment (22–24), and if clinical training is poor and the health care system is weak, high test accuracy alone will not necessarily translate into improved clinical outcomes (20, 25–28).

Epidemiological modeling (29) has suggested that in Asia alone, implementation of a POC dipstick test for TB could prevent 39 million TB cases between 2015 and 2050 and 8 million TB-related deaths, which is almost double the number of deaths preventable with the implementation of accurate centralized nucleic acid amplification testing (NAAT). There is therefore an urgent clinical and humanitarian need for simple, accurate, and rapid tests to

inform the management of TB and thereby scale-up of case detection, which is integral to the World Health Organization (WHO) post-2015 global TB strategy (30). If new tests are successfully implemented at the POC or near care, and the supporting health care infrastructure is strengthened, case detection, same-day treatment initiation, pretreatment loss to follow-up, treatment delays, and patient economic loss can all be improved. This will ultimately result in more patients getting better quicker, a reduction in transmission, and fewer deaths.

Chapter Outline

This chapter will first briefly outline laboratory capacity where POC or near-care testing is likely to be implemented, such as in peripheral-level laboratories. It will summarize recently defined "wish lists," or target product profiles (TPPs), of POC or near-care TB tests, which are essential characteristics that need to be considered by scientists interested in developing new tests. The chapter will also describe which commercially available tests for TB qualify as either POC or near-care because, of the tools presently available, they have the most potential to inform same-day clinical decision making. These include smear microscopy, NAATs such as Xpert MTB/RIF (Cepheid, Sunnyvale, CA) and the loop-mediated amplification (TB-LAMP) test (Eiken, Tokyo, Japan), and antigen-detection tests such as the Determine-TB lipoarabinomannan (LAM) Ag urine dipstick test (Alere, Waltham, MA). Using Xpert MTB/RIF as an example, some of the challenges involved in the POC and near-care implementation of tests, which laboratory workers and test designers should anticipate, will also be highlighted. Other nascent technologies and products at an early state of evaluation or in development which have the potential to be deployed at POC or near-care will be described, including several "fast-follower" NAAT platforms. This chapter will not describe tests for the immunodiagnosis of active TB, which meta-analyses have demonstrated to have no clinical value (31) and poor cost-effectiveness (32), nor tests for latent TB infection, which is poorly understood (33).

NEAR-CARE OR POC TESTS SHOULD WORK AT INTERMEDIATE- OR PERIPHERAL-LEVEL LABORATORIES

Most health systems possess laboratories specializing in the diagnosis of TB at the referral, intermediate, and peripheral levels. Referral laboratories typically receive specimens from a wide catchment area, possess strong infrastructure and highly skilled personnel, and routinely conduct culture, speciation, and drug susceptibility testing. They often perform expensive, high-throughput diagnostic tests, such as BACTEC Mycobacterial Growth Indicator Tube liquid cultures (BD Diagnostic Systems, Cockeysville, MD). Intermediate-level laboratories are more decentralized and routinely perform rapid testing such as light-emitting diode microscopy or Xpert MTB/RIF. In urban centers, intermediate-level laboratories may be located within hours of primary care clinics. Thus, for the purposes of this chapter, tests that may be performed at intermediate-level facilities will be considered near-care, because these facilities could receive a specimen on the day of collection, perform a rapid test, and send the result to the clinic that same day, thereby potentially resulting in the rapid clinical management of patients.

Laboratories at the peripheral level are the most numerous in high-burden countries (HBCs) yet have the weakest infrastructure and testing capability. In the developing world, peripheral laboratories conduct on-site sputum smear microscopy and are physically attached to a primary care clinic. Tests that can be performed in these peripheral laboratories, as well as in clinics or health posts that do not have an attached laboratory facility, are considered POC. Importantly, the greatest volume of patient testing occurs at the peripheral level (65%), followed by the intermediate level (25%) and the district level (15%) (19). Below peripheral laboratories are health posts, which have no laboratory infrastructure, and at-home testing, but no tests for TB expected within the next 5 years will likely penetrate down to these two levels. An overview of current and expected tests for TB, including the level of the laboratory system in which they are expected to operate, is shown in Fig. 1.

WHAT INFRASTRUCTURE IS AVAILABLE IN PERIPHERAL-LEVEL LABORATORIES AT THE POC?

Peripheral-level laboratories are where most POC TB testing will be performed. Figure 2 shows examples of such facilities in HBCs (34). These centers generally perform smear microscopy and are staffed by technicians, but there was, until recently, little data on the expertise, infrastructure, and environmental challenges associated with performing diagnostic testing in these environments. A recent survey of experts in 22 HBCs showed that the conditions, equipment, and expertise present in these laboratories are challenging for laboratory workers and test developers (Fig. 3) (35). High indoor temperatures and humidity were problematic in nearly all of the countries (77%), and two in five (41%) countries reported peripheral laboratories to have no access to running water. Furthermore, "lack of stable power supply," "harsh weather conditions," and "lack of skilled labour" were all stated as challenges to the implementation of POC tests in these settings. On a positive note, nearly all countries performed quality-assured Ziehl-Neelson microscopy on site at peripheral laboratories (95%), most had surgical gloves available (82%), and most had access to mobile phone networks (82%).

TPPs OF RAPID TESTS FOR TB

The WHO STOP-TB Partnership has developed a series of TPPs for new TB tests, which define the required minimum and optimal operating characteristics of new tests for TB (36). Abridged TPPs for a sputum-based test that can replace microscopy and a rapid, biomarker-based, non-sputum-based test are shown in Table 1. Tests of the first type (sputum-based test), with an optional ability to perform DST, are a short-term priority and are expected to be available by the end of 2016. In the long term, a simple and accurate non-sputum-based test that can be performed by unskilled personnel in the field is needed (36, 37). Such a test could be performed on urine (38), breath (39), or blood (40) at facilities without access to a laboratory and could be done on patients who cannot produce sputum (sputum-scarce) or who have extrapulmonary TB. These patients, in whom sputum-based diagnostics have little utility, represent 15 to 25% of all TB patients (1). These tests should be highly sensitive and specific, be user-friendly, be usable by

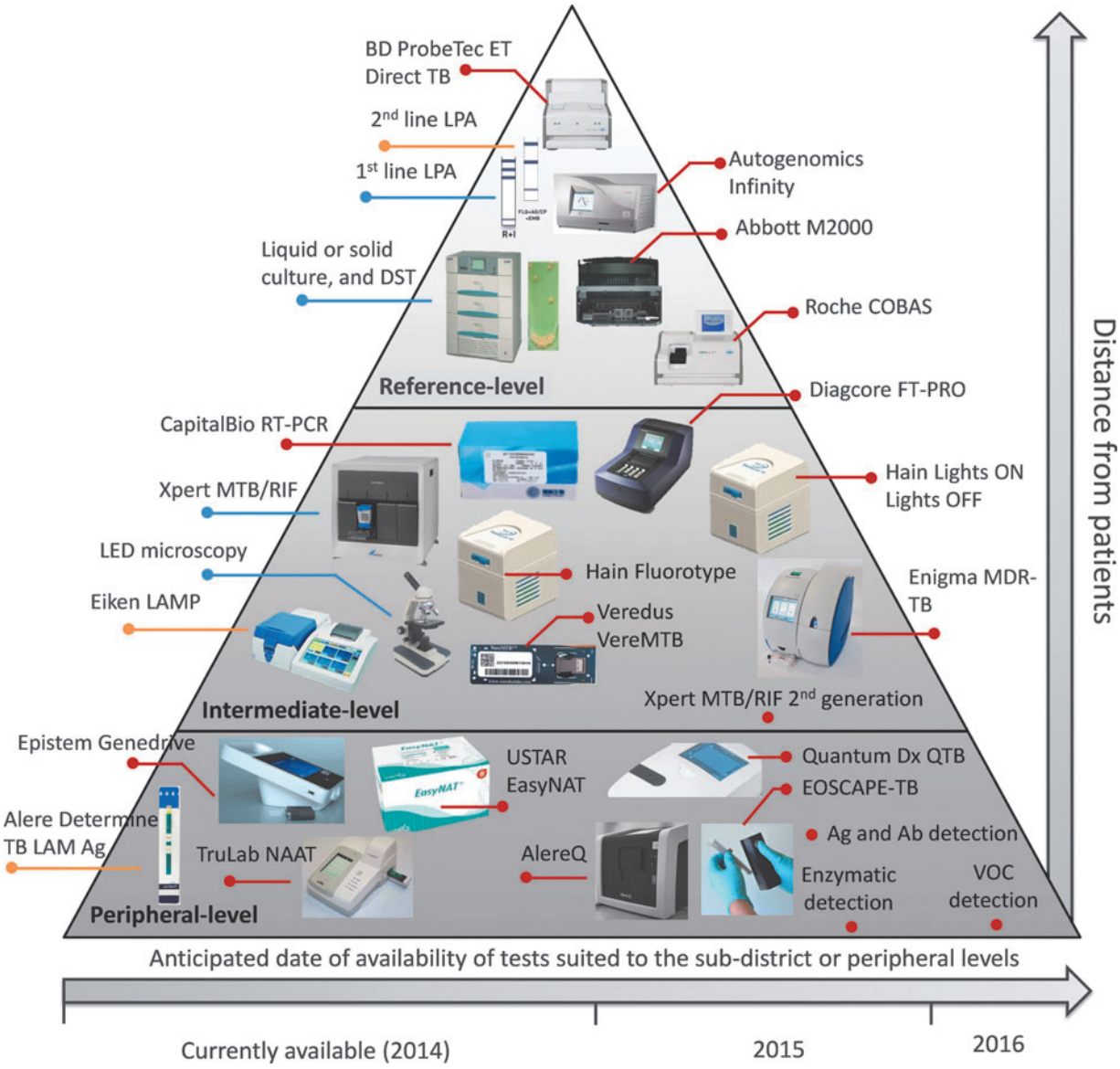


FIGURE 1 Pipeline of new and emerging commercial tests for tuberculosis. Only selected tests are shown. Tests marked with a blue line are endorsed by the WHO, whereas those marked with an orange line have been reviewed but not endorsed, and those marked with a red line have not been reviewed nor endorsed. Adapted from references 19 and 41. Abbreviations: Ab, antibody; Ag, antigen; DST, drug susceptibility test; LAM, lipoarabinomannan; LED, light-emitting diode; LPA, line probe assay; RT-PCR, real-time PCR; MDR-TB, multidrug-resistant tuberculosis; TB, tuberculosis; VOC, volatile organic compound.

minimally trained care workers, require hardly any manual sample processing steps, and be rapid (ideally <20 minutes). Ideally, they should not require an instrument. There is no test currently available or within the diagnostic pipeline that satisfies these criteria in the short term (41).

SPUTUM SMEAR MICROSCOPY

Despite being over 125 years old, sputum smear microscopy is the most commonly used test for TB. It is performed routinely at most intermediate- and peripheral-level laboratories. It is the only routinely available test in some HBCs. Microscopy is rapid, inexpensive, and highly specific. It does not, however, have high sensitivity (40 to 60%) (42), and performs very poorly in paucibacillary specimens, such as those from children or extrapulmonary sites (4). Microscopy also does not test drug susceptibility. The sensitivity of direct microscopy can be improved by treating the specimen with NaOH-NALC or bleach and concentrating it by centrifugation. The use of a fluorescent dye such as auramine-O instead of the Ziehl-Neelson stain offers improved sensitivity, and slides stained with auramine-O are easier to read (43, 44). The replacement of mercury vapor bulbs with light-emitting diodes, which have lower cost and power requirements, improved life span, and similar sensitivity (45), has allowed fluorescent microscopy to penetrate down into peripheral laboratories, where weak infrastructure was previously preventing

FIGURE 2 Examples of peripheral tuberculosis microscopy centers in Uganda (A), India (B, C), and Kenya (D). Point-of-care or near-care tests for TB in most of the 22 high-burden countries will need to be performed in such facilities. Republished with permission from reference 34.

adoption. Two commonly used light-emitting diode microscopes are the Primo Star iLED (Zeiss, Germany) and the CyScope (Partec, Germany).

The performance of microscopy will always be constrained by its suboptimal limit of detection, which is approximately 100-fold more for concentrated fluorescent microscopy than for NAATs such as Xpert MTB/RIF (10,000 CFU/ml versus 131 CFU/ml) (46, 47). Nevertheless, although several new diagnostic tests are in development, approaches to further optimize microscopy's performance are warranted, given its low cost and the existing microscopy infrastructure in HBCs. Furthermore, microscopy has potential utility as a triage test to rule in patients for further investigation with a more expensive test (e.g., test further with a NAAT if microscopy-negative) (48). New technologies to improve the performance of microscopy include the use of magnetic beads that bind to the mycobacterial cell wall and concentrate bacilli. Although this is a rapid and instrument-free approach, the technology has so far failed to result in significant improvements in sensitivity (49, 50). Another approach to improve microscopy is the small membrane filtration method, which uses a vacuum to concentrate bacilli on a filter prior to visualization; however, two studies that used this method have generated conflicting data (51, 52), which warrants further investigation. Automated staining and slide-reading systems, which are aimed at reducing the requirement for skilled personnel and increasing the speed of reading, are also available or under development, such as the RAL Stainer (BioMérieux, Paris, France), and the TBDx system (Signature Mapping Medical Sciences, Leesburg, VA) (53), but data on their performance are limited.

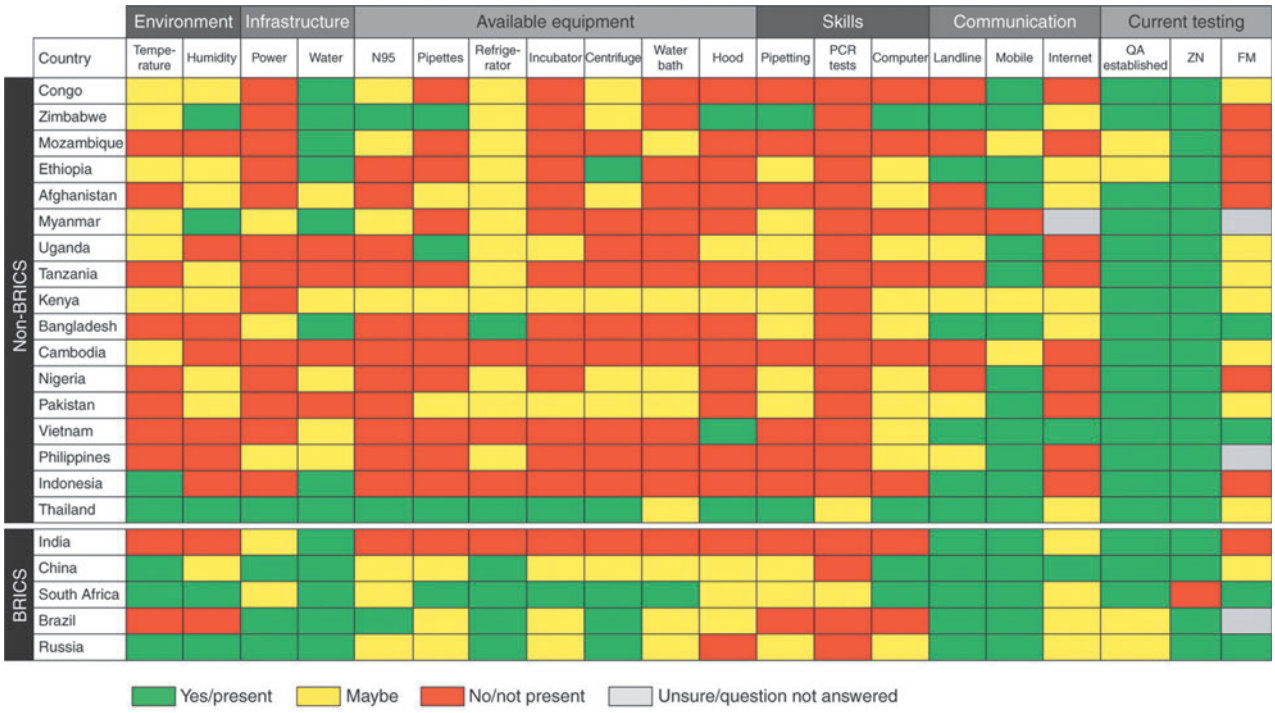


FIGURE 3 Characteristics of peripheral microscopy centers in 22 high-tuberculosis-burden countries. Questions are related to environmental conditions (Is temperature or humidity not a concern?); infrastructure (Is stable power supply, clean water supply present?); presence of equipment (Are N95 respirator, micropipettes, refrigerator, incubator, centrifuge, hot water bath, or biosafety hood present?) and skills (Are staff able to operate a micropipette or computer or perform a PCR test?); and the presence of means of communication (Is landline, mobile network, or Internet present?). Additional questions were asked about whether quality assurance measures were established and which smear methods were currently used. Countries are sorted by increasing purchasing power parity. The BRICS countries are Brazil, Russia, India, China, and South Africa. Republished with permission from reference 169.

Approaches that use low-cost mobile phones to visualize bacilli are also under development (54, 55); however, expensive filters and lenses may still be required (56). These systems require refinement and characterization of their diagnostic accuracy.

NAATS

NAATs are promising tools for the diagnosis of TB. They are rapid, highly sensitive, and due to their ability to multiplex, may simultaneously detect genotypic markers of drug resistance. NAATs for TB detection have traditionally been performed in highly centralized, reference-level laboratories and, mostly due to their lack of automation, have required complex equipment and skilled personnel. This section will focus on the two commercially available NAATs (Xpert MTB/RIF and TB-LAMP) that, of the tools currently available, have the greatest potential to inform rapid clinical decision making in a POC or near-care context.

Xpert MTB/RIF

Xpert MTB/RIF is an automated, real-time NAAT (Fig. 4) that simultaneously detects *Mycobacterium tuberculosis* and mutations that cause resistance to rifampin. Rifampin resistance is strongly but imperfectly associated with resistance to isoniazid (57) and hence MDR-TB. Xpert MTB/RIF comprises an integrated cartridge, microfluidics, DNA extraction technology originally developed for the detection of *Bacillus anthracis* (58), and a series of highly specific molecular beacons (59–61) that span the rifampin resistance-determining region (RRDR) of the M. *tuberculosis* RNA polymerase β subunit (*rpoB*) gene. Since its initial endorsement by the WHO for the diagnosis of pulmonary TB in 2010 (62), Xpert MTB/RIF has been shown to be cost-effective (63, 64) and has undergone scale-up and roll-out at an unprecedented pace: over 3,000 modules were installed, and over 7.5 million cartridges have been procured by the public sector (37, 65). Importantly, the same hardware used by Xpert MTB/RIF can be used to perform cartridge-specific diagnostic tests that detect influenza (66), *Chlamydia trachomatis* and *Neisseria gonorrhoeae* (67), and *Clostridium difficile* (68), among others.

Xpert MTB/RIF Procedure

Xpert MTB/RIF requires a 15-minute sample preparation step, which includes about 2 minutes of hands-on time, and a 2-hour reaction step, which is performed in a GeneXpert real-time PCR instrument (Fig. 4A). Each instrument consists of 1, 4, 16, or 48 modules, which operate independently. Xpert MTB/RIF cartridges, which are available in HBCs for approximately $10 each, are single-use. The cartridges comprise multiple chambers that hold the buffers and lyophilized PCR reagent necessary for the homogenization of sputum, the washing away of debris, DNA extraction, and amplification of the M. *tuberculosis* RRDR. A raw specimen, typically sputum, is processed by adding a 2:1 ratio of the manufacturer-supplied sample buffer (Fig. 4B). This homogenizes the sputum, aids in the release of bacilli from mucus, and after incubation with shaking,

TABLE 1 Abridged optimal and minimal characteristics (target product profiles) of two types of highly desired TB tests[a,b,c]

Characteristic	Target product profile for a rapid, biomarker-based, non-sputum-based TB detection test		Target product profile for a smear replacement test for TB detection	
	Optimal	Minimal	Optimal	Minimal
Scope				
Goal of test	Rapid, biomarker-based test that can diagnose PTB and optimally also EPTB using nonsputum samples (e.g., urine, blood, oral mucosal transudates, saliva, exhaled air) with the purpose of initiating TB treatment within the same clinical encounter (or same day)		Sputum-based PTB for TB detection at the level of a microscopy center with the purpose of supporting initiation of TB therapy within the same clinical encounter (or same day)	
Target population	Countries with medium to high TB prevalence (WHO categories). Target groups are adults and children (including those with HIV) suspected of having active TB—either PTB or EPTB.		Countries with medium to high TB prevalence (WHO categories). Target groups are all patients suspected of having pulmonary TB and able to produce sputum.	
Target user of test*	Health care workers with minimal training	Trained microscopy technicians	Health care worker with minimal training (training ≤ that necessary for performing smear microscopy)	
Setting (health system level)	Health posts without attached laboratories or above	Primary health clinics; peripheral microscopy centers or above	Microscopy center level (primary health centers with attached peripheral laboratories) or above	
Performance characteristics				
Diagnostic sensitivity PTB adults*	≥98% for smear-positive, culture-positive PTB, and ≥68% for smear-negative, culture-positive PTB in adults (i.e., like Xpert MTB/RIF). Sensitivity ≥80% in adults with HIV infection.	≥65% overall but should be >98% among smear-positive, culture-positive PTB (i.e., like smear microscopy). Sensitivity better than smear microscopy in adults with HIV infection.	>95% single test in comparison to culture (smear negative >68%, smear positive 99%)	>80% single test in comparison to cultures (smear negative >60%, smear positive 99%)
Diagnostic specificity*	At least as specific as Xpert MTB/RIF for detection of PTB and EPTB and childhood TB (98% specificity against microbiological reference standard). Able to distinguish between active TB versus latent and past infection.		>98% specificity in comparison to culture	
Operational characteristics				
Manual sample prep (total hands-on steps after obtaining sample)	Integrated sample preparation or not required	Limited number of steps required. No (precise) measuring needed for any step (e.g., volumes or time).	0 or 1 (no precision volume control and precision time steps)	2 steps maximum (no precision volume control and precision time steps)
Time to result*	<20 min, with less hands-on time	<1 h, with less hands-on time	<20 min	<2 h
Instrumentation and power requirement	No instrument	Small, portable or handheld instrument (<1 kg) that can operate on battery or solar in places with interrupted power supply	Battery operated with recharging solution and circuit protector	

(*Continued on next page*)

TABLE 1 Abridged optimal and minimal characteristics (target product profiles) of two types of highly desired TB tests[a,b,c] (*Continued*)

Characteristic	Target product profile for a rapid, biomarker-based, non-sputum-based TB detection test		Target product profile for a smear replacement test for TB detection	
	Optimal	Minimal	Optimal	Minimal
Maintenance/ calibration*	Disposable, no maintenance required	Preventative maintenance at 1 year or >1,000 samples; only simple tools/minimal expertise required; include maintenance alert; remote calibration or no calibration	Preventative maintenance/ calibration at 2 years or 5,000 samples; only simple tools/ minimal expertise required; maintenance alert included; remote or no calibration	Preventative maintenance at 1 years or 1,000 samples; only simple tools/minimal expertise required; maintenance alert included; remote calibration or auto-calibration or no calibration required
Operating temperature/ humidity	Between 5°C and 50°C at 90% humidity	Between 5°C and 40°C at 70% humidity	Between 5°C and 50°C at 90% humidity	Between 5°C and 40°C at 70% humidity
Internal quality control	Internal control (for process and detection)	Internal control (for process)	Full process control including control for sample processing and amplification (for NAAT)	
Pricing				
Pricing of individual test* (reagent and consumable costs only; at scale; ex-works)	< $4	< $6	< $4 just for TB detection	< $6 for TB detection

[a]Asterisks indicate characteristics ranked by stakeholders to be the most important.
[b]Developed by the WHO StopTB Partnership and adapted from reference 36.
[c]Abbreviations: PTB, pulmonary TB; EPTB, extrapulmonary TB.

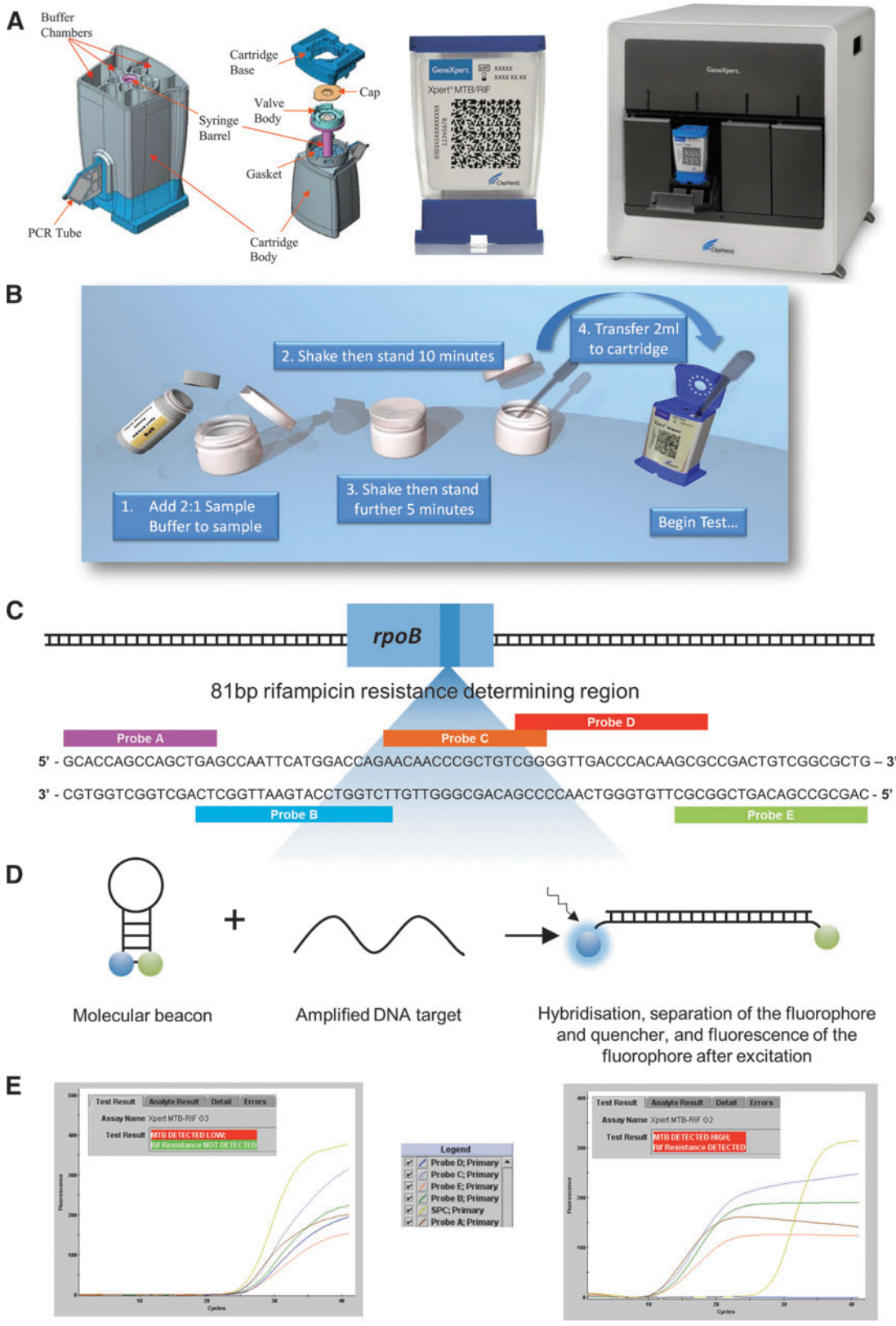
reduces the infectious risk posed by the procedure by causing a 10^{-6}-fold reduction in the viability of the bacilli (61). Next, 2 ml of the sputum-sample buffer mix are transferred to the test cartridge, which is loaded into the machine by hand. This manual portion of the Xpert MTB/RIF procedure generates fewer aerosolized bacilli than smear microscopy (69).

The remaining steps are automated. First, a plunger engages with the syringe barrel within the cartridge to sequentially draw the specimen-sample buffer mix, lysis, wash, and elution buffers into different chambers at the bottom of the cartridge (70, 71). Intact cells, which may be alive or dead, are trapped on a filter in the base of the cartridge and are exposed to a sonicator horn. The cells undergo ultrasonic lysis, which is estimated to be about 70% efficient (70). After the lysis debris is washed away, the freed template DNA passes through the filter membrane and is mixed with the PCR reagents before the start of thermal cycling. Amplification with the real-time monitoring of fluorescence occurs in a transparent chamber attached to the rear of the cartridge.

Xpert MTB/RIF Mechanism of Detection

In addition to lyophilized buffers and reagents, each Xpert MTB/RIF cartridge contains a primer pair for a 192-bp region of the M. *tuberculosis rpoB* gene and five M. *tuberculosis* complex-specific molecular beacons that are complementary to the wild type (i.e., drug susceptible) *rpoB* RRDR (Fig. 4C). A constant quantity of *Bacillus globigii* spores together with a molecular beacon are included in each cartridge. These serve as an internal positive control for both DNA extraction and amplification (46) and allow inhibition to be quantified (4). The PCR is hemi-nested and multiplexed: after initial amplification of the 192-bp *rpoB* region, the molecular beacons bind the 81-bp RRDR and amplification occurs. Each beacon comprises a central

FIGURE 4 The Xpert MTB/RIF system for the detection of TB and resistance to rifampin, which is the first automated nucleic acid amplification platform endorsed by the WHO. (A) Detailed illustration of the Xpert MTB/RIF cartridge body, showing the reagent reservoirs and the PCR amplification chamber, the front-view of an Xpert MTB/RIF cartridge, which is single use, and a GeneXpert four-module machine. (B) Specimen preparation procedure. (C) Five molecular beacons span the 81-bp rifampin resistance-determining region within the *rpoB* gene of *Mycobacterium tuberculosis*. (D) The stem-loop structure within each beacon hybridizes to its complementary region and, after each amplification cycle, the quencher separates from the fluorophore, which after excitation emits light. (E) Examples of two results from the GeneXpert system. The first result is positive for M. *tuberculosis* and, because all beacons successfully bound to their amplicons, is found not to contain any rifampin-resistance-causing mutations, and is hence called rifampin-susceptible. The second example shows a failure of probe B to hybridize and amplify, presumably due to the presence of a mutation. This specimen is therefore detected as M. *tuberculosis*–positive but rifampin-resistant. The bacillary load in the specimen is judged by the software to be "low," "medium," "high," or "very high" based on the cycle threshold values generated by the reaction. The cartridge diagram in (A) is republished with permission from reference 164. Other images in (A) and (B) are republished with permission from Cepheid.


A
Buffer Chambers
Cartridge Base
Cap
Valve Body
Syringe Barrel
Gasket
PCR Tube
Cartridge Body
GeneXpert
Xpert® MTB/RIF
B
2. Shake then stand 10 minutes
4. Transfer 2ml to cartridge
1. Add 2:1 Sample Buffer to sample
3. Shake then stand further 5 minutes
Begin Test...
C
rpoB
81bp rifampicin resistance determining region
Probe A
Probe C
Probe D
5' - GCACCAGCCAGCTGAGCCAATTCATGGACCAGAACAACCCGCTGTCGGGGTTGACCCACAAGCGCCGACTGTCGGCGCTG – 3'
3' - CGTGGTCGGTCGACTCGGTTAAGTACCTGGTCTTGTTGGGCGACAGCCCCAACTGGGTGTTCGCGGCTGACAGCCGCGAC - 5'
Probe B
Probe E
D
Molecular beacon
Amplified DNA target
Hybridisation, separation of the fluorophore and quencher, and fluorescence of the fluorophore after excitation
E
Test Result
Analyte Result
Detail
Errors
Assay Name Xpert MTB-RIF G3
Test Result MTB DETECTED LOW; Rif Resistance NOT DETECTED
Legend
Probe D; Primary
Probe C; Primary
Probe E; Primary
Probe B; Primary
SPC; Primary
Probe A; Primary
Assay Name Xpert MTB-RIF G2
Test Result MTB DETECTED HIGH; Rif Resistance DETECTED
Fluorescence
Cycles

stem-loop structure to which a fluorophore and quencher molecule are covalently attached at each terminus. Following hybridization of the stem-loop, the amplicon, fluorophore, and quencher separate and, after excitation, a fluorescent signal is detected. Importantly, if one or more of the *M. tuberculosis*–specific probes exhibit a delay or failure to bind, defined as a >3.5 difference between the cycle threshold (C_T) values of the earliest and latest probes, the software algorithm assumes this is due to the presence of resistance-causing mutations that have prevented or delayed probe hybridization. This specimen will hence be classified as rifampin resistant. Probe-specific amplification curves are provided by the GeneXpert instrument as part of the standard result readout, two examples of which are shown in Fig. 4D. If the Xpert MTB/RIF internal positive control does not amplify (C_T > 38), the test is classified as having failed and not providing a meaningful result. Importantly, the *M. tuberculosis* molecular beacons are highly specific (they do not bind to closely related nontuberculous mycobacteria) but do bind to DNA from geographically diverse *M. tuberculosis* strains (61, 72), ensuring that Xpert MTB/RIF can be used in different regions.

Diagnostic Accuracy of Xpert MTB/RIF for Pulmonary and Extrapulmonary TB

The accuracy of Xpert MTB/RIF for the detection of *M. tuberculosis* in sputum is well validated: it has sensitivities of 98% and 75% for smear-positive TB and smear-negative TB, respectively, compared to culture as a reference standard (73). However, in settings where paucibacillary TB is common, such as in HIV-infected patients with a low CD4 count, or in patients from low-burden settings who do not have extensive disease, its sensitivity for smear-negative TB can range between 30 and 50% (74–76). In children, its sensitivity for pulmonary TB (~55%) is less than in adults (77), but it is superior to microscopy, which has a sensitivity of ~15% (7). The WHO has endorsed Xpert MTB/RIF for extrapulmonary TB (78), for which its sensitivity varies according to the type of extrapulmonary fluid (4). For example, Xpert MTB/RIF performs comparatively well on lymph node tissues or aspirates (sensitivity of 85% versus culture), gastric lavages and aspirates (84%), and cerebral spinal fluid (82%, provided a concentrated specimen is used) (79, 80) but performs suboptimally on pleural fluid (23%) and pericardial fluid (64%), for which biomarker-based approaches targeting host molecules such as unstimulated interferon-γ will likely provide superior sensitivity (81, 82). Studies on HIV-infected patients have shown that an Xpert MTB/RIF test performed on concentrated urine has a sensitivity of 40 to 50% (83, 84), which underscores the potential utility of urine as a diagnostic specimen. Importantly, many of the patients in studies that used urinary Xpert MTB/RIF were sputum-scarce and thus could not use traditional, sputum-based approaches. Overall, Xpert MTB/RIF had high specificity (~98%) when performed on pulmonary fluids and extrapulmonary fluids (78).

Considerations for Xpert MTB/RIF Implementation in Intermediate- or Well-Resourced Peripheral Laboratories

Xpert MTB/RIF requires a stable electricity supply, security, storage space, and dedicated staff and is recommended for use by the WHO in reference and intermediate-level laboratories (85). While Xpert MTB/RIF does not meet the optimal target product profile for a microscopy-replacement test (36), the far-patient placement of this technology may undermine its potential benefits. For example, programmatic data from South Africa, which uses a highly centralized laboratory system, showed that Xpert MTB/RIF results took approximately twice as long to reach clinicians as microscopy results (6 days versus 3 days), with the longest delays due to laboratory processing times and, alarmingly, the time to transfer the result to the clinician (24). These bottlenecks clearly need to be addressed if centralized testing with next-generation technologies is to have a substantive benefit.

Although Xpert MTB/RIF will never substitute for an accurate, cheap, and instrument-free test, a case can potentially be made for its rational placement in well-resourced primary care clinics in urban centers, including those in prisons or TB hotspots (86). First, the first large-scale evaluation of Xpert MTB/RIF (87) showed that technicians required minimal training to perform the test, that it is not prone to cross-contamination, and that an extensive biosafety infrastructure is not required. Second, the waste generated by the procedure is no more hazardous than that produced by smear microscopy (69). Third, many well-resourced primary care clinics, especially in urban settings such as in Africa and Asia, already have an attached peripheral laboratory in place with some or all of the requirements to perform Xpert MTB/RIF. Indeed, Xpert MTB/RIF comes close to meeting the specifications of a POC test for TB that have been developed by organizations such as Médecins Sans Frontières (88).

The feasibility and impact of Xpert MTB/RIF in primary care were recently examined across five sites in southern Africa (27). Xpert MTB/RIF, when performed in a clinic by a nurse with a single day of training, had an almost identical sensitivity, specificity, and failure rate as the same test performed by an experienced laboratory technician in a well-resourced central laboratory. Notably, most of the failed tests were due to an unstable power supply. The operating temperature only periodically exceeded the recommended maximum (30°C), but this did not affect performance. Indicative of the importance of POC testing, having Xpert MTB/RIF on site almost doubled the number of patients with a positive test result on their first visit compared to microscopy (24% versus 13%), improved the proportion of patients initiating same-day treatment (23% versus 15%), and reduced the proportion of culture-positive patients who did not start treatment (8% versus 15%). Pilot programmatic data suggest that POC Xpert MTB/RIF testing results in fewer clinic visits, quicker results, and more rapid screening of household contacts (89). Importantly, the POC suitability of Xpert MTB/RIF may be improved by the recent advent of the Xpert Omni system, which is a portable, single-module, battery-operated unit.

Several operational considerations need to be factored in when deciding to implement Xpert MTB/RIF in intermediate- or peripheral-level laboratories. Sites must be selected based on their infrastructure, strength of supply chain, ability to communicate results back to clinicians, and whether they have the capacity to initiate on-site treatment. Decentralization will require more instruments (a four-module machine costs approximately $17,500) and greater upkeep and will hence be more costly than centralized approaches (90). It is estimated that to conduct 15 tests a day, POC Xpert MTB/RIF will require two personnel to collect sputum, process specimens, perform the test, and document the results (89); however, whether POC Xpert MTB/RIF is more cost-effective than centralized testing in

terms of the incremental benefit it provides remains to be ascertained, especially because staff for microscopy, who could instead perform POC Xpert MTB/RIF, are already available at primary care clinics in many settings. Xpert MTB/RIF Ultra, which is successor to Xpert MTB/RIF, is currently undergoing evaluations and purportedly will possess a sensitivity similar to a single culture (~10 CFU/ml) when done on sputum (165). Xpert MTB/RIF Ultra achieves improved sensitivity by using a bigger reaction volume than Xpert MTB/RIF (50 µl rather than 25 µl), targeting a multicopy insertion element gene, and optimizing thermal cycling. Importantly, emerging evidence suggests that Xpert MTB/RIF has diminished specificity in retreatment patients due to the presence of old mycobacterial DNA persisting since their previous episode (166; Theron G, et al, *Clin Infect Dis*, in press), and Xpert MTB/RIF Ultra may be more prone to false positivity in these patients.

Manual Loop-Mediated Isothermal Amplification Assay

Tests using LAMP are available for a variety of diseases, including severe acquired respiratory syndrome (91) and *Plasmodium falciparum* infection (92). They have relatively light infrastructure requirements and are designed to be affordable NAAT-based solutions for intermediate-level microscopy laboratories. In analytical studies, the TB-LAMP test has been shown to detect a single-copy of *M. tuberculosis* DNA (93), and has been found to have a limit of detection of 100 CFU/ml (167). Although the test is commercially available, it has been reviewed by the WHO and is not yet endorsed for the diagnosis of TB (94). Unlike Xpert MTB/RIF, the current iteration of the TB-LAMP technology does not detect drug resistance.

TB-LAMP Procedure

TB-LAMP is a simple and rapid manual NAAT system that uses isothermal amplification (i.e., the incubation temperature is constant) and a visual readout (95). Raw sputum (60 µl) is collected in a wide-bore pipette supplied by the manufacturer and is transferred to a heating tube containing extraction solution (94). After mixing by inversion, the tube is incubated for 5 minutes at 90°C in a heating block and allowed to cool down for 2 minutes. The heating tube is then manually attached to an absorbent tube, and the buffer mixed with the lysate. Next, an injection cap is attached to the bottom of the absorbent tube, breaking the seal and allowing between 30 and 35 µl of the solution to be dispensed into reaction tubes that contain the lyophilized PCR reagents. The extraction procedure takes 10 to 20 minutes, is followed by a 40-minute incubation at 67°C, and the reaction products are finally visualized under UV light for 1 minute.

TB-LAMP Mechanism of Detection

TB-LAMP uses *Bst1* DNA polymerase, which has a high strand displacement activity that obviates the need for thermal cycling, plus two pairs of inner and outer primers (Fig. 5B) (95). The inner primers correspond to the sense and antisense sequences of regions flanking the target DNA. Upon binding of the inner primers, the polymerase initiates synthesis of the complimentary strand. The outer primers, which are present in a lower concentration than the inner primers, thereafter hybridize and start to displace the strand synthesized using the inner primers. This displaced strand, which now contains an additional, primer-inserted sequence that is self-complementary to the downstream amplified flanking region, is able to form a stem-loop structure on both ends of the strand, resulting in a dumbbell shape. The inner primers then again hybridize to their complementary regions on each stem-loop structure on the ends of the dumbbell. The free terminus of the inner primers, which is complementary to the regions flanking the amplicon, hybridizes to its complement, and strand synthesis occurs again, resulting in a branching effect.

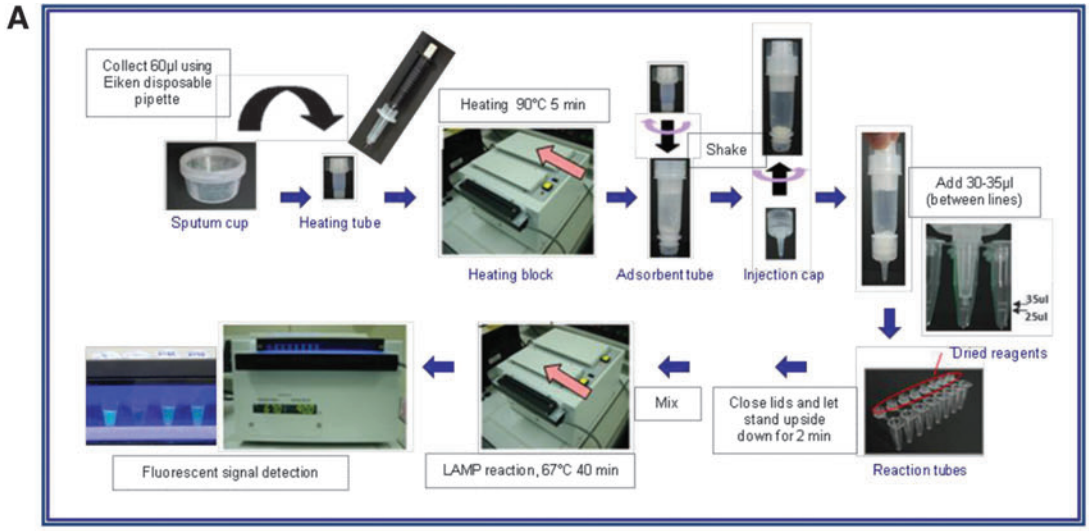
The final product is a mixture of high-molecular-weight DNAs, consisting of stem-loop structures, and cauliflower-like structures that contain multiple stem-loop structures and inverted repeats of the target amplicon. TB-LAMP does not require thermal cycling. A fluorescent agent called calcein is included in the lyophilized reagent and is quenched by manganese, which is included in the reaction (96). Pyrophosphate, which is produced by the TB-LAMP reaction, chelates the manganese and forms a visible manganese pyrophosphate precipitate which, together with the fluorescent calcein, can be visualized by the naked eye (Fig. 5C). As an alternative, SYBR green dye can be used to visualize the double-stranded DNA product (94). The reaction is relatively insensitive to these complexes, so the reaction proceeds until large amounts of amplicon are generated. The technology can achieve amplification in the range of 10^9- to 10^{10}-fold within 15 to 20 minutes.

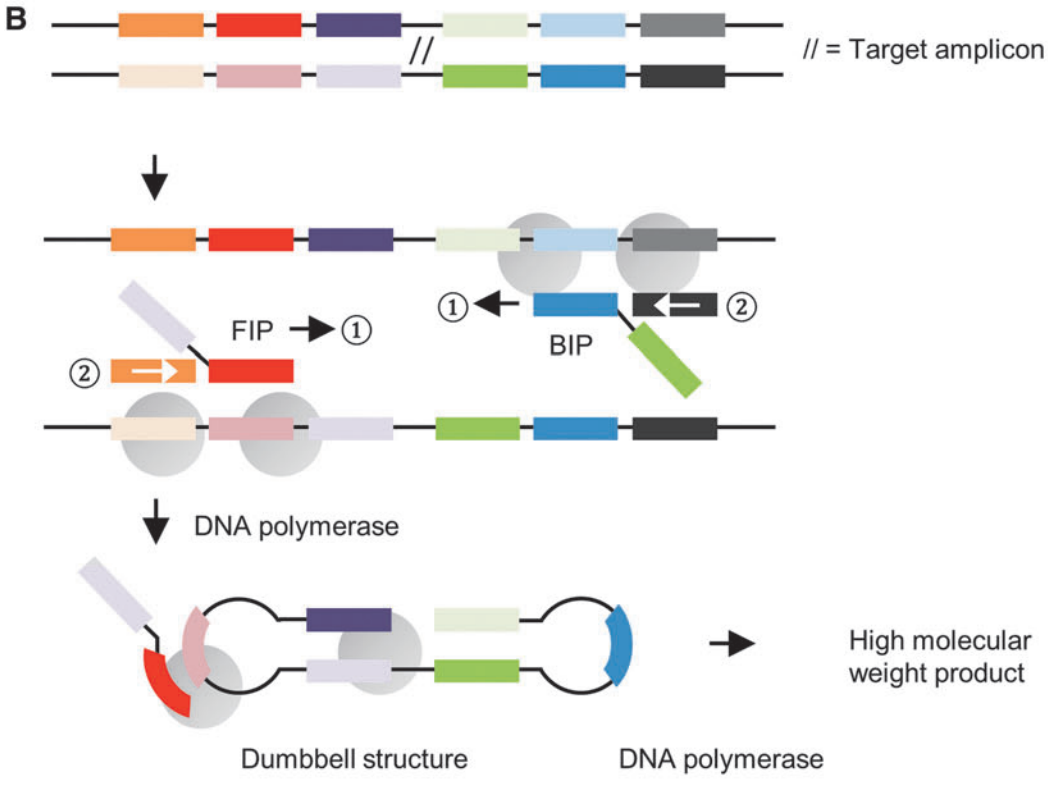
Diagnostic Accuracy of TB-LAMP for Pulmonary TB

Evidence of TB-LAMP's accuracy is growing (96–98), and a recent meta-analysis showed it to have a pooled sensitivity of 80% and a pooled specificity of 96% (99). Validation work conducted by industry and the Foundation for Innovative and New Diagnostics (FIND) has shown TB-LAMP to detect almost all smear-positive cases (98%) and 56% of smear-negative TB cases, with an overall specificity of 94% (94). These studies were conducted in different facilities, including reference-, intermediate-, and peripheral-level laboratories, and demonstrated that TB-LAMP was feasible in resource-poor settings in Africa, Asia, and South America. There are, however, key challenges associated with TB-LAMP. For example, the performance of the test in the FIND-sponsored multisite evaluation varied on a site-by-site basis and appears to be heavily dependent on the skill of the user, which might preclude its use by staff with little technical training. The specificity of the test is also suboptimal: at a TB prevalence of 5%, only 33 to 56% of positive results will be true positive. TB-LAMP hence requires further research to improve its robustness to user variation, improve its specificity, and determine its operational feasibility.

Other Commercially Available NAATs for TB

Other commercially available NAATs for TB have similar diagnostic accuracy to Xpert MTB/RIF or TB-LAMP. These include the Cobas TaqMan, Amplicor, and LightCycler TB detection kits (all made by Roche, San Diego, CA), the Amplified MTB Direct Test (Genprobe, San Diego, CA), the Probe Tec ET system (Becton Dickinson Diagnostic Systems, Sparks, MD) (100–103), and the MTBDR*plus* assay (Hain LifeSciences, Nehren, Germany) (104, 105). Compared to Xpert MTB/RIF and TB-LAMP, these systems require batching, are relatively expensive, rely on specialized laboratory infrastructure, and often require "open" sample processing procedures, which are prone to cross-contamination if not conducted properly.


A
Collect 60μl using Eiken disposable pipette
Sputum cup
Heating tube
Heating 90°C 5 min
Heating block
Adsorbent tube
Shake
Injection cap
Add 30-35μl (between lines)
35ul
25ul
Dried reagents
Reaction tubes
Close lids and let stand upside down for 2 min
Mix
LAMP reaction, 67°C 40 min
Fluorescent signal detection


B
// = Target amplicon
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DNA polymerase
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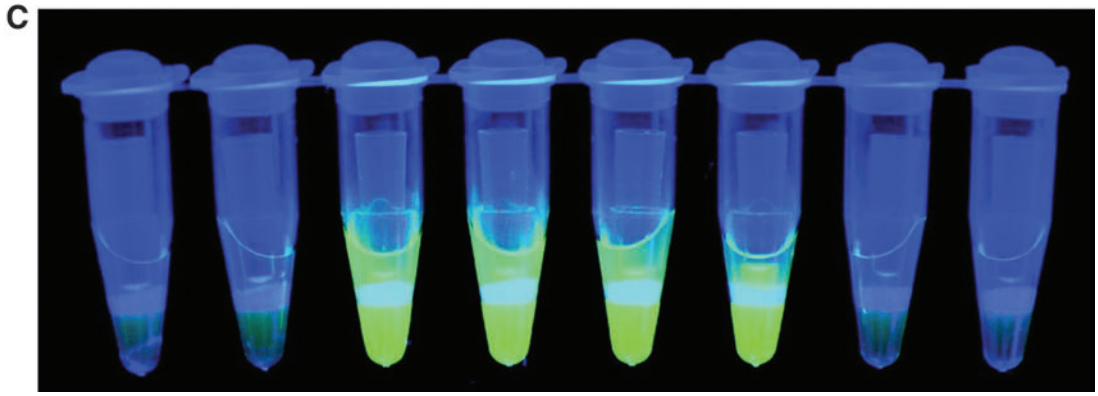
C

These platforms are consequently unlikely to be found in a near-care environment in their present form.

ANTIGEN-DETECTION TESTS

Antigen-based tests hold promise as POC tools for the diagnosis of TB: they do not require a DNA extraction step, are easy to operate and read, and can likely provide results within minutes. Antigen-based tests are routinely used for the diagnosis of HIV and malaria (106). Unlike NAATs that amplify TB DNA, these tests could potentially detect antigen in fluid distant from the site disease. LAM is perhaps the best documented biomarker for active TB presently available in commercial test formats; however, it does not work well in HIV-uninfected patients. Other mycobacterial antigens, such as CFP10, ESAT6, and the Ag85 complex, all have suboptimal sensitivity and specificity when used in an enzyme-linked immunosorbent assay (ELISA) or lateral flow immunochromatographic assay format and therefore should not be used for the diagnosis of active TB (107). Importantly, no matter how good the underlying detection technology is, a biomarker-based test will only ever be as accurate as the presence or absence of the molecule it targets. Several large-scale biomarker discovery efforts are currently in development (108), including those that rely on the detection of multiple transcriptional or serological signatures (77, 109). From a technical viewpoint, these biomarker signatures will be detection technologies capable of a high level of multiplexing, some of which are detailed later.

Urine LAM ELISA and Lateral Flow Test

LAM is a group of 17.3-kDa lipopolysaccharide glycolipids that form part of the *M. tuberculosis* cell wall. LAM, either in its free form or as part of intact bacilli, is detectable in the urine of HIV-infected patients with advanced immunosuppression. An ELISA that uses a monoclonal anti-LAM antibody (Alere) was the first commercially available test that used this antigen. In HIV-infected patients the test has poor overall sensitivity (21%) (110), which is higher in patients with a CD4 count <50 cells/ml (67 to 85%), and high specificity (99%) (111). A key disadvantage of the LAM ELISA is a sample preparation procedure that precludes its use in peripheral laboratories: the urine must be heated to 95 to 100°C for 30 minutes followed by centrifugation at 10,000 rpm for 15 minutes at room temperature (112). The supernatant is then tested in a 96-well-plate format ELISA, which is not suited to low-volume settings.

More recently, a lateral flow immunochromatographic test (or dipstick) for the detection of urinary LAM has been developed, partially validated (3, 113–116, 168), and made commercially available. The Determine TB LAM Ag test does not require any sample processing steps and may be performed and read by nontechnical personnel (Fig. 6). An aliquot of urine (60 µl) is applied to the sample pad and moved through the nitrocellulose matrix via capillary action (112). After colloidal gold-conjugated anti-LAM antibodies attach to the antigen, the immunological complex passes into the conjugate pad, where it is captured by immobilized anti-LAM antibodies, and the presence of the colloidal gold label is visualized chromatographically. A positive result (a visible purple line) indicates that LAM antigen is present in the sample, whereas a negative result (no visible purple line) indicates it is not present or is below the limit of detection. A control bar is incorporated in the assay device. The test strip is read after 25 minutes of incubation at room temperature.

Although the absolute sensitivity of the LAM dipstick is dependent on the patient population (117), it has similar sensitivity to the ELISA version in head-to-head comparisons (3). Early research on the dipstick (3, 118) indicated that use of the manufacturer cut-point (grade 1) resulted in suboptimal specificity (~90%) and, due to subjective user-dependent visual interpretation of the faintest band, decreased inter-reader variability. Consequently, the manufacturer has revised its recommended cut-point for the classification of LAM to grade 2 because, although this will reduce the sensitivity of the test, the specificity and inter-reader agreement will be improved. The visual cut-point issue is noteworthy in the context of test development, where both the clinical significance and the user's interpretation of faint chromatographic readouts can be challenging. This is especially complicated for TB, because there is a spectrum of disease which includes subclinical forms (33), and TB biomarkers such as LAM may share similarity with molecules of commensal or environmental nonpathogenic mycobacteria and fungi (110). Test developers interested in developing antigen-based tests for TB may therefore wish to consider using an electrochemical-based approach to generate an objective, digital readout of the result instead of a visual signal. Importantly, POC testing using the urine LAM dipstick test has recently been found to reduce mortality in hospitalized HIV-positive patients with symptoms of TB in southern Africa (Peter J, et al, *Lancet*, in press).

FUTURE POC OR NEAR-CARE TESTS

Research has illustrated the global TB diagnostics market to be potentially lucrative (119, 120). For example, the 22 HBCs are estimated to perform 61.7 million smears, at an

FIGURE 5 The TB-LAMP system for the detection of TB. (A) TB-LAMP test overview. Bacilli are first lysed using temperature and an extraction buffer before the lysate is mixed with the buffer in the absorbent tube and injected into reaction tubes, which contain the PCR reagents. The mixture is then incubated and the amplified product visualized by fluorescence under UV light. (B) TB-LAMP amplifies DNA using a novel strand-displacement polymerase and specially designed primers that contain oligonucleotides that hybridize to both the sense and antisense strands of the regions flanking the target sequence. The forward and back inner primers (FIP and BIP) first amplify the target sequencing and add a 5′ region that is complementary to the sequence downstream of the primer hybridization site. Once these strands are displaced by the DNA polymerase, they form stem-loop structures, which the FIP and BIP can hybridize to and, after elongation, serve as a template for further amplification. (C) The amplified DNA, which has a high molecular weight due to its complex secondary structure, is visualized by the titration of manganese by pyrophosphate, which is produced during the reaction, which allows the calcein marker within the reaction tubes to fluoresce. In this example, the middle four tubes are positive. (A) is republished with permission from reference 94, (B) is republished with permission from references 71 and 95, and (C) is republished with permission from reference 96.

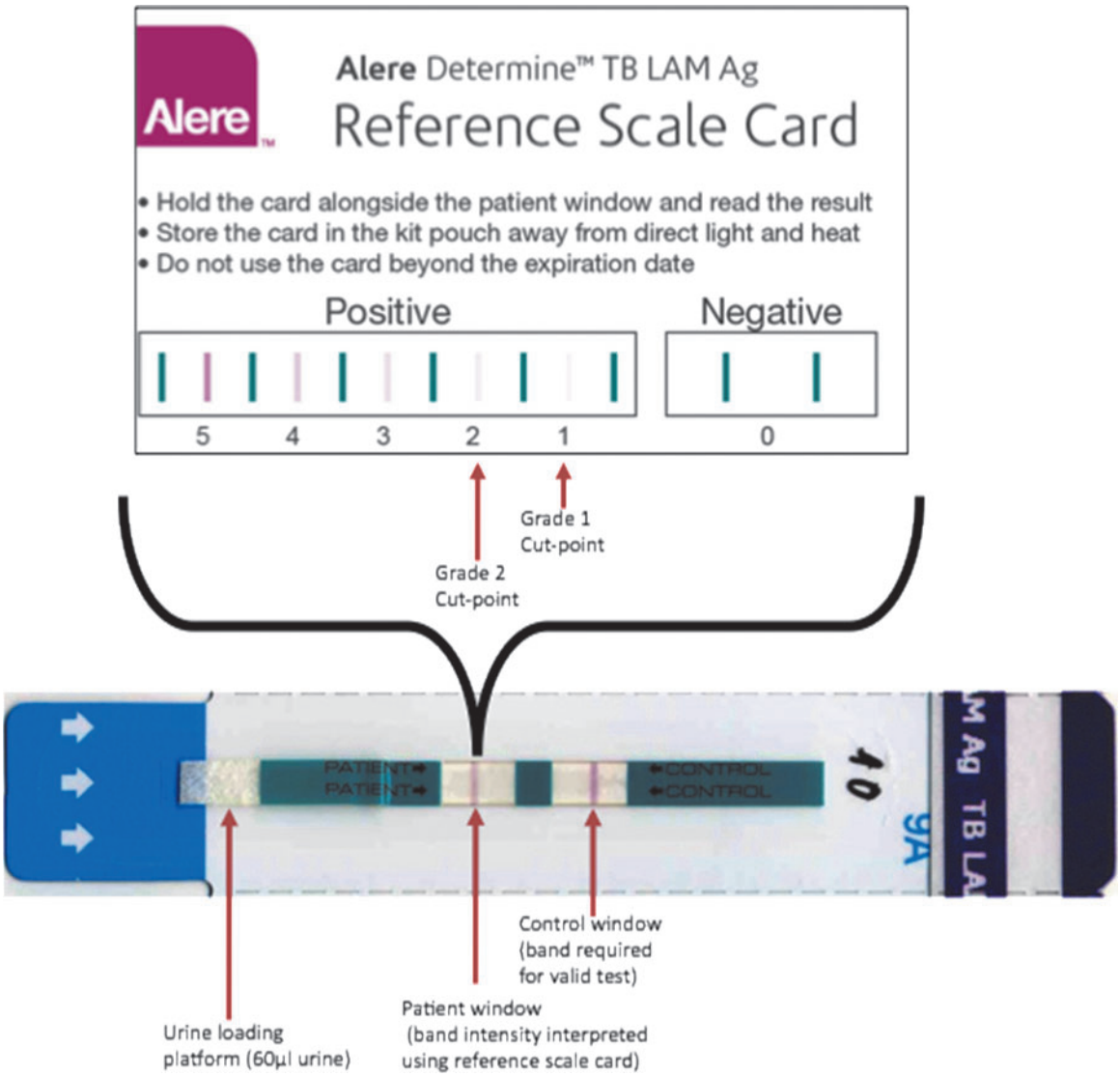

FIGURE 6 Urine lipoarabinomannan (LAM) strip test and reference scale card. The reference scale card, provided with each 100-strip packet, illustrates six cut-off points (visual grades 0 to 5) categorized by different band intensities appearing in the patient window. To optimize the specificity of the test, it is recommended that the grade 2 cut-point is used (118). Republished with permission from reference 3.

expenditure of $109 million. Assuming that a replacement test for microscopy would be performed once on a single specimen, this translates into a potential replacement volume of 30.8 million units or, assuming a test price of $5, $154 million per annum (119). Commercial interest in the TB diagnostic pipeline (Fig. 1, above) has also been stimulated by several initiatives by organizations such as FIND and the Bill and Melinda Gates Foundation, and there are now more than 50 companies involved in the development of new diagnostic tests (41). This section will briefly describe some of the molecular NAAT-, antigen-, and volatile organic compound (VOC)–based technologies in the pipeline (Table 2). Other diagnostic technologies, such as portable chest X-ray machines and those that improve specimen collection, are reviewed elsewhere (37).

NAATs

NAATs are the most common type of test in the TB diagnostic pipeline. In addition to their sensitivity, NAATs have the added advantage of being able to detect mutations associated with drug resistance. This section will briefly describe key considerations for designing new NAAT-based devices for TB detection and will give an overview of several NAATs in the pipeline that have potential for deployment at intermediate- or peripheral-level laboratories (Table 2). The NAATs described use several novel amplification technologies (PCR, TMA, LAMP, NASBA, SDA), which have been reviewed in detail (71, 121).

NAAT Design Considerations

Sample Processing and DNA Extraction

Sputum is the specimen most commonly used for TB diagnostic testing. However, sputum represents a challenge for the extraction of DNA because it is infectious, often thick, can contain a broad, variable concentration of TB bacilli, and the quality and quantity, which are highly patient-dependent, can vary. Furthermore, the thickest, most viscous component of sputum, which is the most difficult to

TABLE 2 Key features of selected NAATs currently in development or undergoing evaluation that are suitable to use in intermediate- or peripheral-level laboratories[a]

Manufacturer	Test name	Extraction, amplification and detection technology and estimated level of training required	Integrated	Dedicated instrument	Multiplexed	Throughput	Drug resistance detection	Lowest intended use setting	Market release
Cepheid	Xpert MTB/RIF	Manual treatment of sputum with buffer, automated capture of bacilli on a filter, lysis, washing, and amplification then occurs via fluorescent molecular beacons and RT-PCR. Minimal training required.	Yes	Yes	Yes	2 h per module	RIF	Intermediate	2009
Eiken	TB-LAMP	Heat lysis of mycobacteria. Manual clean-up of the extraction and dispensing into reaction tubes. Amplification occurs via LAMP, and reaction products are visualized by the naked eye using fluorescence and UV light. Moderate training required.	No	Yes	No	42 tests per day	No	Peripheral	2011
Epistem	GenedriveMycobacterium iD Test-kit	Paper-based DNA extraction method that uses a small volume of specimen. Amplification occurs via real-time PCR, and the product is visualized fluorescently. Expected to require a low to moderate level of training.	No	No*	No	1.5 h per test device	RIF	Peripheral	2013
Insilixa Inc.	HYDRA	PCR, microarray	No	Yes	Yes	8 tests per device per day	RIF, INH, PZA, EMB, FLQ, AMG	Peripheral	2015
Molbio	Truelab TB Assay	Uses magnetic beads to perform solid phase extraction of DNA via a semi-automated process, which is then amplified via real time PCR. Expected to require a low to moderate level of training.	No	Yes	No	12 tests per test device in 8 h	No	Peripheral	2013
Ustar	EasyNAT TB	Manual heat lysis of mycobacteria followed by solid phase extraction of the DNA, which is then amplified using CPA and detected using a lateral flow strip test within a cartridge.	No	No	No	30 tests per day	No	Peripheral	2013
Wave80	EOSCAPE-TB System	NASBA, fluorescence	No	Yes	No	50 tests per day	No	Peripheral	2016

[a]Xpert MTB/RIF, which is currently available, is included as a reference. Adapted from the UNITAID Annual Diagnostic Landscape for 2015 (37), which contains an exhaustive list of products applicable to all laboratory settings, and their regulatory status. Abbreviations: CPA, cross-priming amplification; NASBA, nucleic acid sequence-based amplification; RIF, rifampin; INH, isoniazid; PZA, pyrazinamide; EMB, ethambutol; FLQ, fluoroquinolone; AMG, aminoglycoside.

extract DNA from, contains approximately 32-fold the concentration of bacilli than the watery components (72). Also, mycobacteria have thick and waxy cell walls, which makes them difficult to lyse. These factors can limit the release of template DNA. POC or near-care NAAT platforms should therefore have automated and integrated systems for homogenization, lysis, and DNA extraction.

Sample processing is a bottleneck for POC and near-care NAATs, because most tests require separate sample preparation steps with the manual addition of reagents before transfer to the amplification and detection platform (122). Xpert MTB/RIF and TB-LAMP use chemicals such as NALC-NaOH and isopropanol to homogenize the specimen. To lyse M. *tuberculosis* bacilli, cells can be treated with a combination of heat, enzymes such as lysozyme and proteinase K, chemicals such as phenol, or mechanical action, such as sonication or bead-beating. There are few head-to-head comparisons of the different methods of mycobacterial DNA extraction from sputum, but the use of small (0.2 mm) glass or zirconia (0.1 mm) beads appears to be the most efficient (123, 124). Bead-based methods of lysis are undergoing incorporation into prototype cartridge- and microfluidic-based NAAT devices (125–127), which use solid-phase extraction to purify the DNA. This approach involves capturing nucleic acids onto a solid support, followed by washing and elution (71, 128). Wash-free methods have recently been demonstrated (129). Avoiding or removing inhibitors of DNA amplification and detection, which may be added during sample processing and DNA extraction, is another key consideration. Recently, PCR assays that are less prone to inhibition have been demonstrated (130, 131), and it appears that the isothermal amplification technologies are less affected by inhibitory compounds (132). Importantly, an added advantage of these closed, self-contained cartridge- and microfluidic-based systems is that the chance of cross-contamination is minimized, and the requirement for amplicon-free areas for testing, which are challenging to implement and maintain in intermediate- and peripheral-level laboratories, is diminished (37).

Drug Susceptibility Testing

All patients diagnosed with TB should receive DST (1). Ideally, this should be integrated within the TB detection test, such as in the case of Xpert MTB/RIF, but it can also be performed as a reflex (follow-up) test. DST should be done for both rifampin and isoniazid. Although resistance to rifampin is strongly associated with MDR-TB, most countries that have implemented Xpert MTB/RIF require a DST for isoniazid too, although the patient may be started on treatment for MDR-TB in the interim. This is because patients can harbor rifampin-susceptible, isoniazid-resistant strains (133, 134). There are also several new drug regimens for new first-line therapies undergoing evaluation (135). Their introduction, which is expected to start within the next 2 to 3 years, will mean new technologies are needed to rapidly detect drug-resistance to these new drugs, which include pyrazinamide and fluoroquinolones such as moxifloxacin (37).

NAATs Under Evaluation or in Development

NAATs are the most common types of tests in the TB diagnostics pipeline. There are several in evaluation or development that are targeted at intermediate- or peripheral-level labs or both. Pictures of some of these devices are shown in Fig. 7 and Fig. 8, and are described below. The UNITAID TB Diagnostics Landscape Technology Report offers an exhaustive summary of future NAAT tests (37).

Genedrive

Genedrive (Epistem, Manchester, United Kingdom) can extract DNA from bacilli in sputum or urine for the real-time PCR-based detection of TB and rifampin resistance. The bench-top instrument uses a novel method of simultaneous sample processing and cell lysis that uses chemically treated paper containing an organic moiety (136). Paper discs (1 mm) soaked in cellular lysate are punched from the lysis tool and are placed in each of the three reaction wells in the cartridge, which contains lyophilized reagents for PCR amplification, before the cartridge is inserted into the Genedrive instrument for analysis. A study using Genedrive found it to have a similar limit of detection as Xpert MTB/RIF when performed on spiked sputum specimens and high sensitivity (91%) and specificity (100%) (136). The system is modular and is targeted for use at a peripheral level yet requires further validation.

EasyNAT TB

The EasyNAT TB test (Ustar Biotechnologies, Hangzhou, China) uses cross-priming isothermal amplification in combination with a lateral flow cassette to detect the amplified product and minimize cross-contamination (137). It is not automated or integrated, and the user has to provide a heat block, vortexer, centrifuge, pipette, and tips, but the manufacturer is also developing a syringe-driven extraction device akin to that used by TB-LAMP (138). A cold chain for the storage of test reagents is also required. The test is targeted at microscopy centers and also requires the XCP Nucleic Acid Device (Ustar) for the detection of amplified DNA. It has undergone a large multicenter evaluation of its diagnostic accuracy in China, where it was found to have an overall sensitivity and specificity of 84% and 98%, respectively, with a sensitivity for smear-positive TB of 60% (139). Given its current requirements for manual sample processing and lack of integration, it is unlikely to be usable in its current format at peripheral-level laboratories.

Truelab Real Time Micro PCR System

The Molbio Diagnostics group (India) has developed the Truelab Real Time Micro PCR System, which is targeted as a low-cost, semi-automated, and quantitative NAAT platform for peripheral health care settings (138). Sputum is expectorated directly into a collection cup, which contains lyophilized reagents to liquefy the sample and kill bacteria. A 1-ml sample is transferred to the Trueprep-MAG system, which performs paramagnetic bead-based DNA extraction. Extracted DNA is manually transferred into a cartridge containing lyophilized PCR reagents, which is inserted into the Truelab Uno instrument for real-time PCR detection system, which contains a rechargeable battery that can run the system for up to 8 hours and is attached to an Android phone. Unlike the other technologies, the Truelab cartridge itself performs the thermocycling, rather than the instrument into which it is inserted. Two studies have been conducted using the Truelab device (140, 141). The sensitivity for smear-positive TB was 99%, whereas the sensitivity for smear-negative TB was 76% with overall an excellent specificity of 100%

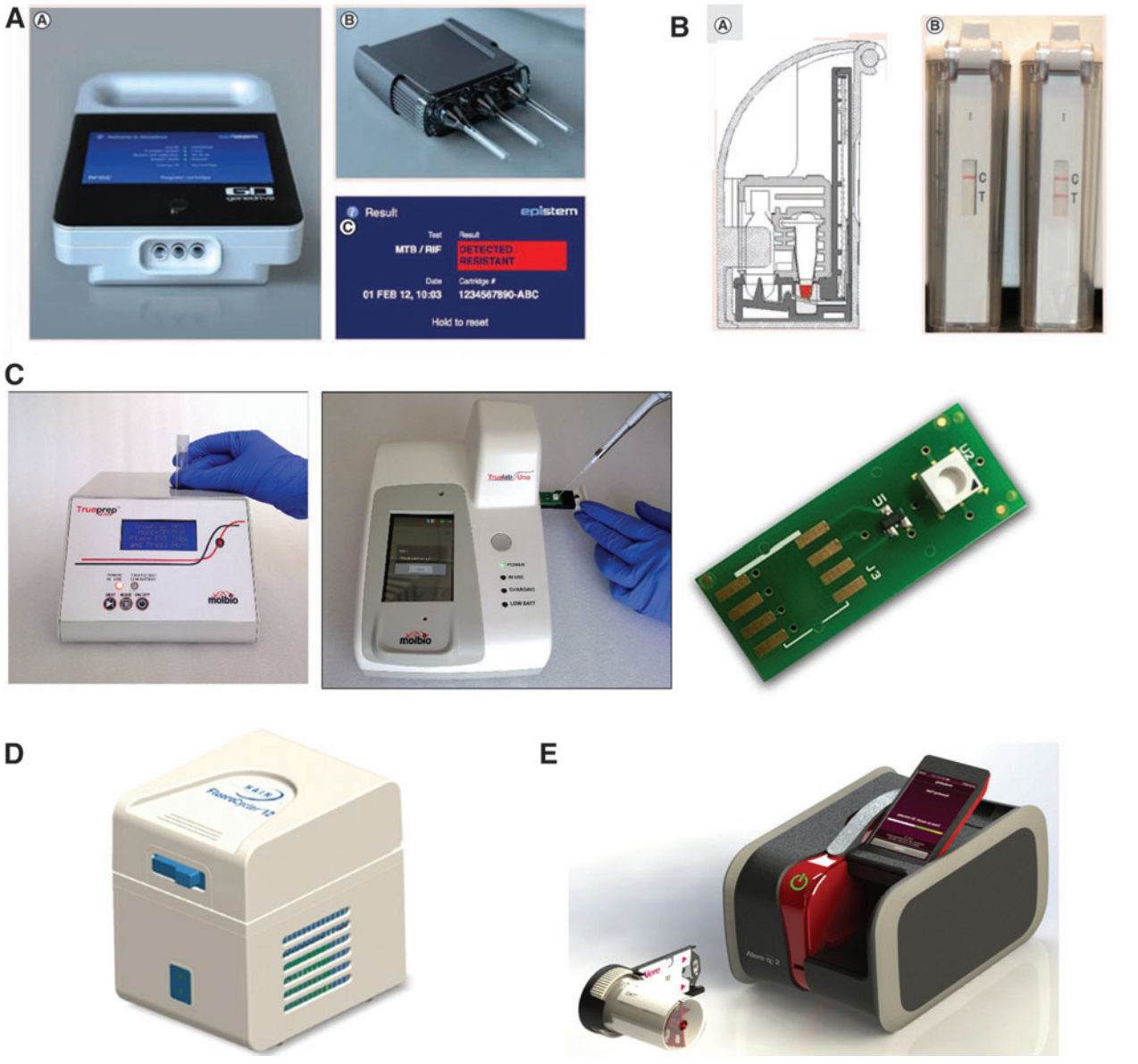

FIGURE 7 Selected NAAT platforms currently under evaluation or in development. (A) The Epistem Genedrive system for the detection of *Mycobacterium tuberculosis*, into which the cartridge with three ports that serve as reaction tubes are inserted. The results screen is also shown. (B) A schematic of the XCP Nucleic Acid Device (Ustar Biotechnologies), which is used in conjunction with the EasyNAT TB test (Ustar Biotechnologies) cartridge. The cartridge contains a plastic bulb with both the reaction mix and a lateral flow running buffer. This is inserted into the detection chamber holding the lateral flow test strip. After 5 to 10 minutes the result is read. Examples of negative and positive test results with control bands are shown. (C) The equipment made by the Molbio Group (India) required to process specimens and extract DNA (Trueprep) and monitor amplification (Truelab UNO real-time PCR analyzer) and the chip used for the detection of DNA. (D) The Fluorocycler (Hain Lifesciences) which is used for the semi-automated detection of TB using the Fluorotype MTB test. (E) The Alere Q system and cartridge, which are currently being developed for the detection of TB. It performs on-board sample processing, lysis, DNA extraction, and TB detection. Sputum is collected in a special container that is attached to the test cartridge, which is then inserted into the machine. A and B are republished with permission from reference 138. C, D, and E are republished with permission from reference 37.

(140). The manufacturer is developing a follow-up cartridge for downstream DST.

Fluorotype MTB

The Fluorotype MTB test (Hain Lifesciences) is a semi-automated platform for TB detection targeted at intermediate-level laboratories (37, 142). It uses the Fluorocycler instrument and allows amplification and detection in a closed system using the manufacturer's proprietary HyBeacon probe technology and melt curve analyses. DNA extraction still needs to be done manually, or it can be done using a separate instrument, and then the PCR mixes are manually

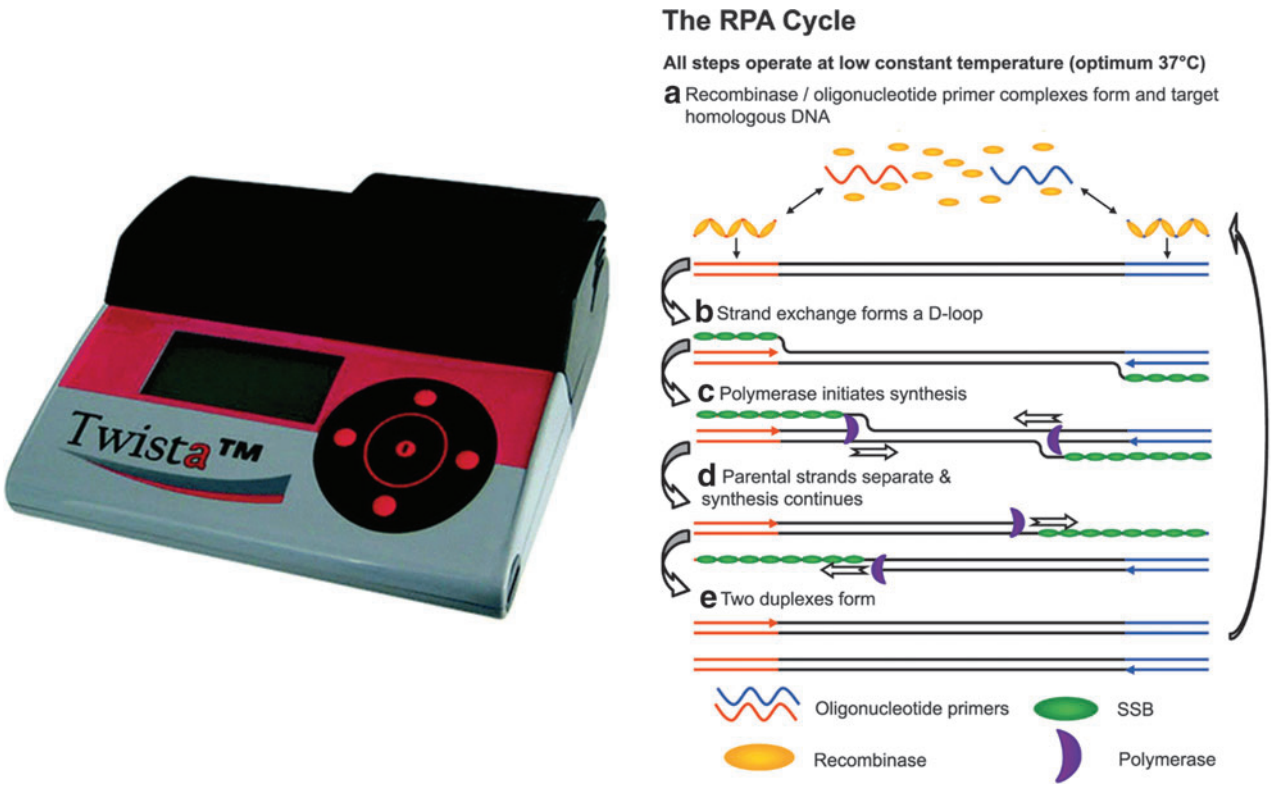

FIGURE 8 The Twista diagnostic platform. The left panel shows the battery-powered portable fluorometer for monitoring the progress of the recombinase polymerase amplification reaction. The right panel shows the mechanisms of amplification, in which three core proteins (a recombinase, single-strand DNA binding protein [SSB], and strand-displacing polymerase) isothermally amplify DNA (143). The right-hand panel was created by TwistDx Ltd (http://www.twistdx.co.uk/our_technology/) and is licensed under a Creative Commons Attribution 3.0 United States License.

prepared, and this likely precludes its use at a peripheral level in resource-poor settings. The entire procedure takes approximately 4 hours and, in the first study that used this device, it had sensitivities of 100% and 56% for smear-positive and smear-negative specimens, respectively, with an overall specificity of 99% (142). A separate version of the test that will use the same equipment for the detection of multiple drug resistance mutations in a single reaction using "lights-on-lights-off" probes is under development (37).

Alere Q

Alere is developing a standalone, battery-powered diagnostic system (Alere Q) that is targeted at microscopy centers. It will use the entire sputum volume available and, if the test is positive for TB, perform a follow-up DST using a separate cartridge. After the collection of sputum into a custom vessel, the container is attached to the cartridge and the cartridge is inserted into the Alere Q instrument. The device then performs on-board sample processing, lysis, DNA extraction, purification, amplification, and detection. It is one of the few completely integrated solutions in the TB diagnostics pipeline and hence holds considerable promise as a POC test. Amplification is performed using an isothermal nicking enzyme amplification reaction technology. The manufacturer is targeting a total time to result of 20 minutes. The device is expected to enter field trials in late 2016.

TwistDx Recombinase Polymerase Amplification Assay

TwistDx (Cambridge, United Kingdom) has, in collaboration with the Program for Appropriate Technology in Health (PATH), developed a system that uses low-temperature isothermal recombinase polymerase amplification (RPA) to detect the IS*1081* and IS*6110* *M. tuberculosis*–specific targets in less than 20 minutes (Fig. 8) (143). The reaction requires the battery-powered Twista RT-PCR reader, which can simultaneously measure eight reactions (144). RPA is novel among the isothermal amplification technologies because it does not require a precise or uniform temperature and can operate at a range of 25 to 42°C (144). The technology is already integrated into commercially available kits for *Salmonella* detection and has been used for the detection of HIV and Rift Valley Fever (145, 146). The molecular mechanism of DNA amplification is novel: a recombinase enzyme first binds to oligonucleotide probes. This forms a complex with recombination filaments that recombines with homologous DNA flanking the target site. A second enzyme binds to the strand of DNA displaced by the probes and forms D-loop structures, thereby preventing the dissociation of the primers. A third enzyme, which is a DNA polymerase with strand displacement activity, synthesizes the sense strand and "unwinds" the DNA double helix. Specific nucleases, which only recognize the bound probes, are then added. Cleavage of the

probes results in fluorescence, which is detected using the Twista RT-PCR reader (144).

Manual NALC-NaOH sputum decontamination and DNA extraction using beads and heat lysis are required in the current iteration of this technology (143). In the first study of the technology's accuracy for the detection of TB in clinical specimens, the IS*1081*-targeted RPA assay had sensitivity and specificity of 91% and 100%, respectively, but further validation, especially in smear-negative specimens, is required. The manufacturer is targeting the device at peripheral-level laboratories, but this will first require the platform to be fully integrated and perform on-board sample preparation and DNA extraction.

Antigen Diagnostic Tests

Although they are unlikely to be commercially available before the previously described NAAT technologies, several promising antigen-based tests are in development. Many of these are multiplexed and target multiple antigens, because most TB antigens discovered to date offer suboptimal sensitivity and specificity when measured individually (147). For example, FIND, in collaboration with MBio Diagnostics (United States), has developed a test based on *M. tuberculosis* proteins that elicit a distinct antibiotic signature in the serum of patients with TB (37, 148). This signature, which relies on 57 *M. tuberculosis* antigens, is detectable using multiplexed ELISA with fluidic channels integrated within a single disposable cartridge. The time to result is less than an hour. The test can use serum, whole blood, or plasma and does not require any off-board sample processing. TB Biosciences (United States) is also developing a test for an *M. tuberculosis*–specific antigen immune signature that relies on three proteins and their reactivity with synthetic peptides (149). These synthetic peptides interact with each target protein in the serum, and the result can be visually scored using an immunochromatographic strip test. Both these tests are currently undergoing clinical evaluation.

A portable TB breathalyzer device (Rapid Biosensor Systems, Cambridge, United Kingdom) which detects TB antigens in coughs has been described (150, 151). The patients' cough aerosols are captured in a collection tube and subsequently distributed onto fluorescent dye–bound peptides that bind *M. tuberculosis* Ag85 antigen. Fluorescence is monitored by inserting the collection tube into a battery-powered reader, and the whole test can be performed in under 10 minutes. An initial validation study found the test to have sensitivity of 64% and a specificity of 80% (150).

Recently, the development of a sputum-based test that detects the activity of the beta lactamase (*bla*C) enzyme, which is produced by *M. tuberculosis*, was described (152, 153). This approach uses an engineered fluorogenic molecule that resembles beta lactamase but cannot be cleaved by the *bla*C enzymes of microbes other than *M. tuberculosis*. A new reporter molecule, resembling cephalosporin, was subsequently developed, and the refined assay can detect 10 CFU/ml of *M. tuberculosis* in sputum (152). A preliminary validation study found the test to have 90% sensitivity but a suboptimal specificity of 73%, with results being obtainable within 1 hour (152). The device will use a portable, battery-powered reader. It is currently undergoing further development, with clinical trials expected to start in 2016.

VOC Tests

M. tuberculosis produces various chemicals as part of its metabolism that can be used as diagnostic markers of infection. These include VOCs arising from oxidative stress, such as alkanes, and metabolic products, such as derivatives of cyclohexane and benzene (154). These chemical signatures are detectable in patients' breath. Gas chromatography, mass spectrometry, or novel sensor-based approaches, which are more amenable to miniaturization, can be used as the basis of detection. The sampling of the breath is particularly attractive as a diagnostic medium, because it is minimally invasive, does not require sample preparation, poses little risk of cross-contamination, and works for patients who may otherwise be unable to provide a diagnostic specimen (37). There are nevertheless challenges for the breath-based sampling of VOCs, such as the low concentration of the metabolites and the specificity of the compounds produced (86).

Studies using giant African pouched cane rats (*Cricetomys gambianus*) which have been trained to smell VOCs associated with TB in sputum show sensitivity comparable to smear microscopy (155–157). There are also several companies working on field-friendly breathed-based detection devices of *M. tuberculosis*–specific VOCs, each of which uses a different rapid detection technology (37). To date, these have demonstrated suboptimal diagnostic accuracy. For example, the Breathscanner (Menssana Research, Newark, NJ), which uses gas chromatography in conjunction with flame ionization and surface acoustic wave detection, has a sensitivity and specificity of 71% and 72%, respectively (37, 154). The Aenose (eNose, The Netherlands) has a sensitivity and specificity of 77% and 87%, respectively (158). This device, which is handheld and portable, uses an initial "flush-out" period in which environmental contaminants can be expelled from the lungs. These devices are currently undergoing multicenter clinical trials.

WHERE DO TECHNOLOGIES FOR THE DIAGNOSIS OF TB FALL SHORT?

As of 2016, there is still no commercially available accurate, rapid, and inexpensive POC test for TB. Xpert MTB/RIF represents a major advance in the diagnosis of TB, and its automated sample processing, DNA extraction, and amplification technology are currently unmatched by any other products on the market and many NAATs in development, which still require manual sample processing and DNA extraction. Xpert MTB/RIF is, however, expensive, requires an instrument, and requires infrastructure such as a stable electricity supply, which is not available in poorly resourced peripheral-level laboratories in HBCs (the need for this infrastructure may be reduced somewhat by the Xpert Omni system). The urine-based Determine TB LAM Ag test is another promising technology, but it is only useful for patients living with HIV who have a low CD4 count. Ironically, these patients are most frequently found in hospital settings, which usually have comparatively well-resourced diagnostic laboratories located on-site. Pediatric TB and extrapulmonary TB also continue to be neglected, with both Xpert MTB/RIF and the Determine TB LAM Ag test performing suboptimally for these conditions and there being no tests in the TB diagnostics pipeline targeted specifically at these patients nor a well-defined reference standard for assessing their performance.

A key bottleneck in the development of non-NAAT-based tests for TB is a lack of suitable biomarkers (108), which is why few antigen-based tests for TB are close to commercialization. Donors such as the National Institute of Allergy and Infectious Diseases and the European and

Developing Countries Clinical Trials Partnership have funded the creation of biobanks consisting of fluids such as sputum, serum, and urine which are collected from clinically well-characterized patients. These have already begun to pay dividends, with a variety of promising RNA (40, 77, 159, 160) and proteomic (38, 149, 161, 162) signatures being identified. There remains a critical shortage of biomarkers for evaluating treatment response and predicting TB treatment failure. Importantly, the most commonly used biomarker of sputum smear conversion at 2 months has poor sensitivity (57%) and suboptimal specificity (81%) for treatment failure (163).

All of the technologies in the TB diagnostic pipeline will require extensive independent evaluation to gain widespread adoption; the few small studies that are available are inadequate for informing policy. Test developers therefore need to budget and plan for field evaluations as part of the test development process, and schemes need to be in place to assist small companies who are seeking regulatory approval.

CONCLUSIONS

Research on new diagnostics for TB has traditionally been underfunded (106), but concerted efforts by donors such as the WHO and FIND have stimulated the TB diagnostics pipeline and helped develop the frameworks necessary to shepherd a test from conception through to prototyping, clinical evaluation, approval, and hopefully, market-wide uptake. New tests such as Xpert MTB/RIF and the Determine TB LAM Ag test are already showing promise and helping to transform the diagnosis of TB. The benefit of both these tests and future technologies in the TB diagnostic pipeline will only be enhanced as they are deployed in an increasingly decentralized manner. Furthermore, the next 2 to 3 years should see a rapid, sputum-based smear replacement test designed specifically for peripheral-level laboratories and, within the next 5 years, ideally the advent of a rapid, biomarker-based, non-sputum-based test. These tests should possess the ability to conduct simultaneous or follow-up DST, which is essential for limiting the emergence of MDR-TB.

Considerable obstacles remain in the design and deployment of new tests for TB. Given the diverse manifestations of the disease and the broad spectrum of patients it infects, it is unlikely that a single test will optimally perform in all settings. Automated and integrated sample processing and DNA extraction remain a crucial challenge of POC tests. The new sample preparation technologies in development, which are closed systems that may use the entire specimen volume, will help NAATs penetrate further into under-resourced primary care clinics in HBCs. This will be driven further by the imminent coming-of-age of several isothermal amplification technologies, which are inherently less complex, require less equipment, and have been successfully used for the POC diagnosis of other diseases such as malaria. The success of these tests will also be facilitated by HBCs improving their testing infrastructure in decentralized laboratories. Finally, it is worth noting that the impact of diagnostics will always be modulated by the capacity of the health care system into which they are embedded.

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Molecular Diagnostics for Use in HIV/AIDS Care and Treatment in Resource-Limited Settings

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41

While access to care and treatment for HIV/AIDS in the developed world is good, in resource-limited settings there still is a need to increase basic access to diagnostics for early detection and treatment. The most persistent challenges in the developing world are molecular tests for early infant diagnosis (EID) and viral load testing for monitoring patients on antiretroviral therapy (ART). The majority of molecular testing options available globally are laboratory-based tests performed on sophisticated instruments requiring dedicated laboratory space and trained technicians. With respect to both viral load and EID testing, there are a good number of platforms available, but their cost and complexity are barriers in resource-limited settings. In the interest of improving the accessibility and affordability of high-quality ART, there is a growing demand for simple, affordable, reliable, and quality-assured point-of-care (POC) diagnostics for use in resource-limited settings. Many contend that POC diagnostics can make ART more scalable and will allow ART service delivery to be significantly decentralized to the community level. At the same time, simplifying diagnostic technologies may reduce the cost of diagnosing and monitoring HIV/AIDS patients without diminishing the quality of care.

To understand the benefits that viral load and EID testing at the POC may offer, it is necessary to understand the current diagnostic technology landscape. With an eye to maintaining high standards of patient care, it also is important to consider the future landscape of such diagnostics and what efficiencies might be achieved with respect to test algorithms, the cost of testing, and decentralized service delivery, especially with respect to the introduction of diagnostics performed at or near the point of patient care. This chapter reviews current diagnostic platforms and pipeline technologies for viral load and EID testing.

In most countries improved access likely will be achieved through a mix of diagnostic services that combines sophisticated, high-volume, low-unit-cost laboratories in high-density areas and lower-volume, simpler, POC or near-POC platforms in less densely populated regions. Determining the optimal mix of centralized, high-volume viral load/EID testing and POC diagnostics based on each country's unique needs is a challenge but is critical to ensuring efficient access to quality-assured HIV diagnostic services in resource-limited settings.

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OVERVIEW OF THE FIELD

Diagnostics for HIV/AIDS can generally be divided into three test categories: (i) tests to facilitate initial diagnosis, (ii) tests to stage the patient, and (iii) tests to monitor the patient, both before and after initiation onto ART. There are generally accepted algorithms and tests used at each stage.

Initial Diagnosis

A number of tests are available to determine whether a person is infected with HIV, the virus that causes AIDS. Of these, HIV antibody tests are most commonly used for routine diagnosis of patients over 18 months old because they are inexpensive and accurate. For such patients, HIV rapid disposable tests are most commonly used for screening in decentralized settings without a laboratory infrastructure (1).

However, these antibody tests are not effective for infants less than 18 months old. The most widely used test for EID is the DNA PCR molecular test, a laboratory-based test. It also is possible to use viral RNA detection methods or p24 testing for this purpose, but these methods are rarely used. To reach the broader population in resource-limited settings, blood collection for the DNA PCR test has been decentralized to clinics, prevention of mother-to-child transmission centers, and the like. The infant's blood is collected on filter paper (known as dried blood spots [DBS]), which is transferred via couriers to the laboratory for testing, and test results are then returned to the clinic for dissemination to caregivers. Unfortunately loss to follow-up is a persistent problem.

Patient Staging

HIV disease involves a continuum of progressive damage to the immune system from the time of infection to the manifestation of opportunistic infections, wasting, or CD4 lymphocyte count that marks the development of full-blown AIDS (2). The period of time from infection to the development of AIDS can vary significantly from person to person. It is generally quite long (years) compared to the short period common to many other viral infections (3). Clinical disease progression depends on the relationship between the viral load and CD4+ T-cell counts (4).

The extended incubation period of AIDS means that laboratory tests are required to identify people at high risk of disease progression to guide clinical decision-making in asymptomatic seropositive patients. Determination of the

CD4 lymphocyte count (or percentage) has been the most important laboratory marker of disease progression.

CD4 testing is used together with clinical staging to determine whether the patient is eligible for treatment (5). After a primary HIV infection, the virus directly attacks CD4 T lymphocyte cells and begins to destroy them while using them for replication (6). Clinicians therefore seek to routinely test an individual's CD4 count to monitor disease progression and to determine when an individual should be initiated onto ART. If the absolute CD4 count of an adult or a child 5 years or older is below a defined threshold (currently ≤500 cells/mm^3), then ART should be initiated (1).

Patient Monitoring

Tracking the course of the HIV virus itself by accurate measurement of the quantity of viral RNA in the patient's plasma has become as important a laboratory marker as CD4 lymphocyte count and is considered the best marker to use for ART decision-making. Viral load testing is used to determine whether the virus is "undetectable" in the patient's blood (below the limit of detection of currently available technologies) and is considered to be the most effective means of identifying treatment failure. Especially in resource-limited settings, clinical signs and immunological (CD4) monitoring are generally lagging indicators of treatment failure, with misclassification of ART failure by these methods as high as 45% (7–9).

Identifying treatment failure early enables patient adherence counseling and may enable patients to stay on first-line ART longer than otherwise, thereby avoiding unnecessary switches to more expensive second-line regimens. Viral load testing also enables clinicians to switch failing patients early to new drugs, thereby reducing the spread of highly resistant virus.

As a result, the World Health Organization (WHO) now recommends routine viral load testing as the preferred monitoring approach for patients post-initiation onto ART. Viral load testing quantifies the HIV viral burden in plasma, and the WHO now defines treatment failure as persistent viral load readings above 1,000 copies (cp)/ml.

However, while routine viral load testing is widely implemented in the developed world, due to the cost and complexity of the test, the implementation of viral load testing in resource-poor settings has been limited. Virtually all viral load testing is laboratory-based. Most testing is performed using sophisticated, high-throughput instruments. With the exception of a single POC platform, there are no viable POC testing options currently available, although several are in development. Blood samples have to be collected and transported to central laboratories for viral load testing and, although DBS has recently been introduced for several of the viral load platforms, uptake to date has been limited.

OVERVIEW OF VIRAL LOAD TESTING TECHNOLOGIES

Viral Load Testing Complexities

The first molecular assay for quantifying HIV viral RNA was approved by the U.S. Food and Drug Administration (FDA) in 1999. Since then, a number of assays have been developed and are considered in this chapter. First, it is worth considering some of the complicating factors that characterize viral load assays and platforms, which should inform the choice of platforms for a given setting. These include HIV diversity and certain practical challenges, including laboratory infrastructure and transport of samples.

HIV Diversity

In 1985, several years after HIV was recognized, a genetically similar virus causing AIDS was discovered in West Africa. As a result, two types of HIV have been classified and characterized: HIV-1 and HIV-2. Of the two types of HIV, HIV-1 is predominant and has been most responsible for the HIV pandemic that exists today (10). HIV-1 is divided into four groups, designated M, N, O, and P, the main group being group M. In addition, there are multiple clades, and within each clade, there are subclusters of individual strains that have been isolated around the world. Finally, mutation of the virus and different evolutionary rates have led to extensive genetic diversity below the clade level. The high level of genetic heterogeneity of HIV-1 and the emergence of recombinant strains of the virus complicate viral load assay development (11, 12). In an ideal world, viral load assays would detect and quantify all known HIV-1 subtypes, as well as intersubtype recombinants and emerging variations thereof. But currently, that is not the case. Therefore, it is important to consider the prevalence of HIV-1 and HIV-2 groups and subtypes in a particular region when choosing an assay.

Sample Transport

Most methods of viral load determination require venous blood collection, processing of that blood to obtain plasma within a certain timeframe, cold chain, and storage of specimens by trained personnel. In resource-limited settings where viral load testing will generally take place only in a national reference, or comparable, laboratory, this means that patient samples have to be transported from urban, peri-urban, and rural settings to the laboratory for processing. This is done using sample transport networks in-country, taking advantage of courier or similar services to take samples to the laboratory and to return results at a later date. But frequently, these services are not well developed, leading to long delays in returning sample results to patients and loss to follow-up. Therefore, the ability to use DBS samples for viral load is an important consideration because it simplifies transport, providing enhanced stability, ease of use, and cost effectiveness. However, there has been some concern about the correlation of viral load measures using DBS as opposed to plasma. Although several studies have demonstrated good correlation between the two using different viral-load methodologies, with sensitivity ranges close to 3 log HIV-RNA cp/ml (13–15), other studies failed to find such a correlation (16–18). As a result, because of the possibility of reduced sensitivity of DBS for viral load measurement at 1,000 cp/ml, the WHO 2013 guidelines suggest that programs relying on DBS technology for viral load testing may consider retaining a higher threshold (3,000 to 5,000 cp/ml) (1, 19).

EXISTING VIRAL LOAD TECHNOLOGIES

HIV viral load technologies can be categorized broadly as nucleic acid–based test (NAT) and non-NAT-based technologies. The technologies differ in the methods used to quantify HIV virions circulating in the body. NAT technologies detect and quantify viral RNA, whereas non-NAT technologies detect and quantify HIV viral

enzymes and proteins that can be correlated to the amount of viral RNA. This chapter focuses only on NAT-based technologies. All such technologies incorporate amplification techniques because levels of nucleic acids are otherwise too low to be detected directly (10). Currently, the bulk of commercially available viral load assays are based on target amplification, as opposed to signal amplification.

Preamplification methods are critical to the viral load testing process. Protocols for the preamplification steps include the use of purification methods for cells and virion centrifugation or a capture step for RNA in plasma, followed by a nucleic acid extraction step (10). Although HIV nucleic acids are relatively stable, molecular detection methods require prompt processing of samples, a rapid extraction method, and appropriate storage of plasma or cells prior to assessment.

Finally, postamplification methods require the detection and/or quantification of either the amplification products or the increased detection of signals that have been amplified (10). Detection can be achieved using any one of a number of reagents, for example, colorimetric, radioactive, or fluorescence. Detection can either be done at the endpoint of the process or in "real time." Real-time techniques, in which amplification and detection occur simultaneously, are now commonly used. Most of the assays are available in quality-assured kits, and clinicians are comfortable interpreting the results. The assays vary in terms of sample preparation and amplification/detection methodologies, among other things.

Platforms Based on RT-PCR

Currently, there are four commercially available real-time reverse transcriptase (RT)-PCR-based viral load assays: (i) cobas AmpliPrep/cobas TaqMan version 2.0 (Roche), (ii) RealTime HIV-1 (Abbott), (iii) Versant HIV RNA 1.0 (kPCR) (Siemens), and (iv) Artus HIV-1 QS-RGQ (Qiagen). There also are a number of in-house procedures and test systems that have good sensitivity and reproducibility that are used in various countries but which will not be described in detail in this chapter.

Roche cobas AmpliPrep/cobas TaqMan System

Roche manufactures a single real-time PCR assay, the cobas AmpliPrep/cobas TaqMan version 2. It is an *in vitro* diagnostic (IVD) test that uses the AmpliPrep instrument for automated viral nucleic acid extraction and the cobas TaqMan analyzers (TaqMan 48 or TaqMan 96) for automated amplification and detection of the viral nucleic acids. The cobas AmpliPrep/cobas TaqMan version 2 test was designed specifically to address certain HIV-1 mutations for which a dual-target approach is used. The dual-target technology provides additional confidence in results in the event of mutation. The assay is able to coamplify two target regions of HIV-1, which were specifically chosen because they are not HIV drug targets. By targeting both regions of the genome simultaneously, the test increases the probability of detection of virus particles. The cobas AmpliPrep/cobas TaqMan HIV-1 test version 2 is intended for use in conjunction with clinical and laboratory markers of disease progress for the management of HIV patients. The assay can be run using DBS in addition to plasma specimens. It is able to quantify HIV-1 group M (subtypes A through H) and HIV-1 group O and has a linear range of between 20 cp/ml and 10×10^6 cp/ml. The cobas AmpliPrep/cobas TaqMan HIV-1 test version 2 for TaqMan 48 and TaqMan 96 is prequalified by WHO. The test is also FDA-approved for plasma but is "research use only" for DBS. The test has a good correlation with the Amplicor HIV-1 Monitor version 1.5 assay, which was generally considered to be the "gold standard" (20).

The cobas AmpliPrep instrument is an automated sample preparation technology for use in conjunction with the Roche cobas TaqMan analyzers. The company considers the AmpliPrep to provide "walk-away" sample handling capability, which can significantly reduce hands-on time for laboratory technicians. Roche manufactures two versions of its TaqMan analyzer, the cobas TaqMan 48 analyzer and the cobas TaqMan 96 analyzer. Each of the analyzers is a fully automated, closed-tube system. The TaqMan 48 is relatively compact and can run from 6 to 48 samples at a time. The instrument is equipped with two thermal cyclers that operate independently and provide run times of 90 to 120 minutes.

Abbott *m*2000 System

Abbott manufactures the Abbott RealTime HIV-1 assay, which is an RT-PCR assay for the quantification of HIV-1, on its automated *m*2000 and *m*24 systems and the Abbott RealTime HIV-1 qualitative assay (collectively, the *m*2000system) for qualitative detection of HIV-1 in plasma and DBS used as an aid in the diagnosis of HIV-1 infection in pediatric and adult subjects. The primers and probes of the assays are targeted to the conserved integrase region of the *pol* gene as opposed to the gag region targeted by the Roche assays. The combination with the unique probe design and cycling conditions ensures a high mismatch tolerance to detect HIV-1 groups and subtypes. The Abbott RealTime HIV-1 assay uses an external calibration strategy. It can be automated using the Abbott *m*2000*sp* (or *m*24*sp*) for sample preparation and the *m*2000*rt* for amplification and detection. The assay introduces an RNA sequence that is unrelated to the HIV-1 target into each specimen at the beginning of sample preparation. The amount of HIV-1 target sequence that is present at each amplification cycle is measured through the use of fluorescent-labeled oligonucleotide probes on the *m*2000*rt* instrument. The probes do not generate a signal unless they are specifically bound to the amplified product. The amplification cycle at which the fluorescent signal is detected by the *m*2000*rt* is proportional to the log of the HIV-1 RNA concentration of the sample. The RealTime HIV-1 assay has a linear range of 40 cp/ml to 10 million cp/ml and can detect HIV-1 group M (subtypes A through H, including recombinant forms), group O, and group N. The limit of detection is 40 cp/ml for 0.6 ml input and 150 cp/ml for 0.2 ml input. Performance has been assessed with good results (21).

The Abbott RealTime assay is designed to be used with the *m*2000rt amplification and detection instrument as well as with one of three methods of sample preparation: (i) manual (for laboratories with low throughput), (ii) the *m*24*sp* instrument (low to medium throughput), or (iii) the *m*2000*sp* instrument (medium to high throughput). The *m*2000*sp* by Abbott is a larger and more automated sample preparation device than its sibling, the *m*24*sp*. With complete automation comes increased walk-away time. It is a high-throughput system with a maximum batch size of 96 samples per run.

Versant kPCR Molecular System

The Versant kPCR molecular system and the Versant HIV RNA 1.0 assay (kPCR) are manufactured by Siemens.

Because they conform to the requirements of European Union directives (i.e., they are Conformité Européenne [CE]-IVD marked), but are FDA-approved, they are only available outside of the United States. The Siemens HIV assay is an automated amplification method based on reverse transcription and real-time PCR. The system consists of the sample preparation module used to extract nucleic acids from plasma as well as other samples, and the amplification detection module, along with Versant kPCR software. The system has no need for clean room operations. The Versant kPCR sample preparation module along with the Versant sample preparation 1.0 reagents kit are used to extract RNA from plasma. The reagents kit includes proprietary magnetic silica beads that provide for extraction of nucleic acids. Extraction consists of a lysis step with proteinase K and a chaotropic buffer and several washes to remove non–nucleic acid components of the sample and elution. The Versant kPCR sample preparation module also pipettes the purified RNA onto a PCR plate containing an HIV-1 primer/probe mix and the enzyme mix. The plate is then transferred to the amplification detection module, where the HIV and internal control RNA molecules are reverse-transcribed and simultaneously amplified and detected using the kPCR technique. The RT-PCR step targets a conserved pol region.

The Versant kPCR molecular system provides the flexibility to process samples in batch sizes of 1 to 96 tests per run. The HIV assay provides patient results for up to 89 samples per run with a total time to result of less than 6 hours. The linear range of the assay is between 37 HIV-RNA cp/ml and 11 million cp/ml. The assay can detect HIV-1 group M (subtypes A through G) and group O variants. Its performance is comparable to assays from other manufacturers (22). The Versant HIV-1 RNA 1.0 assay has been prequalified by WHO.

Artus HIV-1 RG/QS-RGQ RT-PCR System

Qiagen has recently introduced the Artus HIV-1 RG/QS-RGQ RT-PCR kit. The assay is CE-IVD-marked and targets the long terminal repeat region. The kits can be used in combination with either a manual (Artus HIV-1 RG RT-PCR kit) extraction and sample preparation system (QIAamp DSP virus kit) or an automated (Artus HIV-1 QS-RGQ RT-PCR kits) extraction and sample preparation system (QIAsymphony SP/AS). The assay must then be run on one of the Qiagen Rotor-Gene Q thermocyclers for amplification and detection. The Artus HIV-1 QS-RGQ assay has a linear range of 45 HIV-1 RNA cp/ml to 45 million cp/ml (using automated extraction) and can detect HIV-1 group M (subtypes A through H) down to a limit of detection of approximately 35 cp/ml. The time to result is from about 5 to 6 hours for 24 samples. Performance of the Artus assay is comparable to that of the Abbott RealTime system (23). Sample preparation for the Artus HIV assay can be conducted manually using the CE-IVD-marked Qiagen QIAamp DSP virus kit, which provides silica-membrane-based RNA purification using a vacuum process. The SP/AS system includes touch-screen controls and bar code–labeled sample tubes containing prefilled reagents and allows for continuous loading in batches of up to 24 samples plus internal controls. The QIAsymphony SP/AS instruments can be integrated into laboratory information management systems. The Artus HIV assay can be run on the Rotor-Gene Q, which has a unique centrifugal rotary design in which each sample tube spins in a chamber of moving air, which keeps all samples at precisely the same temperature. As each tube aligns with the detection optics in the device, the sample is illuminated and a fluorescent signal is quickly collected.

Platforms Based on Alternative Technologies

Besides the technologies described above, several other available products perform well and are based on different technologies. The most important ones in this field are discussed below.

NASBA Platform

The Nuclisens HIV solution is manufactured by bioMérieux. The Nuclisens EasyQ HIV-1 version 2.0 assay targets a well-conserved region of the *gag* gene and is based on nucleic acid sequence-based amplification (NASBA). Following sample extraction with proprietary magnetic Boom technology, the highly efficient real-time NASBA amplification reaction ensures very sensitive test results in only 1 hour. NASBA is an isothermal transcription-based amplification method which amplifies RNA from an RNA target. The amplicons produced through this process are detected in real time by molecular beacons (24). Kinetic analysis of the fluorescent signals reveals the transcription rates of both the HIV RNA target and a calibrator RNA. This transcription rate is used to determine the quantity of HIV-1 RNA in the original specimen. The linear range of the EasyQ HIV assay version 2.0 is from 10 to 10 million cp/ml. The assay can detect HIV-1 group M (subtypes A through J) as well as CRF01_AE and CRF02_AG. Performance of the assay correlates well with assays from Roche, Abbott, and Siemens (25, 26). The Nuclisens Minimag and Nuclisens Easymag extraction systems make up part of the Nuclisens HIV solution but can be used for any other molecular diagnostic assay requiring the purification of nucleic acids from clinical samples. For higher throughput needs, the Easymag is an automated benchtop nucleic acid extraction device that can perform 24 extractions in 40 minutes. The instrument has one generic DNA/RNA extraction protocol and one set of reagents for all applications, which together with touch-screen technology, make the process relatively simple. Nuclisens EasyQ amplification and detection is a closed system made up of a real-time NASBA amplification step with automated data analysis.

Real-Time Transcription-Mediated Amplification Platform

Hologic Gen-Probe has introduced the random access Panther system. The platform brings the flexibility of clinical chemistry instrumentation to molecular diagnostics. The company has developed a fully quantitative viral load assay, the Aptima HIV-1 Quant Dx assay. The Aptima assay involves three main steps, all of which take place in a single tube on the Panther system: target capture, target amplification by transcription-mediated amplification (TMA), and detection of the amplicons by the fluorescent labeled torch probes. During target capture, viral RNA is isolated from samples. The sample is treated with a detergent to release viral genomic RNA. Oligonucleotides capture and hybridize to highly conserved regions of HIV-1 RNA. The hybridized target is then captured onto magnetic microparticles that are captured by magnetism. Finally, washing removes extraneous components. Transcription-mediated amplification is a transcription-based nucleic acid amplification method that uses two enzymes: RT and T7 RNA

polymerase. The Aptima assay amplifies two regions of HIV-1 RNA for HIV-1 groups M, N, O and P. Torches hybridize specifically to the amplicon in real time. As more torches hybridize, more fluorescence is generated. The time taken for the fluorescent signal to reach a threshold is proportional to the starting HIV-1 concentration. Each reaction has an internal calibrator/internal control.

The Panther's intuitive task-driven software with touch-screen interface simplifies setup, adding or removing reagents or samples, and onboard inventory management of reagents and consumables; a bidirectional laboratory information system interface is included. Low-volume dilution options allow quantitative results to be obtained on as little as 240 µl of plasma. The system can be programmed to perform automated maintenance outside of laboratory hours. Reagent management is simplified with 48-hour onboard stability or 30 days' refrigeration for assay reagents. DBS is being validated for use with the system. The assay is now CE-IVD-marked and is available for sale in the European Union and in other countries that observe CE-IVD marking.

POC Viral Load Testing Platforms: SAMBA

A semiquantitative assay for use on the SAMBA system has been launched. Developed by Diagnostics for the Real World, four SAMBA NAT-based HIV assays are available: (i) a semiquantitative test with a cutoff of 1,000 cp/ml for monitoring ART using plasma, (ii) a semiquantitative test with a cutoff of 1,000 cp/ml for monitoring ART using whole blood, (iii) a qualitative test based on plasma or whole blood for the detection of acute HIV infection before the appearance of antibodies, and (iv) an EID test based on whole blood. There are two SAMBA systems, with SAMBA I being capable of semiautomated batch testing with a throughput of 30 to 42 samples per day. It automates extraction (SAMBAprep) and integrates amplification and detection (SAMBAamp) into a benchtop analyzer with amplification and detection taking place in a hermetically sealed cartridge. SAMBA II is a fully automated "sample-in, result-out" system. It is suitable for both low- and high-volume testing sites, with a throughput of 4 to 64 samples per day. A display unit controls the assay module, and each display unit can control up to 16 assay units. It is a modular, random-access system that allows the throughput to be adjusted according to the requirements of the site. The amplification and detection processes are integrated in a closed cartridge to prevent contamination and target the long terminal repeat. Amplification is based on target and signal amplification (Fig. 1).

A capture probe is used to capture the target sequence, and a detector probe with multiple hapten labels is subsequently attached to the target sequence, enabling amplification of the signal to improve sensitivity and allow visual reading. The lattice structures (Fig. 1) ensure visual detection of the RNA or DNA target, which can be visually read on a nitrocellulose, lateral flow test strip within 25 minutes. Based on an assessment with the WHO international standard HIV RNA genotype panel containing 400 cp/ml, the SAMBA assay was able to detect all HIV-1 subtypes. At present, the total assay time is 2 hours for the SAMBA EID assay and 90 minutes for the semiquantitative viral load assay. In addition to regulatory approval of the SAMBA viral load assay in Malawi, both the SAMBA EID and viral load assays have recently received product approval in Uganda and Kenya. The assays are currently being evaluated in a number of additional countries in sub-Saharan Africa, including Nigeria. The SAMBA system is best suited for use at district hospital facilities or in large clinics in sub-Saharan Africa where laboratory technicians and electricity are available.

Alere q (Alere)

The Alere q system is a generic platform for the implementation of nucleic acid testing. The first test to be commercialized (in 2014) is an integrated test for the qualitative detection of HIV-1 and HIV-2 simultaneously from 25 µl of blood, which can be used for EID. This will be followed by the release of a test for the quantitative measurement of HIV-1 and HIV-2 viral load from 500 µl of plasma and a test for the quantitative detection of HIV-1 and HIV-2 simultaneously from 25 µl of blood. The device on which the assay is run has a small footprint, is portable, contains an integrated uninterruptible power supply, can be run either on mains power or from a dedicated battery pack, and withstands harsh environments. The Alere q tests are disposable cartridges that contain all reagents required for the assay in a stabilized form. The cartridges provide for sample collection, lysis, target capture, reverse transcription, RT-PCR and real-time fluorescence detection based on competitive reporter probe hybridization on an integrated microarray. The viral load cartridge uses the same principles and chemistry as the whole-blood cartridges but requires a separate plasma preparation step. The system will detect HIV-1 groups M, N, and O and HIV-2.

The Alere q whole-blood tests are designed to require no manual sample preparation or pretreatment. The required 25 µl of blood can be collected via finger prick, heel prick, or venipuncture. The disposable cartridge, once

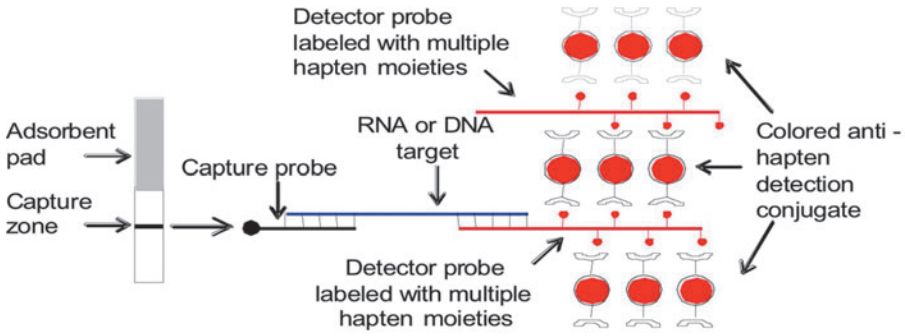

FIGURE 1 SAMBA amplification process showing lattice structures.

capped, cannot be reopened. At no time does the sample or the reagent come into contact with the analyzer, thus reducing the chances of cross-contamination. The results can be disseminated using GSM mobile telephone network infrastructure.

GeneXpert System

The Cepheid GeneXpert HIV viral load assays were commercially launched in early 2015. The quantitative HIV-1 assay using plasma was CE-IVD-marked as of December 2014, and the qualitative HIV-1 assay using either whole blood or DBS is expected to be CE-IVD-marked at launch. The assay targets one genomic region of HIV-1 that is proven both *in silico* and *in vitro* to detect the vast majority of all HIV-1 strains independent of group and subtype. The forward and reverse primer and the TaqMan probe are located in the most conserved region of the long terminal repeat. To be able to detect group O as efficiently as groups M and N, an additional TaqMan probe was designed. The forward primer and the two probes included in the assay incorporate Cepheid's proprietary chemistry to maximize inclusivity and exclusivity at the sequence level. The assay detects all strains of HIV-1, including HIV group M subtypes A, B, C, D, F, G, H, J, K, AB, AE, and AG and groups N and O. The quantitative assay has a limit of detection of approximately 20 cp/ml and a limit of quantitation of approximately 40 cp/ml with a 1-ml plasma sample input volume. The quantitative assay includes two internal quantification standards to provide accuracy and precision in quantitation. The qualitative assay has a limit of detection of approximately 300 cp/ml for 100 µl of whole blood sample input volume.

The workflow for the quantitative assay consists of (i) collecting whole blood in an acid citrate dextrose or EDTA tube, (ii) centrifuging the tube, (iii) transferring 1 ml (pipette provided) directly into the GeneXpert cartridge, (iv) scanning the cartridge bar code, and (v) loading the cartridge into the GeneXpert module and closing the door, with an approximately 95-minute time to result. An early termination step is included to shorten the time to a positive result. The qualitative viral load assay has similar ease of use. GeneXpert cartridges can handle a variety of sample volumes within macrofluidic chambers and then concentrate the target material down to microfluidic volumes, which can increase the sensitivity of the assays.

Furthermore, the GeneXpert system is modular. Individual modules contain solid-state circuitry that controls temperature, pressure, rotation of the valve that moves the liquid between reservoirs, and the detection software. These individual modules are packaged in units of 1, 2, 4, 16, 48, or 80; the latter two systems are fully automated, walk-away robotic instruments developed for high-throughput laboratory applications. The system can be powered by a 12-V DC/120-V AC voltage converter in mobile laboratories or even solar batteries.

VIRAL LOAD TECHNOLOGIES IN THE PIPELINE

As indicated above, there are three viral load assays currently on the market. There are also a number of additional platforms/assays in development, which will likely be launched in 2016 and beyond. Described below are viral load assays in the pipeline.

cobas Liat System

The cobas Liat system, manufactured by IQuum for Roche, is an automated sample-to-result NAT platform that performs sample nucleic acid extraction, purification, reverse transcription, PCR, and real-time detection for the detection and/or quantification of pathogens. The test procedure is straightforward, with no sample manipulation or reagent loading steps other than inputting the plasma or whole-blood samples directly into the Liat tube. The Liat system is a closed system and allows testing to be performed near the patient. It incorporates a variety of intelligent features: bar code data entry prevents errors in sample or assay coding, and onscreen prompts provide easy-to-follow directions to guide the operator through sample loading and tube insertion. Sample metering capabilities ensure the correct test volume and warn if the sample volume is insufficient. A sensor further monitors system operations in real time and automatically recovers from errors or aborts the assay to prevent incorrect results from being reported. An internal control contained in each Liat tube is processed and detected with the sample. The Liat system is small and portable and executes all required assay steps and reports a qualitative or quantitative test result within 15 to 35 minutes. If the user wants to measure viral load from a plasma sample down to 80 cp/ml, then the device takes about 30 minutes to produce a result; if measuring down to 1,000 cp/ml, the device takes about 35 minutes. The Liat system has six independent optical detection channels for real-time monitoring and quantification, allowing for the detection of multiple targets and providing future expandability for detection of multiple diseases at lower per test cost. It can be powered by AC mains or by battery.

Eoscape-HIV HIV Rapid RNA Assay System

Based on its liquid micropiston technology, Wave 80 Biosciences is developing the Eoscape-HIV, a rapid HIV NAT-based POC viral load test designed for use in resource-limited settings. The company describes the cartridge as incorporating automated sample metering with filtration for removal of proviral DNA, a no-spin, cartridge-integrated nucleic acid extraction process, and tuned isothermal nucleic acid amplification technology (iNAAT) coupled with a proprietary ultrasensitive bipartite luminescent signaling/detection system. The system processes finger-prick whole blood in a single-use, enclosed cartridge. The cartridge contains all reagents necessary to run the test and does not require cold-chain transport. The system is made up of an analyzer consisting of a microprocessor, communications hardware, and a battery, as well as an optics module in a rugged, swing-down cartridge enclosure. The assay detects HIV RNA and quantitates HIV viral load over the range from 400 to 50,000 cp/ml without the need for external calibration. Product launch is planned for 2016–2017.

Truelab Real Time Micro PCR System

Molbio Diagnostics has developed a comprehensive, rapid, near-patient real-time PCR platform called the Truelab Real Time Micro PCR system. The system is portable and includes all instrumentation, reagents, and essential accessories from sample preparation to final result reporting, which is all accomplished within 1 hour. A Truelab Micro PCR printer also is available. The system works on ready-to-use Truenat disease-specific assays that are stable at room temperature. Assays for HIV viral load are in

development. The testing process begins with sample collection (blood, serum, or plasma) followed by extraction, which uses the Trueprep MAG sample prep device and Trueprep Mag sample prep kits. The extraction process takes about 20 to 25 minutes per sample. From there, 6 µl of the extracted nucleic acid is dispensed into the reaction well of the disease-specific Truenat Micro PCR chip. The chip, which contains all of the chemistry required to complete an assay, is then inserted into the Truelab Uno Real Time Micro PCR analyzer. During amplification, the Truenat Micro PCR chip exponentially releases fluorophores. These signals are captured by sensors and are displayed as an amplification curve on the Truelab screen. Test results are compared to lot-specific standard values preset into the Truenat chip, which enables quantitative estimation of the test analyte and display as RT-PCR results in approximately 30 minutes. Test results are automatically stored (up to 5,000 results) and can be printed and transported wirelessly to any server or compatible device by Wi-Fi, GPRS, Bluetooth, or spatial multiplexing. The assay is currently undergoing laboratory-based trials in India.

NWGHF Savanna Viral Load Test and Platform

Northwestern Global Health Foundation (NWGHF), in collaboration with Quidel Corporation, is developing a POC rapid RT-PCR testing platform called Savanna that will be both easy to use and low cost. The product can accommodate 13 tests in an 8-hour day. The proposed viral load assay will achieve a limit of detection of 1,000 cp/ml of plasma, using ~150 µl of whole blood that is converted into plasma with simple sample preparation using materials provided by NWGHF. For low- and middle-income countries, the system is expected to cost $12,000, with a cartridge cost of $10 per test. NWGHF/Quidel Corporation expects to launch the Savanna viral load test and platform in 2016 or 2017.

BART (Bioluminescent Assay in Real-Time) Technology

Lumora has introduced BART, a bioluminescent reporter system for molecular diagnostics that can reduce instrument costs and open up new applications for diagnostics and disease monitoring in resource-constrained settings. Requiring only a single-temperature heating block and a photo-diode light detection system, BART is designed for use with iNAAT. It combines simple and robust chemistry and technology in real-time, closed-tube analysis and less demanding sample preparation. Through the utilization of Lumora's proprietary heat elution sample preparation technology (Fig. 2), it is possible to extract a sample from whole blood or DBS in 10 minutes. The processed sample can then be added directly to freeze-dried amplification reagents.

Lumora has patented improvements to loop-mediated isothermal amplification, enhancing performance not only for viral detection and difficult bacterial targets, but also for speed and ease of development. Lumora's stem primer technology has facilitated the development of a fully inclusive HIV viral load test. Lumora uses stem primers that offer new strategies for dealing with the sequence diversity of HIV. iNAATs generally used in molecular diagnostics produce pyrophosphate exponentially. BART employs pyrophosphate as the start of a reaction driven by firefly luciferase enzymes to generate light. The enzymes are modified to be thermally stable and function at 60°C. The time taken to reach the peak light signal reflects the amount of target nucleic acid in the samples, and BART can quantify the target in an amount of time similar to fast PCR systems. BART is well suited to high-throughput applications, making it equally useful in both highly decentralized settings and centralized laboratories requiring high-throughput technologies. The hardware is portable and is powered by mains or a battery at low cost with a small footprint. The first-generation assays with either heat elution or manual sample extraction are now ready to license. The fact that BART and the associated technologies from Lumora are easy to use and relatively low cost means that wider adoption of this technology could be expected. Lumora believes that BART will offer a simple and effective method for monitoring viral load in developing countries and could support current efforts to increase the effectiveness of and adherence to ART regimens.

RT CPA HIV-1 Viral Load Test

Ustar Biotechnologies has developed cross-priming amplification (CPA), a novel iNAAT with multiple iterative designs that can address a wide variety of key obstacles to traditional amplification technologies such as PCR. By using multiple crossing primers and probes, target DNA sequences can be rapidly and precisely amplified at a uniform temperature (typically 63°C) in an easy-to-use protocol with high sensitivity and specificity. Recent work at Ustar and the University of Victoria has shown that RT CPA can effectively amplify an RNA template with performance similar to existing DNA-based assays. Results indicate that the use of an RNA template does not alter the overall performance of CPA compared to the use of a DNA template. Additionally, by using novel enzymes together with inherent RT activity, as little as 0.1 pg of RNA can be detected in less than 30 minutes. Ustar also possesses a proprietary process that stabilizes enzymes for

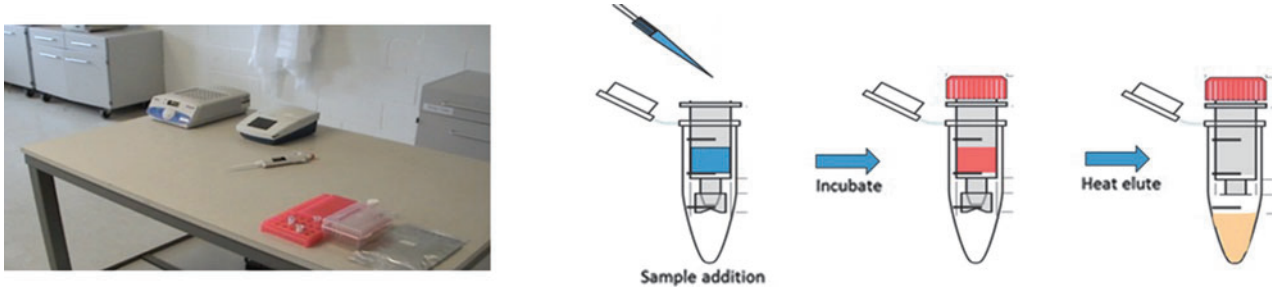

FIGURE 2 BART heat elution sample preparation technology.

ambient temperature transport and storage. Ustar's goal is to develop a quantitative RT CPA HIV viral load assay and test cartridge in conjunction with a robust and user-friendly portable instrument. Ustar plans to modify the commercially available Genie, a portable instrument developed by OptiGene. A fully quantitative viral load measure will be available in as little as 20 minutes, and the sample can be run for 45 minutes to ensure a viral load measure of <1,000 cp/ml. Ustar is now actively working on the development of its viral load assay with completion and launch expected in 2016 or 2017.

Gene-Radar Platform (Nanobiosym Diagnostics)

Nanobiosym has developed a portable nanotechnology platform called the Gene-Radar. This chip-based system, which is about the size of a laptop computer, uses approximately one drop of specimen to recognize pathogen DNA or RNA. About 20 to 100 µl of sample is collected and transferred to a disposable chip, which is inserted into the platform. The Gene-Radar then extracts DNA/RNA present in the sample and determines whether it matches the DNA/RNA of a particular pathogen. The Gene-Radar platform does not require sophisticated laboratory infrastructure, trained laboratory technicians, continuous power, or running water. Nanobiosym has developed a viral load assay for the Gene-Radar version 1.0 platform, which can give a fully quantitative viral load measure in real time. Because Gene-Radar is a flexible and reconfigurable platform, the company is continuously incorporating innovations to optimize its performance metrics.

Genedrive

Epistem, a biotechnology company headquartered in the United Kingdom, has developed a new molecular diagnostic platform, the Genedrive, which uses endpoint PCR detection. The Genedrive is a highly portable POC platform weighing about 550 g and is approximately the size of an iPad mini. The platform accommodates both electric mains (110- to 240-V AC) and battery (12-V DC) power. Test results are available in less than 60 minutes. The Genedrive platform is integrated with a simple extraction process based on an advanced composite paper technology that allows extraction and decontamination in a single step and is suitable for use in low-resource settings. The sample is manually transferred with one pipetting step into the Genedrive reaction cartridge.

EID TESTING

Because of the persistence of maternal antibodies in infants under 18 months old, the use of antibody tests, such as commercially available HIV rapid disposable tests, cannot be used to accurately screen infants for HIV. Instead, viral testing or ultrasensitive p24 antigen testing should be used to determine the HIV status of infants in that age group (1). Current WHO guidelines call for all HIV-exposed infants to have viral testing at 4 to 6 weeks of age or at the earliest opportunity thereafter (1). The most widely used test for EID is DNA PCR. The qualitative HIV-1 DNA test detects the presence of HIV proviral DNA. Unlike the quantitative HIV-1 RNA tests discussed above, the DNA PCR molecular test does not provide a quantitative measure of a patient's viral load but rather provides a "yes" or "no" answer with respect to whether the infant is infected with HIV.

There are currently two HIV-1 DNA assays available in resource-limited settings that are used for EID: the Roche cobas AmpliPrep/cobas TaqMan HIV-1 qualitative test and the Abbott RealTime qualitative HIV-1 test, both of which have CE-IVD marking. Each of these assays must be performed on laboratory-based instruments as described in the previous section, and even with the use of DBS, there is significant loss to follow-up of infants. Because RNA PCR testing can be used for the detection of HIV in infants under 18 months old, the new POC technologies discussed in the previous section on viral load testing should be considered viable options for EID. In addition, Alere q has a dedicated DNA assay for EID, and SAMBA, the cobas Liat system, and GeneXpert, among others, will also have qualitative assays specifically for EID.

FUTURE DIRECTIONS FOR VIRAL LOAD TESTING

With the exception of Brazil and South Africa, where millions of viral load tests are conducted annually, routine viral load testing has not been widely implemented other than in high-income settings. Other countries that have established viral load testing on a relatively large scale include Botswana and Thailand. Beyond that, there is very little viral load testing done in the public health sector in resource-limited settings. A few countries, including China, Kenya, and Lesotho, are increasingly using viral load. As indicated earlier, the reasons for this limited use include cost, infrastructure requirements, and the need for trained laboratory technicians. It is expected, however, that since the current WHO guidelines recommend routine viral load testing as the preferred monitoring approach to diagnose and confirm ART failure, more countries will begin to scale up viral load testing.

To reach patients in peri-urban and rural settings, it is necessary to set up sample transport networks to transfer patient blood samples to the reference laboratory for testing and to return results to the patient. Since viral load tests generally require plasma for extraction, there is a requirement to centrifuge the whole blood samples from patients, usually within 6 hours of the blood draw. In addition, plasma must be transported and stored under refrigeration. These demands put pressure on the sample transport system and add costs to the process. The introduction of the use of DBS with some of the laboratory-based viral load platforms (Roche TaqMan, Abbott RealTime, and bioMérieux EasyQ), and its use for EID testing, help to make the sample transport process more manageable, removing some of the time pressure.

Given the consensus on the importance of viral load testing for detection of treatment failure for patients on ART, it is likely that testing algorithms in resource-limited settings will migrate toward routine viral load testing, which already exists in high-income settings. The frequency of testing remains to be determined. The purpose of global ART should be the effective, long-term management of chronic patients to ensure the successful treatment of as many people for as long as possible. Patient management algorithms in low- and middle-income countries will need to be upgraded to accommodate the effective use of viral load information.

The level of access required for viral load testing likely will necessitate centralized testing facilities and, at the same time, a drive toward the use of both DBS and POC testing for low- and middle-income countries. As indicated

above, there was a limited launch of the SAMBA viral load technology in 2013 and a launch of Alere q for EID in 2014; additional viral load and EID POC technologies are in development, with possible launch of additional products in 2015 and beyond. It is too early to predict the exact pricing of the POC devices and tests, but it is hoped that the price will be at or below $15 per test.

Further details on this subject can be found in the original UNITAID reports which formed the basis for this chapter (http://www.unitaid.eu/images/marketdynamics/publications/UNITAID-HIV_Diagnostic_Landscape-4th_edition.pdf).

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Rapid Point-of-Care Diagnosis of Malaria and Dengue Infection

LIESELOTTE CNOPS, MARJAN VAN ESBROECK, AND JAN JACOBS

42

MALARIA AND DENGUE: COMMONALITIES OF CHALLENGING TROPICAL DISEASES

Malaria and dengue are both mosquito-borne diseases and are endemic in many subtropical and (sub)tropical regions (1, 2). They have a major impact on public health, and prompt diagnosis is important in endemic settings as well as in travel medicine. Early diagnosis, timely treatment, and case management prevent severe disease and complications (3, 4). Point-of-care (POC) (or bedside) testing offers timely diagnosis in the acute phase of infection for both diseases (5–7), and the POC diagnostics used are mostly referred to as "rapid diagnostic tests" (RDTs). Over 20 definitions are given for POC (8), but most essentially, RDTs should comply with certain diagnostic and performance criteria and—because they are deployed in endemic and low-resource settings—meet the ASSURED criteria (affordable, sensitive, specific, user-friendly, rapid and robust, equipment-free, and delivered) (9).

This chapter will discuss most available RDT formats for rapid diagnosis of malaria and dengue, with a focus on handheld cassette formats. RDTs have been used for malaria diagnosis for decades (10–12) and have already demonstrated their global impact. This chapter will cover the current challenges, pitfalls, and in-depth experience-based facts. This is in contrast to the more recent advances in the development and use of dengue RDT testing, about which current basic information will be presented.

MALARIA AND DENGUE: GEOGRAPHY AND BURDEN OF DISEASE

The Microorganisms and Their Geographic Distribution

Dengue and malaria are vector-borne diseases, and both are spread through the bite of an infected female mosquito. Both diseases are widely spread in many tropical and subtropical regions and demonstrate overlapping distributions, although dengue fever is more prevalent in urban areas than malaria. The highest prevalence of malaria is seen in sub-Saharan Africa, while dengue is seen most in Southeast Asia (Fig. 1) (13–15).

Malaria is caused by a parasite of the genus *Plasmodium* and transmitted to humans by a bite of an infected *Anopheles* mosquito. After a short passage through the liver, the *Plasmodium* parasite invades and multiplies in red blood cells. Five *Plasmodium* species are known to infect humans: *Plasmodium falciparum*—the most lethal species—occurs in tropical regions and is the dominating species in sub-Saharan Africa. *Plasmodium vivax* is adapted to lower ambient temperatures and is mainly prevalent in South America and Asia, where it co-occurs with *P. falciparum*, as well as in the horn of Africa (16). *Plasmodium malariae* is present in most malaria-endemic regions. *Plasmodium ovale* is mainly confined to sub-Saharan Africa, New Guinea, and the eastern parts of Indonesia and the Philippines, but rare cases have been observed in the Middle East and Asia (17). The fifth species, *Plasmodium knowlesi*, is mainly observed in Malaysian Borneo but also in other Southeast Asian countries (18).

The *Aedes aegypti* mosquito is the primary vector of dengue. The secondary vector of dengue is *Aedes albopictus*, also known as the Asian tiger mosquito. The latter is not confined to tropical and subtropical regions and has recently spread into North America and parts of Europe, largely due to the international trade of tires and other goods such as lucky bamboo. Unlike the *Plasmodium*-transmitting *Anopheles* mosquito, which has a night-biting habit, *Aedes* mosquitoes are daytime feeders, with peak biting periods mainly in the morning and late afternoon.

Dengue virus (DENV) belongs to the flaviviruses, which are enveloped, single-stranded, positive-sense RNA viruses that contain 10 genes encoding 3 structural proteins (capsid [C], envelope [E], membrane [M]), and 7 nonstructural proteins (NS1, NS2a, NS2b, NS3, NS4a, NS4b, NS5) (4). The DENV complex consists of four antigenically distinct serotypes (denoted DENV1 to DENV4), and within each serotype there is considerable genetic variation between phylogenetically defined genotypes (19). The discovery of a probable fifth DENV type, presumable DENV5, in Malaysia was recently reported by Vasilakis et al. at the Third International Conference on Dengue and Dengue Hemorrhagic Fever (October 2013, Bangkok, Thailand).

Dengue is the most important mosquito-borne viral infection worldwide (4, 20). Before 1970, only nine countries had experienced severe dengue epidemics, while to date,

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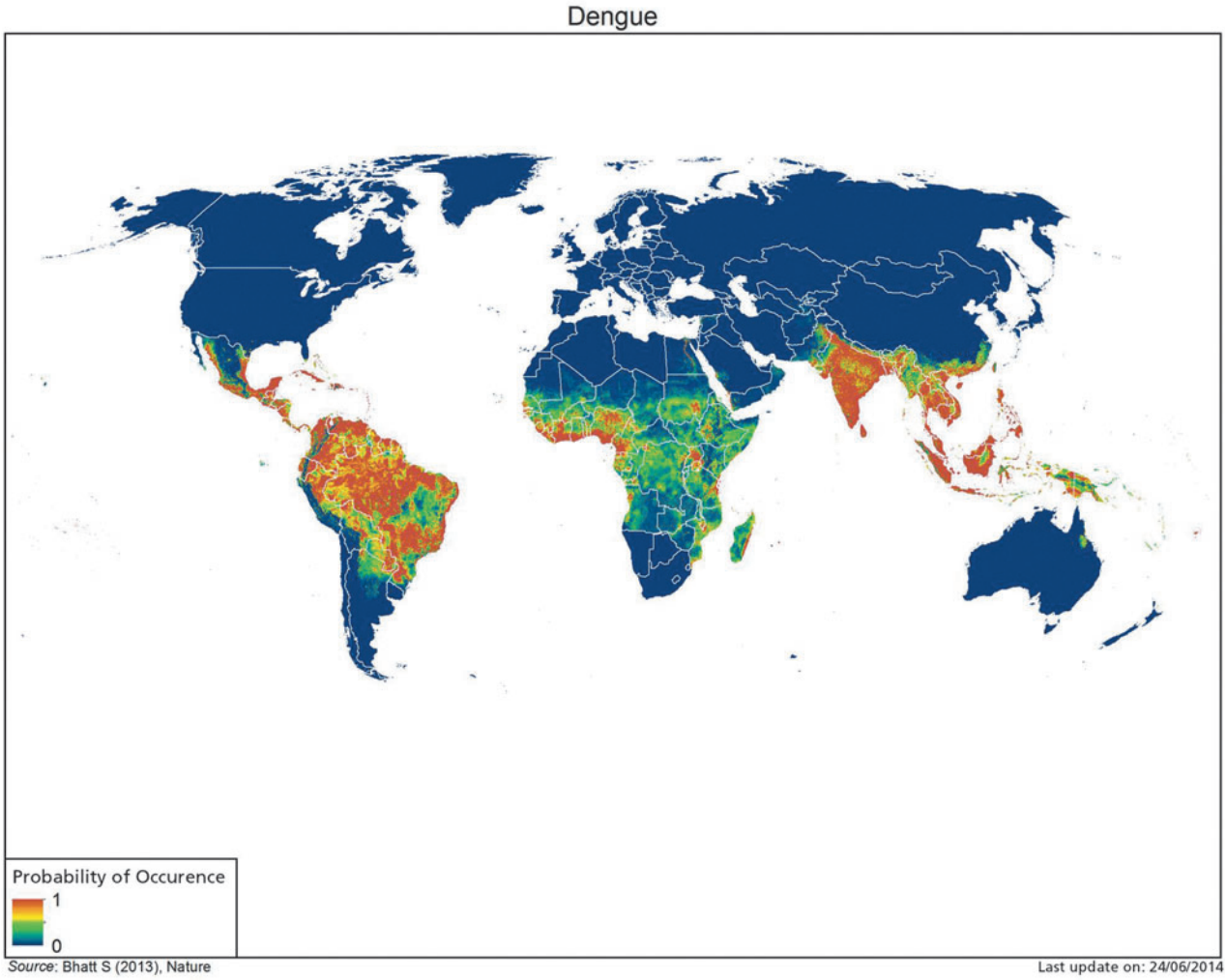

FIGURE 1 Areas at risk of dengue infection (14). Reprinted from the *Atlas of Human Infectious Diseases* (15) with permission of the publisher.

more than 125 countries in Africa, the Americas, the eastern Mediterranean, and Southeast Asia are known to be areas of dengue endemicity (21, 22). Cocirculation of different serotypes in a single region exists, with the dominant serotype fluctuating over time and from place to place (4, 20).

Clinical Symptoms

Clinical presentation of malaria is associated with fever, and in the case of uncomplicated malaria, it includes vomiting, headaches, chills, muscle aches, anorexia, abdominal pain, and diarrhea and may be indistinguishable from gastroenteritis, respiratory tract infections, or influenza (23). In the case of *P. falciparum* malaria in vulnerable patients (such as children and nonimmune travelers), infected red blood cells may create sludge and block the capillaries in deep organs, evolving rapidly to severe malaria with profound anemia, convulsions, coma, respiratory distress, and shock (24), mimicking other life-threatening diseases such as invasive bacterial diseases (25). In regions with cases of so-called stable malaria, transmissions occur all year round and populations are continuously exposed to a high rate of infected mosquito bites. As a result, partial immunity to clinical disease is built up during early childhood, and serious clinical illness is mostly confined to young children. In the case of unstable malaria, inoculation rates vary over time and season and acquisition of immunity is delayed (26).

In settings of endemicity as well as low transmission, there are asymptomatic carriers, i.e., symptom-free infected people carrying malaria parasites including the sexual stages (gametocytes). These carriers play a role in malaria transmission (27). In the case of *P. ovale* and *P. vivax*, dormant liver stages (so-called hypnozoites) may cause infections or relapses months after a person has left malaria-endemic regions or after successful treatment of blood stages, whereas *P. malariae* may persist subclinically in the blood and cause disease many years after inoculation. For *P. falciparum* malaria in travelers, median delay of onset is 5 days after return, but clinical disease up to 9 years after return from a country where malaria is endemic has been described (28).

DENV infection has a wide clinical spectrum, ranging from asymptomatic infection to potentially fatal dengue hemorrhagic fever and dengue shock syndrome (29). The classical course of the dengue infection is characterized by a sudden onset of fever, headache, rash, and serious joint and muscle pain (1, 20). Due to the latter, dengue fever is

also known as breakbone fever. Dry cough and vomiting may also occur (30).

Infection with one serotype provides lifelong immunity to that serotype but only partial and temporary protection against the other serotypes. A person can therefore have up to four dengue infections in his or her lifetime.

Burden of Disease

In 2012, approximately 3.4 billion people were at risk of malaria, with an estimated 207 million cases (uncertainty interval of 135 million to 287 million) and 627,000 (473,000 to 789,000) deaths, most of them occurring in children in sub-Saharan Africa. Over the past decade, the burden of malaria has decreased: worldwide, estimated malaria mortality rates fell by 47%, and 12 out of 97 malaria-endemic countries are in the pre-elimination phase of malaria control (31). Among international travelers upon return from endemic countries, approximately 10,000 cases of imported malaria are notified annually, but the actual number may be as high as 30,000 (32).

The global incidence of dengue has grown dramatically in recent decades, and over 2.5 billion people are now at risk (21). In 2013, the WHO ranked dengue as the fastest spreading vector-borne viral disease, with an epidemic potential all around the world due to climate changes, globalization, travel, and viral evolution. Dengue is estimated to cause 50 to 100 million cases annually, with 500,000 severe presentations that require hospitalization, a large proportion of whom are children (33). According to Bhatt (14), the numbers are even higher, and it is estimated that 390 million dengue infections occur annually, of which 96 million have an apparent manifestation. Accurate numbers are lacking due to poor disease surveillance and low levels of reporting and the lack of inexpensive POC tests (22).

Besides the increasing dengue prevalence in endemic settings (21), DENV (34) is an emerging pathogen among tourists traveling to tropical and subtropical regions (35). Dengue fever is (together with schistosomiasis) the second most common cause, after malaria, of hospitalization of travelers returning to the European Union (36–38). About 84% of the dengue cases imported into Europe represent tourists, while only 9% are imported due to immigration (39). This is in contrast to imported malaria, for which migrants and the so-called visiting friends and relatives group account for an increasingly high proportion (up to 83% in Europe), with a particular risk among children (28, 40).

Diagnostic Needs

Malaria can be lethal, and progression to severe malaria with fatal outcome can only be prevented by timely diagnosis and treatment (3). Diagnosis by clinical presentation leads to overdiagnosis and overtreatment (41–43), so the WHO recommends parasitological confirmation of malaria before initiation of treatment (26). Globally, the rate of diagnostic testing in the public sector at the global scale has increased from 67% in 2005 to 77% in 2011, with the lowest rate (47%) in sub-Saharan Africa, exactly where the need is high (44).

Although there is no specific antiviral treatment for DENV infections, early detection and access to proper medical care lower fatality rates in severe DENV infections from 50% to below 1% (21, 30). Early dengue diagnosis can assist patient triage, clinical management, and early supportive fluid treatment and can prevent complications and unnecessary treatments and interventions (45). In addition, early and rapid dengue diagnosis can be useful for surveillance purposes and early epidemic detection. Early notification of dengue cases is crucial for identifying outbreaks and initiating an early response (4, 45).

POC TESTING OF MALARIA AND DENGUE: CURRENT SITUATION

Microscopy: Cornerstone of Malaria Diagnosis

If available, microscopy is still considered the primary method for malaria diagnosis: apart from confirming the diagnosis, microscopy allows differentiation of the *Plasmodium* species (needed to select appropriate treatment) and determination of parasite density and stages with recognition of signs of severity (presence of schizonts or hemozoin pigment in white blood cells). Parasite density is expressed as the number of red blood cells infected with asexual parasites (trophozoites and schizonts) per microliter of blood. The limit of detection of expert microscopy is close to 50/μl, but for inexperienced microscopists it is up to 10-fold higher (i.e., 500/μl) (46).

In endemic settings, microscopy has its drawbacks: it is labor-intensive and its quality suffers from problems with electricity, microscopes, and stains and from a shortage of competent staff (47–51). Malaria RDTs can overcome many of the limitations of microscopy and are largely accountable for the scale-up of parasitological diagnosis (31, 52, 53). Ironically, in nonendemic settings, where resources and infrastructure are available, microscopic competence for the diagnosis of malaria is often lacking due to the low exposure to malaria-positive samples (54). Also, travelers or long-term expatriates are facing the problem of availability of competent malaria diagnosis. The use of standby emergency treatment is increasingly recommended (55) and would ideally be preceded by a high-quality self-diagnostic test.

Dengue Diagnosis: a Combination of Direct and Indirect Methods

Diagnosis of dengue fever may involve direct detection of the virus by culture, nucleic acid, or antigen (Ag) and the detection of antibodies. Because of the dynamics of the DENV infection, the phase of illness has a large effect on the selection of the diagnostic assay (antigen/PCR versus antibodies). Direct methods can be used up to about 1 week after symptom onset, while antibody testing is usually the only available diagnostic option after this initial stage (Fig. 2) (4, 9).

Molecular tests such as reverse transcriptase (RT)-PCR and antigen detection methods directly target viral nucleic acid and NS1 (nonstructural protein 1) antigen, respectively, at a similar window of detection and can provide early diagnosis in febrile patients. However, viral RNA can be detected without detection of antigen and vice versa (56, 57): such differences may be explained by the formation of antibody-antigen complexes reducing free NS1 antigen, the level of viremia (i.e., the numbers of viral particles in the blood), and previous DENV infections. The duration of dengue viremia is extended in patients with primary infections compared with those experiencing secondary infections (5.1 days versus 4.4 days, respectively) (58). In contrast, detectable NS1 levels persist longer in secondary than in primary infections (6 to 12 versus 5 to 6 days after symptom onset) (59). Real-time RT-PCR has the advantage over classical virus isolation that it is

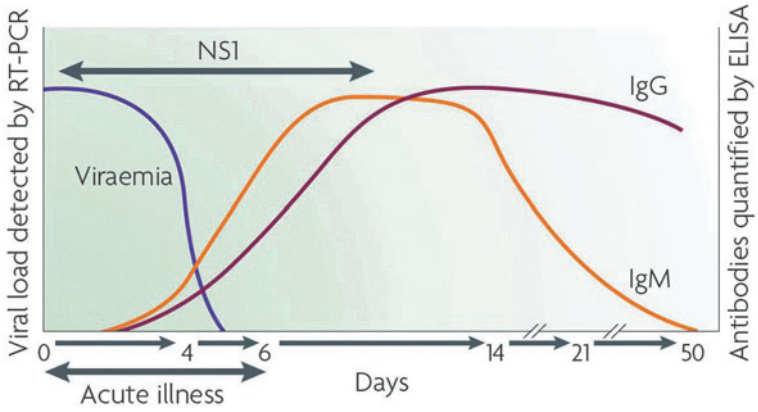

FIGURE 2 Timing of diagnostic tests in a primary dengue infection. Reprinted from reference 4 with permission of the publisher.

very sensitive and fast, allows for serotyping and relatively precise viral load quantification, and has a wider window of detection (4).

After the virus has been cleared from the blood, laboratory diagnosis relies on the detection of circulating antibodies. The acquired immune response to DENV infection consists of the production of IgM, IgG, and IgA that are mainly directed to the virus envelope (E) protein (45). IgM antibodies are usually detectable between 5 and 6 days after onset of symptoms and peak around 2 weeks later. IgM antibodies persist from 2 up to 6 months in primary infections, while in secondary infections, IgM can be detected earlier, demonstrates lower peak levels (60), and persists in the circulation for up to 4.5 months (61). IgG antibodies are detectable starting from about 10 to 15 days of illness for a primary infection and about 4 to 5 days post symptom onset for a secondary infection. IgG antibodies remain detectable for many years. Although less widespread, anti-dengue IgA can also be used as a diagnostic marker. IgA antibodies have a shorter persistence (up to 45 days) in circulation than IgM antibodies and a higher positivity rate in secondary infections. Serological tests exist in several formats, of which the enzyme-linked immunosorbent assay (ELISA) format is still the most widely used. Serological cross-reactions between dengue viruses and other, closely related flaviviruses have been reported and should be taken into account in regions where these flaviviruses are endemic (56). The best diagnostic approach is to combine PCR and/or antigen detection in the early phase and antibody testing in the subsequent phase because this approach makes it possible to broaden the diagnostic window.

Due to the requirement for specialized equipment and experienced personnel for the performance of nucleic acid detection and conventional serological tests, growing attention is being given to RDTs, which are especially of interest for diagnostics in dengue-endemic settings (62, 63). Since RDTs are mainly of interest for diagnosis of acute dengue infections, the focus in this chapter will be on dengue NS1 antigen and IgM antibody detection.

Current Dengue and Malaria RDTs: Lateral-Flow Immunochromatographic Assay in a Cassette Housing

The most widespread platform for dengue as well as malaria RDTs is the lateral-flow immunochromatographic assay: antigens (or in the case of dengue: antigens and antibodies) are detected by an antibody-antigen reaction on a nitrocellulose strip. This strip is mostly embedded in a plastic cassette or occasionally in a cardboard format. In some cases, this strip may present as a dipstick (self-standing strip) to be dipped in a tube or a hybrid format (Fig. 3). Reactions on the nitrocellulose strip are mostly generated with colloidal gold as the indicator and visible cherry-red lines within 20 to 30 minutes. The term "lateral flow" refers to the diffusion of specimen and buffer along the nitrocellulose strip, as opposed to the "flow through" test, in which the specimen moves through the membrane. Figures 4 and 5 show the details of the cassette device and describe the sequence of events of a lateral-flow immunochromatographic test. In resource-limited endemic settings, tests are carried out on capillary blood (obtained through a finger-prick and requiring lancets and alcohol swabs), and a small volume of blood (usually 5 to 10 µl) is applied to the specimen well by means of a transfer device (pipette, straw, capillary, inverted cup) (Fig. 6). Lancets, alcohol swabs, and transfer devices are called accessories and are standard components of most diagnostic kits (Fig. 7).

Several dengue antigen-based RDTs are commercially available.The three leading manufacturers are Bio-Rad Laboratories (France), Standard Diagnostics (SD; Korea), and Alere (United States). These manufacturers market NS1 antigen ELISA formats, from which they recently developed corresponding antigen-detecting RDT assays. Due to changes in company ownership, it may be difficult to trace a particular brand when reading study reports. For example, Inverness acquired Alere in 2007, Panbio in 2008, and SD in 2010. In addition, Inverness changed its company name globally to Alere in 2010. As a consequence, the Panbio dengue early rapid test from Alere (57) and the Panbio dengue NS1 antigen strip from Inverness (64) are basically identical RDTs. The first commercially available and most widely studied antigen RDT is the Dengue Ag Strip test (Bio-Rad Laboratories) (59, 65). Most evaluations using this RDT were done on serum samples (65–67) and/or plasma samples (68, 69). Only SD states that its RDT is designed to detect NS1 in whole-blood samples in addition to serum and plasma. In comparison to the drop of blood (~20 µl) needed for malaria RDT assays, a larger sample volume is needed for dengue antigen RDT assays, ranging from 50 µl (Bio-Rad) to 100 µl (SD). The time to result varies from 15 to 30 minutes. Only SD's

FIGURE 3 Different RDT formats (malaria rapid diagnostic tests). From left to right: cassette; dipstick; auto-transfer cassette (in which the nitrocellulose strip is uncovered at the indentation and can be applied directly to the blood); hybrid format.

antigen-based RDT can be stored at ambient temperature (1 to 30°C), while the RDT assays from Bio-Rad and Alere should be stored at a cool temperature (2 to 8°C) (59).

Antibody-detecting RDTs use recombinant antigens from all four DENV serotypes (70). They performed poorly until the mid-2000s, while second-generation devices have improved sensitivities (59). In general, antibody RDTs have a lower sensitivity than ELISA tests, but they provide results faster, score higher for user-friendliness (70), and are field applicable. Compared to dengue antigen RDTs, many more companies produce or distribute antibody RDTs, which show substantial differences in test performance (71). IgM-detecting RDTs have moderate specificities with false-positive results in patients with malaria, a related flavivirus infection, leptospirosis, rickettsiosis, a previous dengue infection, or the presence of rheumatoid factor (70, 71).

IgM-based RDTs exist in different formats including particle agglutination, lateral flow, and wicked-style immune-chromatographic strips, with or without a plastic cassette. The volume of sample needed is relatively low and varies between 1 µl and 25 µl (59). Specimen type can be serum, plasma, or whole blood (70), and reading time varies between 15 and 90 minutes (59, 70). Most antibody RDTs can be stored at temperatures up to 30°C; only the Panbio duo IgM and IgG Rapid Test Strip must be stored cool (2 to 8°C). One study demonstrated that no reduction in sensitivity was seen over time when antibody RDTs were stored at 4°C, while sensitivity gradually decreased over time when the tests were stored at 35°C (71). In addition to the IgM-/(IgG-) RDTs, a rapid test (MP Diagnostics Assure Dengue IgA Rapid Test) is available for detection of antidengue IgA in serum and blood samples (72, 73).

The combined antigen and antibody detection approach (57, 63, 74) may overcome the problem of NS1 levels being masked/cleared from circulation by circulating antibodies. The combined antigen-antibody RDT consists of two collated devices in one cassette, for which 100 µl of sample is needed for the NS1 detection cassette and 10 µl for the IgM and/or IgG detection cassette (Fig. 8). Attention should be paid to the terms "duo" and "combo," which can refer to the combination of antigen and antibody detection as well as to the combination of IgM and IgG in one test. Studies evaluating dengue antibody- (59, 71) and antigen-based (59) RDTs have been reviewed.

FIGURE 4 Inside view of a malaria RDT cassette showing the nitrocellulose strip with the specimen/buffer pad (left), conjugate pad (red because of the colloidal gold), and absorption pad (right). Note that the cassette has a single specimen (sample)/buffer well.

Antigens and Antibodies Targeted

Plasmodium antigens targeted by commercially available RDTs include histidine-rich protein-2 (HRP2) and *P.*

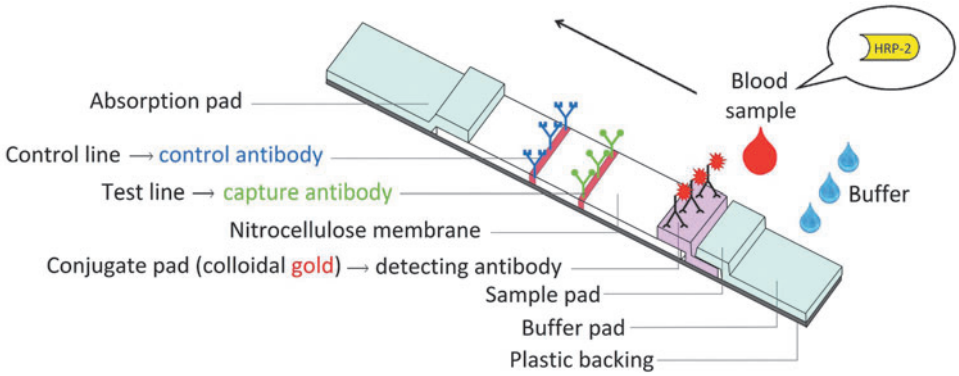

FIGURE 5 Schematic representation of the two-band malaria RDT targeting *P. falciparum* HRP2 antigen. Only the nitrocellulose membrane is displayed. This membrane is glued to a plastic backing. The sequence of events is as follows: (i) whole blood is applied to the specimen (sample) well and is absorbed by the specimen (sample) pad. (ii) Buffer is applied to the buffer well and is absorbed by the buffer pad. (iii) Migration of the blood/buffer mixture starts toward the opposite end of the membrane (attracted by the absorption pad). (iv) The blood-buffer mixture passes the conjugate pad, which contains detection antibodies directed to the target. These detection antibodies are conjugated to colloidal gold. If present in the specimen, the target antigen binds to this detection antibody-conjugate. (v) The antigen-antibody-conjugate complex migrates farther and binds to the capture antibodies present on the test line. These capture antibodies bind to another site (epitope) of the target antigen. (vi) The capture antibodies are applied on a narrow section of the test strip: as a result, the antibody-conjugate with the colloidal gold will be concentrated and become visible as a red line. (vii) The excess of the detection antibody-conjugate that was not bound by the target antigen and the capture antibodies moves farther until it binds to a goat antimouse control antibody. There, the colloidal gold will create a colored control line.

falciparum-specific parasite lactate dehydrogenase (Pf-pLDH), which are both specific for *P. falciparum*; there is also *P. vivax*-pLDH (Pv-pLDH) and pan-pLDH and aldolase (common to all human *Plasmodium* species). HRP2 is a stable protein produced by asexual forms and young gametocytes of *P. falciparum* (75–78). It is slowly cleared from the body and may therefore persist in the blood (and cause positive testing) for more than 5 weeks after effective treatment of infection (79, 80). The enzymes pLDH and aldolase are used in the glycolytic pathway of all *Plasmodium* species and are produced by both sexual and asexual forms (81).

Malaria RDTs are further categorized as two-, three- or four-band products, depending on the number of lines ("bands") next to the control line. Most common are two-band RDTs (detecting a single antigen), whereas three-band RDTs detect *P. falciparum* antigen (HRP2 or Pf-pLDH) and mostly a pan-malaria antigen (pan-pLDH or aldolase) (Fig. 9). Four-band RDTs detect a *P. falciparum*-specific antigen, a pan-malaria antigen, and a *P. vivax*-specific antigen. Although there are malaria RDTs that detect anti-*Plasmodium* antibodies, they have no place in acute patient management, because they do not distinguish between current and past infection. Their use as a tool for surveillance in the scope of malaria elimination is currently being explored (44).

NS1 is a highly conserved nonstructural glycoprotein of about 50 kDa with a high homology among flaviviruses (82). This hexamer protein is secreted from DENV-infected cells and circulates in sera of infected patients during the acute phase (83, 84). NS1 levels in circulation range from several nanograms to micrograms per milliliter of blood (83). Although its function is not yet clear, one study demonstrated that soluble NS1 protein can bind to endothelial cells and could contribute to plasma leakage during severe DENV infection (85). More recently, NS3 was evaluated as a potential diagnostic target antigen, and good agreement between recombinant NS3 ELISA and NS1 antigen detection was observed (86).

Performance Characteristics of RDTs

According to a Cochrane meta-analysis (compiling diagnostic accuracy for 74 studies published up to January 2010) (87), the sensitivity of HRP2-detecting RDTs detecting uncomplicated *P. falciparum* malaria in endemic settings is slightly but not significantly higher than that of Pf-pLDH-detecting RDTs (95.0% versus 93.2%, $p = 0.34$), particularly at low parasite densities (<100 asexual parasites/μl). This higher sensitivity comes at the expense of a lower specificity (95.2% versus 98.5%), in part explained by the persistence of HRP2 after cure of a previous infection. For diagnosis of *P. vivax*, reported sensitivities for pan-pLDH-, Pv-pLDH-, and aldolase-detecting RDTs are, respectively, 76.1 to 100.0%, 76.9 to 100.0%, and 80.0 to 81.4% (5). For *P. ovale* and *P. malariae*, the limited data available indicate low sensitivity of RDTs (88), and although there is cross-reactivity of *P. knowlesi* with pLDH antibodies, current RDTs lack the diagnostic sensitivity to reliably detect infection with this species (89).

For all *Plasmodium* species, RDT sensitivity declines at lower parasite density; in reference settings, there is a sharp decline in sensitivity at parasite densities below 100 asexual parasites/μl (6), a threshold at which vulnerable patients may still be symptomatic (90). The WHO Foundation of Innovative New Diagnostics assesses RDT products in side-by-side comparisons; these product testing rounds give detailed and updated information about the diagnostic performance (including after temperature challenges) of individual RDT brands (91). Factors that may explain the discrepancy between reported results of diagnostic accuracy of malaria RDTs include, apart from the product itself (including lot-to-lot variations) (53), the

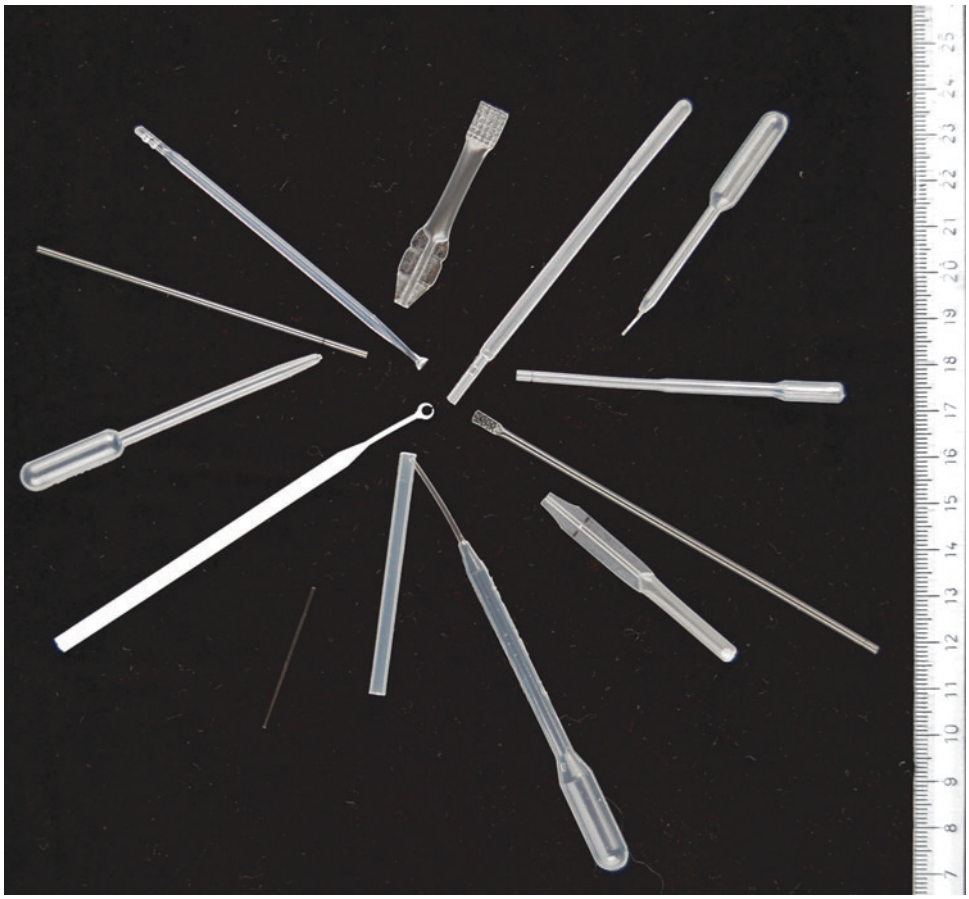

FIGURE 6 Specimen (blood) transfer devices. Most malaria RDTs require only 5 or 10 µl of whole blood. The depicted devices include pipettes, straws and capillaries (some of which are very tiny), a loop (at 8 o'clock) and an inverted cup (at 11 o'clock). Some devices have no volume mark.

local malaria epidemiology, the reference method used (microscopy versus molecular methods), as well as the type of end user, e.g., experienced laboratory worker versus community health care worker (see below).

Since the mid-2000s, advances in the development of DENV NS1 antigen ELISA assays indicated the promising diagnostic potential of NS1 in early dengue diagnosis (83). Commercial NS1 ELISA kits demonstrated excellent specificities but needed further improvement for sensitivity. Only very recently, lateral-flow formats for detection of NS1 antigen became available and offered an even faster path to dengue diagnosis with little loss of sensitivity.

The first published evaluation of a commercially available dengue NS1 RDT appeared in 2008. Dussart et al. (65) tested a panel of 222 serum samples obtained from RT-PCR- or viral isolation-confirmed dengue cases collected in French Guiana. The DENV NS1 Ag Strip rapid test kit (Bio-Rad laboratories) showed a sensitivity of 81.5% after 15 minutes of reading and 82.4% after 30 minutes (for ambiguous results). The specificity was 100%. The higher sensitivity with prolonged reading times was also observed in other studies without affecting the specificity (67, 69). Other factors also influence the sensitivity of the NS1 Strip test. One factor is the day of analysis after symptom onset, with increasing sensitivity seen from day 0 to 3 and on day 6, the latter probably due to late release of NS1 from capillary blood from endothelium or hepatocytes (65). Another study showed a more gradual decrease in sensitivity with increasing days after onset of illness (68). Further, the presence of IgG antibodies has a negative effect, possibly due to substantial amounts of complexes formed by NS1 and IgG during secondary infections (68, 92, 93). No significant difference in sensitivity between the four serotypes was observed in some studies (65, 67), while others showed lower sensitivity for DENV2 serotype (68, 94). Finally, NS1 sensitivity is significantly higher in patients with high viremia levels (68, 92). Overall, the NS1 RDT was demonstrated to be suitable as a first-line test in the field; it only needs a micro-centrifugation device for serum separation, and it can be performed in parallel with an RDT for malaria in areas in which these diseases are coendemic (65).

A study in Taiwan performed between 2008 and 2012 (94) assessed the role of the same NS1 Ag Strip in routine dengue diagnostics in addition to real-time RT-PCR and IgM/IgG ELISA. The overall sensitivity was 68.4% for the antigen RDT and 71.9% for PCR. Diagnosis would have been missed in 4% of the cases when NS1 RDT was not used and in 21% of the cases when RT-PCR was not used. A diagnosis was made in 11% of patients with the NS1 Ag Strip without the need for a second, convalescent sample.

Similar results were observed in a study of Malaysian researchers in 2011 (95), with sensitivities of 72.8%, 52.8%, and 44.0% for antibody, RT-PCR, and antigen testing, respectively. Of particular interest, the detection rate of

FIGURE 7 Lancets for capillary blood samples. Plain metal lancets (below, in paper packaging), "safety-seal" lancets (upper right, blue; the plastic cap has to be unscrewed to free the lancet's point), auto-retractable lancets (upper left).

infection was similar for the combination of RT-PCR and antibody assays (65.9%) and antigen combined with antibody testing. The NS1 RDT can thus be used in peripheral laboratories instead of RT-PCR that is mostly restricted to well-equipped reference laboratories. Another study demonstrated the usefulness of the on-site NS1 RDT in screening imported dengue cases at airports (66).

FIGURE 8 A dengue duo cassette for combined antigen-antibody detection. (Left) An acute-phase serum sample positive for NS1 and dengue IgM. (Right) Serum that is positive for dengue IgM only.

A second well-known antigen RDT is the SD Bioline Dengue rapid test (SD), which is available as a single-cassette device for detection of NS1 alone or as one cassette containing two devices (SD Bioline Dengue Duo kit), with the dengue NS1 antigen test on the left and at the dengue IgG/IgM test on the right. To our knowledge, only one study has described the use of the single SD Bioline NS1 antigen test (64). The SD Bioline showed a sensitivity of 48.0% compared to the Bio-Rad Strip test and Panbio NS1 test, with a sensitivity of 58.6%, which is lower than previously reported, probably due to the high proportion of secondary infections typically seen in an endemic setting such as Sri Lanka (64). In our experience as a national reference center (ITM, Antwerp, Belgium) with parallel testing of the SD Bioline NS1 Ag test and dengue multiplex RT-PCR (96) on acute-phase serum samples, we saw more than 94.0% agreement between dengue NS1 and RNA detection. Using the SD Bioline Dengue Duo kit in a study of Vietnamese patients (92), sensitivity was 62.4% for NS1 detection alone, and specificity was 100.0% with RT-PCR as the reference method, similar to what was observed on the same panel of samples for the Bio-Rad Strip RDT (sensitivity 61.9%, specificity 100%).

The third antigen RDT is the Panbio dengue Early Rapid kit (Inverness), which demonstrated a lower specificity compared to the Bio-Rad and SD NS1 RDTs. False positivity was seen in serum of patients with chikungunya, leptospirosis, scrub typhus, Q fever, and bacteremia (64). Fry et al. (57) evaluated the RDT at two study sites. At the Vietnamese study site, a sensitivity of 69.2% and

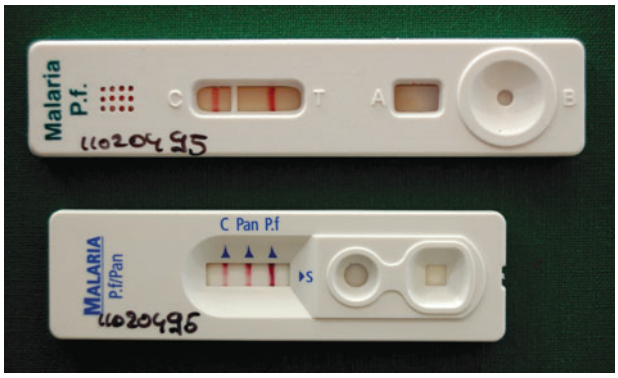

FIGURE 9 Two- and three-band malaria RDT (above and below, respectively) run with whole blood from a patient infected with *P. falciparum*. Control and test lines are visible as cherry-red lines. The three-band test shows a visible test line for the "P.f" (*P. falciparum*) target antigen as well as for the "Pan" antigen (in this case pan-*Plasmodium* lactate dehydrogenase, reacting with all four common *Plasmodium* species).

specificity of 96.0% were observed, similar to the study site in Malaysia, which showed a sensitivity of 68.9% and specificity of 96.7%. The analytical sensitivity determined with recombinant NS1 was <5 ng/ml for all serotypes, with a limit of detection of 0.25, 0.75, 2.4, and 3.49 ng/ml for DENV2, DENV1, DENV4, and DENV3, respectively.

In general, the commercially available IgM-detecting RDTs have a very modest specificity, particularly in samples from patients with other infections such as malaria (70). SD's first-generation IgM-based RDT also had a very poor sensitivity, which was improved in the second-generation SD IgM assay (59). In a study that compared the performances of four IgM-detecting RDTs over seven countries, the Hapalyse dengue-M PA kit (Pentax, Japan), for which the assay principle is based on particle agglutination, performed significantly better for sensitivity (97.7%) but lower for specificity (76.6%) compared to the other three lateral flow IgM-detecting RDTs, for which sensitivity varied between 20.5 and 77.8% and specificity between 86.7 and 90.6% (70). The accuracy quoted by the manufacturer (sensitivity: 97.6%, specificity: 98.3%) might also differ from the observed accuracy as was the case for the Immunoquick dengue fever RDT (Biosyntex) for IgM detection, with a sensitivity of 79.8% and specificity of 46.3% when evaluating samples from Sri Lankan patients with fevers (59). Although many manufacturers claim that antibody-detecting RDTs are able to differentiate primary from secondary infections, it was demonstrated that most RDTs cannot reliably differentiate the infection states (64). More details on antibody-based RDTs for dengue diagnosis can be found elsewhere (64, 70, 71). The accuracy of IgA-RDT is rather high, with a sensitivity range of 86.7 to 99.4% and a specificity range of 86.1 to 100.0% (72, 97).

As mentioned above, the combined use of antigen and antibody rapid testing approaches is the most effective diagnostic application. The sensitivity of the SD Dengue Duo (NS1/IgM/IgG) lateral flow RDT improved significantly from 62.4% when only NS1 was detected to 75.5% when NS1 and/or IgM were detected, and 83.7% when NS1, IgM, and/or IgG were detected (92). Another study showed even better results with an increase in sensitivity from below 70% to above 90% when antigen detection was combined with antibody detection (57). A slightly lower sensitivity (89.9% versus 92.9%) and significantly lower specificity (75.0% versus 88.8%) of the Panbio combined antigen-antibody RDT were seen in comparison to the SD Duo test when evaluated on the same panel of samples (64). In an evaluation of samples of hospitalized patients in Cambodia, the performance was dependent on the setting, with a sensitivity of 85.7% and a specificity of 83.9% in provincial hospital laboratories and 94.4% and 90.0%, respectively, in the Cambodian reference laboratory (63). The SD Dengue Duo RDT, with a sensitivity of 93.9% and specificity of 92.0%, showed no significant differences in performance when testing early/late presenters, primary versus secondary infections, or DENV1 versus DENV2 infections (74).

Rapid Diagnostic Tests in Resource-Limited Settings: Pursuing the ASSURED Criteria

Given the nonspecific clinical picture of malaria and dengue and its overlap with other infectious diseases, diagnostic tools need to be sufficiently accurate. Because most malaria and dengue infections need to be diagnosed in resource-limited, remote settings, diagnosis faces other challenges: a laboratory infrastructure with a stable, uninterrupted power supply and dust-free, climate-controlled rooms is rare, especially in combination with qualified and trained laboratory staff. Moreover, a regular supply chain (transport and storage facilities) may be lacking or deficient, precluding an uninterrupted supply of quality-assured stains and laboratory consumables. The current evolution toward more decentralized diagnosis (community health care workers, private sector, home-based malaria management) is increasing the need for simplicity and robustness but also for user-friendliness and harmonization of rapid diagnostic testing (44). The requirements for RDTs deployed in resource-limited settings have been aggregated in the acronym ASSURED, referring to the following criteria: affordable, sensitive, user-friendly, rapid and robust, equipment-free, and deliverable to end users (98). Table 1 lists the achievements and challenges in meeting the ASSURED criteria for current malaria RDTs.

Only the more recently published papers on dengue diagnostics using RDTs (either antigen- or antibody-based) (59, 74) mention the ASSURED criteria and highlight that the characteristics of an ideal dengue diagnostic test is defined by these criteria. The price per dengue RDT ranges from $3 to $15 (70). Dengue RDTs will probably be more *affordable* when the production scale increases and their use has become widespread. Dengue RDT assays demonstrate sufficiently high *sensitivity*, especially when antigen and antibody detection is combined. Antigen RDTs are very *specific*, while antibody RDTs lack specificity. Specificity must be considered in the context of the setting in which a test is to be used (70). The challenges for dengue assays are the combined need for sensitivity for all four serotypes and restricting cross-reactivity with other infectious agents that circulate in the same setting (57). Dengue RDT assays score well for *user-friendliness*. Andries et al. (63) observed differences in antibody RDT result interpretation between experienced personnel in a reference laboratory and untrained hospital technicians and health care workers, while good agreement in the NS1 RDT results was observed in both groups. The same type of end-user errors as seen for malaria RDTs (weak signal misinterpretation, RDT result reading before/after the recommended time, etc.) can occur. Most dengue RDTs give *rapid* results, especially the antigen-based RDTs; some antibody-based RDTs need more than 30

TABLE 1 ASSURED criteria recommended for POC rapid diagnostic tests for malaria in resource-limited settings (98)

Acronym	Meaning	Achievements	Challenges
A	Affordable	The weighted average public sector price for a *P. falciparum* two-band and a *P. falciparum*/pan three-band RDT in 2013 was $0.32 and $0.38, respectively (44)	Small benefit margins and a volume-driven market offer few incentives for investment in quality and innovation
S	Sensitive	Sensitivity: In round 4 of the WHO/FINF Product Testing Programme (2012), no RDT products met the minimal performance thresholds.	Sensitivity and specificity are generally much lower in field versus reference laboratory testing, and most performance data are obtained only from reference settings. This may be due to differences in target population, malaria prevalence, transport and storage conditions, lot-to-lot variations, as well as to end-user errors. The HRP-2 antigen may persist for several weeks after past or treated *P. falciparum* infection. Diagnostic algorithms should include the possibility of (bacterial) coinfections. Concurrent infections (schistosomiasis, hepatitis B, dengue, sleeping sickness, etc.) as well as rheumatoid factor may cause false-positive results Diagnosis of malaria in pregnancy and detection of asymptomatic carriers in the scope of (pre-)elimination requires lower limits of detection
S	Specific		
U	User-friendly	Training of end users is relatively easy Low-skilled end users such as community health care workers and retail shopkeepers have been proven able to perform RDTs accurately and to master treatment strategies and stock management when provided training and supervision.	End-user errors are observed (some of them consistently) related to performance and reading/interpretation. The plethora of commercially available RDT products with a wide variation in procedures (volume of sample and buffer, reading time, instructions) and accessories (lancets, transfer devices, alcohol swabs) present a challenge to malaria control programs when changing from one product to another, a practice dictated by commercial objectives. Shortcomings in packaging and instructions may add to end-user challenges.
R	Rapid and robust	RDTs are environmentally stable; some of them are stable up to 40°C RDTs have a shelf life of 18 to 24 months.	Humidity and heat may exceed limits recommended by manufacturers and cause decreased performance There are currently no tools for quality control in the field or at the bench RDT manufacturing level of quality is not known
E	Equipment-free	No need for laboratory infrastructure nor electricity	
D	Deliverable to end user	Long shelf-life (2 years from production date) No need for a cold chain Temperature stability up to 45°C Globally, diagnostic coverage (% of suspected cases confirmed by parasitological diagnosis) increased to 77% in 2011 (44) In 2012, 205 million RDTs were sold, versus 45 million in 2008.	Weak supply chains may limit the availability of RDTs on the spot. Health care workers, clinicians, and patients may be reluctant to accept RDTs Improved management of nonmalaria febrile illnesses will reinforce use of and adherence to malaria RDT results Diagnostic coverage is lower in Africa (47% in 2011) Diagnostic coverage is still much lower in the private sector where awareness is lower and market penetration will be more difficult The estimated need for RDTs exceeds the current market size, and there is concern about a funding gap

minutes. Improvement is needed for *robustness* because some RDTs need storage between 2 and 8°C. Although the RDT itself is *equipment-free*, it is notable that most RDTs are evaluated on serum, which still needs centrifugation for separation. Dengue antigen RDTs have only been introduced recently and their use is not yet widespread, so dengue RDTs are not yet *delivered* to those who need it most.

The Use of Malaria RDTs Is Expanding

Being instrument-free test devices that are capable of providing results within 30 minutes, handheld lateral-flow immune-chromatographic malaria RDTs offer simplicity, robustness, and environmental stability. The best products among them also display excellent sensitivity and specificity. They require minimal training and expertise and can be performed by less skilled health care workers (26, 52, 99). In endemic settings, sensitivity for diagnosis of *P. falciparum* infections by well-performing RDTs has been shown to be equal or superior to routine microscopy, and malaria RDTs appear to be more cost-effective than microscopy (6, 100–103). Likewise, two prospective studies in nonendemic settings concluded that in routine diagnostic laboratories, RDTs performed equally well or even better than microscopy (104, 105). In addition, the idea of self-testing by travelers (raised 15 years ago but abandoned at that time in view of poor RDT performance by sick travelers [106–109]) has been revived, because simple two-step RDTs are currently marketed through the Internet (110).

As a result, the use of RDTs is expanding in both endemic settings and travel medicine: from less than 200,000 RDTs used worldwide in 2005, the number increased to 205 million in 2012 (44).

In malaria-endemic settings, RDTs are mainly procured through the public sector, with most (78%) of the 108 million RDTs delivered in 2012 being used in Africa (31). Because many countries are still in the process of scaling up, an increase in volumes is expected (52). In addition, to achieve universal diagnostic coverage, investments are being made to introduce RDTs into the private sector, including private clinics and pharmacies, but also retail outlets (31). In Europe, the use of RDTs in travel medicine is also expanding (111), with 80.0% of laboratories declaring that RDTs had improved their malaria diagnosis (54).

RDT Limitations Inherent to Product Design and Engineering

Despite being robust, reliable, and simple, malaria RDTs are not fail-proof and have their limitations, which may be intrinsic to design and engineering, caused by conditions of transport and storage, or linked to end-user errors.

As to the design and engineering of malaria RDTs, there are the inherent limitations of diagnostic performance described above. Of note, the persistence of HRP2 after cured infection is a disadvantage in the endemic setting but may be advantageous in the nonendemic setting, because it allows *a posteriori* diagnosis of malaria in a returned traveler consulting after self-administered or empiric treatment for suspected malaria abroad (6, 112). Further, unlike microscopy, malaria RDTs do not assess parasite densities, they do not differentiate parasite stages, and they do not recognize signs of severity. In addition, the pan-pLDH and aldolase test lines do not differentiate between the different *Plasmodium* species.

False-negative test results are mostly associated with low parasite densities (<100/μl), but faint test lines or—very rarely—absent test lines may also occur at very high parasite densities: this observation is known as the prozone (or high-hook) effect and affects HRP2- but not pLDH-detecting RDTs (113). Further, deletions in the HRP2 gene may result in the absence of production of the HRP2 antigen and cause false-negative HRP2-detecting antigen results, a problem which appears to be confined to the Peruvian Amazon region (114, 115).

Because malaria RDTs do not include a wash step, nonspecific binding may occur. False-positive results can occur, among others, in the presence of rheumatoid factors, antinuclear antibodies, and viral (hepatitis B or hepatitis C, dengue) and parasitic infections (schistosomiasis and human African trypanosomiasis) (112, 116–119). Overall, the prevalence of these false-positive reactions is low, although specificity decreased to 11.3% for one RDT product in a study of patients with human African trypanosomiasis (118). Cross-reactions between *Plasmodium* species and target antigens have been observed in both directions for *P. falciparum* and *P. vivax*, particularly at high parasite densities (115, 120).

Exposure to high humidity or extreme temperatures (both heat and freezing) during transport and storage weakens the binding of the antibodies to the nitrocellulose membrane and affects sensitivity. RDTs that detect pLDH appear to be somewhat more susceptible to heat damage compared to HRP-2-detecting RDTs (121).

A Wide Range of End Users

It is a well-known observation that the diagnostic performance of *in vitro* diagnostics (IVDs) is lower in daily routine or field settings than in reference settings, and this has been observed for malaria RDTs too, in both the endemic and nonendemic settings (5, 122). Discrepancies may be ascribed to differences in patient populations and environmental conditions, product characteristics, or lot-to-lot variations, but the end user is a major determining factor.

In endemic settings, the end user may not be a fully trained laboratory professional, and concerns may arise because many RDTs are labeled "intended for professional use." However, ISO 18113 (123) defines a professional user as "qualified to perform IVD testing through special education and training," thereby allowing enough flexibility to extend the use of malaria RDTs to nonlaboratory health care personnel including community health care workers and actors from the private sector, provided adequate training and supervision are in place. In contrast, in regulatory terms, self-testing refers to testing with IVDs by a lay person—defined as an individual with no formal training in a relevant medical field of discipline—and dedicated regulations for IVDs apply (see below) (123–125).

End-User Errors

Studies of end-user performance are rare; most field studies of malaria RDTs assessed the accuracy of RDTs *per se* rather than during routine unsupervised use. An external quality assessment conducted in a nonendemic setting showed excellent analytical performance of RDTs, but errors occurred in interpretation and reporting: not mentioning the possibility of a mixed species infection, not recognizing invalid test results, reporting "positive" without mentioning the presence of *P. falciparum*, etc. Some of these errors were traced down to inadequacies in the RDTs (126). An external quality assessment of reading and interpretation of malaria RDTs by means of high-resolution photographs in DR

Congo identified most frequent errors as (i) disregarding (or interpreting) faint test lines as negative, (ii) failing to recognize invalid test results, and (iii) incorrectly identifying *Plasmodium* species (48). A compilation of end-user errors reported from field settings can be found elsewhere (5). Reading beyond the recommended time appears to be a frequent error (127): this can cause false-positive results due to the so-called backflow phenomenon, consisting of back diffusion of the excess unbound conjugate with passive deposition at the place of the test line. In addition, application of the correct specimen and buffer volume may cause problems, as also observed in self-testing by expatriates (6).

Compliance with RDT Results and Clinical Algorithms

Compliance of health care workers with the results of malaria RDTs varies widely, with overtreatment (prescription of antimalarials in the case of a negative RDT result) observed in 17% up to half of observed health care workers. Of interest, community health care workers showed good adherence to RDT test results. In addition, the perception of RDTs by the patient and the clinician may influence adherence. Clinicians' confidence may be eroded if diagnosis of malaria has been missed, and patients may insist on treatment despite a negative RDT result (128). Further, implementation of malaria RDTs will have the most impact if they are viewed as a tool for the management of febrile illness, not just malaria (129). Indeed, the widespread use of malaria RDTs has shown that many fevers are in fact not attributable to malaria, and it is expected that improved diagnosis of nonmalaria febrile illnesses will increase adherence to RDT results (128, 130–132). Because most malaria-endemic settings are resource-poor, affordable diagnostic tests for such infections are badly needed. Likewise, clinical algorithms should take into account that a positive malaria RDT result does not necessarily mean the patient's symptoms are due to malaria: besides persistence of HRP2 antigen, there may be invasive bacterial infections (such as nontyphoid salmonellosis), which tend to occur together or shortly after (i.e., when HRP2 is still detectable) severe *P. falciparum* malaria and are clinically similar to severe malaria (131).

Also, for dengue RDT results, a retrospective study (63) showed that physicians did not take the RDT results into consideration for care management or antibiotic therapy.

The Expanding Market for Malaria RDTs: A Need for Harmonization

The expansion of the malaria RDT market has been unprecedented but has created specific challenges. Currently, it is estimated that there are at least 200 RDT products on the market (5, 16, 132, 133), which vary in both type and design of test device (shape of cassette, number of wells), accessories, and procedure (specimen volume, numbers of buffer drops, reading time). In addition, there are many differences in labeling and terminology for the RDT components and accessories (52). This diversity and variation have an impact on user-friendliness and increase the demands for procurement, supply, training, and supervision when national malaria control programs switch from one RDT product to another (which is a common market strategy), particularly during the early phases of RDT introduction at the country level (52). Currently, the Roll Back Malaria Partnership (comprising manufacturers, RDT implementers from national malaria control programs, international procurers, and experts in regulatory affairs) is developing an action plan for enhanced RDT harmonization. Harmonization is expected to have additional beneficial effects, such as improving adherence to manufacturers' recommended procedures and reduction of operational errors.

The Malaria RDT Market: Scale and Price Driven, with Concerns About Quality

The IVD market in resource-limited settings is poorly regulated, with minimal quality standards and postmarket surveillance in place (52). Despite the scarcity of information, there is evidence that substandard RDTs are widespread (9). In addition, international regulations are of little value: as an example, for products not listed in Annex II of the European Community Directive 98/79 on IVD devices (comprising, among others, malaria and dengue RDTs), Conformité Européenne (CE) certification (permission to affix the "CE mark" label) depends only on self-certification by the manufacturer (134).

The malaria RDT market emerged more than 10 years ago in an era of limited regulation. The market is highly competitive and is driven by large volumes, small profit margins, and short lead times. There is concern that this market pressure impedes quality (52). Indeed, shortcomings in design, construction, and labeling of RDTs and accessories have been observed (both in CE-marked and non-CE-marked products), and lot-to-lot variation of performance is a well-known phenomenon (53). Likewise, there is concern about products for self-testing that are marketed through the Internet, because they are of varying quality as well (9). Figures 10 and 11 show examples of shortcomings of malaria RDTs and their accessories. Several initiatives to improve and ensure the quality of malaria RDTs have been developed, such as the WHO-FIND Malaria RDT Product Testing and Lot Testing Programs, the WHO RDT Procurement Guidance, and the WHO Prequalification of Diagnostics Program (16, 91, 135, 136). With these programs, WHO provides quality control by inspection of products and manufacturing sites, systematic assessment of RDT products and their performance, a region-based lot control program, and provision of job aids and training material.

The Role of Packaging, Labeling, and Instructions

Some of the end-user errors in performing malaria RDTs (described above) were traced to shortcomings in RDT instructions and labeling (126). Labeling of IVDs—which includes the instructions—is crucial because it assists performance, quality, and safety. Labeling should be appropriate to the technical knowledge, experience, education, and training of the intended users; this means it should be easily understood by end users in resource-limited tropical settings who may only be basically educated and trained (99, 133, 137–139). Instructions for malaria RDT products have shown shortcomings with regard to content (e.g., discrepancies between depicted versus real cassettes and accessories, not mentioning all line combinations in case of invalid results), style (too high reading levels) and layout (too small font sizes and low interline distance) (126, 140) (Fig. 12). As guidance for manufacturers and with the aim of standardizing malaria RDT products, the Roll Back Malaria Partnership has recently aggregated best practices for labeling and instructions, which can be relevant for other POC tests too (141).

Because the risk of performance errors is even higher in the case of self-testing, clear and well-written instructions

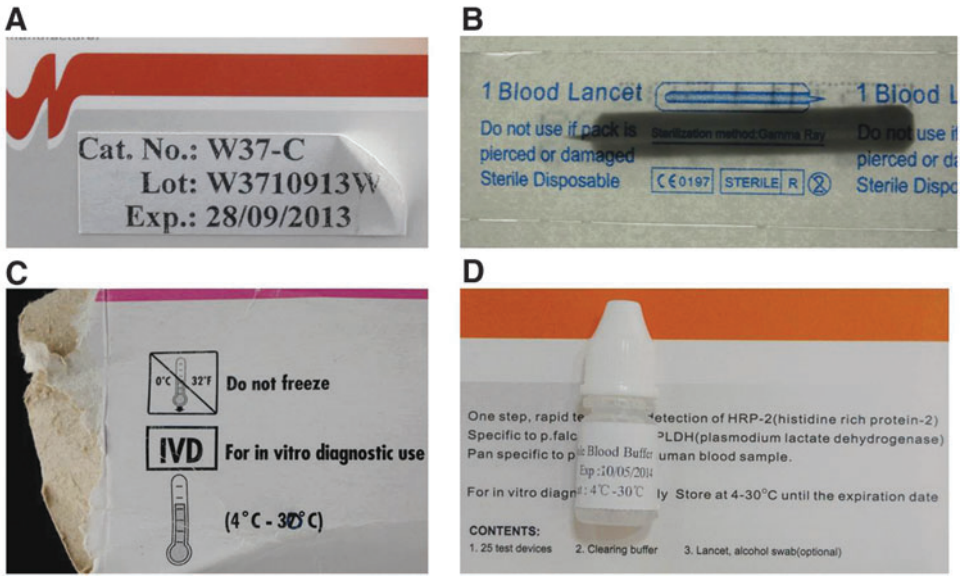

FIGURE 10 Shortcomings of quality in design, construction, and labeling of RDT components and accessories. (A) Non-humidity-proof label poorly glued to the RDT box. (B) Incorrectly packed lancet: point is directed to the end opposite to what is indicated on the package). (C) Poor-quality cardboard RDT box; temperature symbols are not internationally recognized; symbol below is over-written by hand. (D) Terminology: simultaneous use of different terms: "clearing buffer" and "blood buffer."

are even more important: guidance and regulatory documents such as those put out by the International Medical Device Regulation Forum (137) and the European Union (IVDD 98/79/EC) (134) list special requirements for IVDs marketed for self-testing. The International Organization for Standardization (ISO) document ISO 18113 (123) (adopted as the European Union standard) has a section addressing self-testing (ISO 18113-4). Instructions for use of IVDs marketed for self-testing must, apart from being easy to follow and understandable for laypeople, reduce the risk of errors in use or interpretation to the minimum and mention which actions are to be taken in case of positive/negative results, including when to consult a health care professional. In the case of IVDD 98/79/EC non-Annex II IVDs (such as malaria RDTs) marketed for self-testing, conformity assessment for the CE label requires independent certification by a notified body (134). The U.S. Food and Drug Administration (FDA) has approved one RDT (BinaxNOW Malaria test, Alere) for use in the United States by health care facilities, but not by individual clinicians or by patients themselves (142).

Quality Assurance for RDTs in Laboratory Use

To overcome the problem of substandard products and lot-to-lot variation, the WHO provides quality control of products and lots as described above. There are, however, few if any controls at the bench. Of note, the control line only witnesses correct migration of the specimen/buffer/colloidal

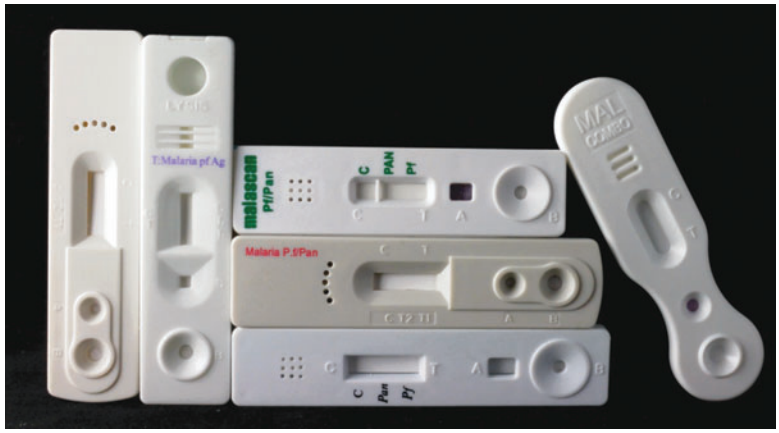

FIGURE 11 Examples of design and labeling of malaria RDT cassettes: different shapes and surface structure (with embossments in the plastic housing impeding writing the patient's name), reading legends as printed symbols displaying the *Plasmodium* species targeted versus embossed universal characters (T and C). Some cassettes have double reading scales. The second cassette from the left has a large evaporation hole (labeled "lysis") which may be mistaken for the specimen well.

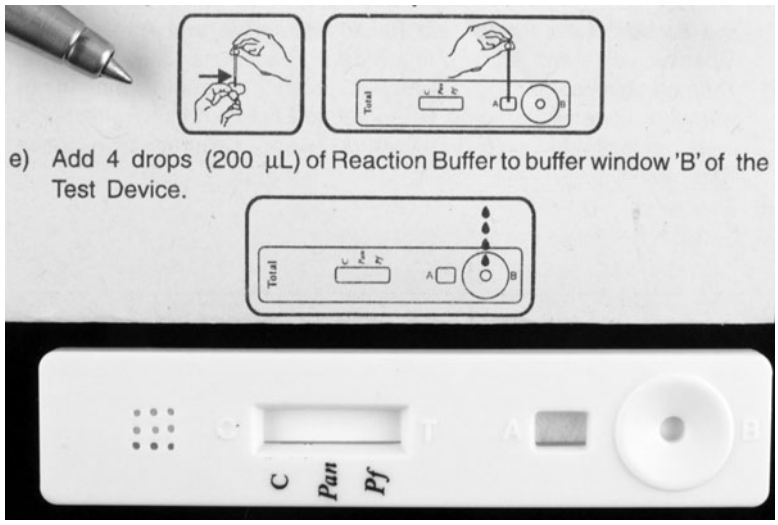

FIGURE 12 Shortcomings in instructions for use of malaria RDTs that detract from user-friendliness. (Top left) Shows sampling without gloves. (Top right) Left-handed application of the sample. (Bottom) Depicted cassette is different from the actual cassette (photograph below): evaporation holes are missing and labeling at the distal end is not displayed on the cassette; the reading legend at the reading window is shown on the opposite side. On the photo, note the double reading legend (printed characters as well as "C" and "T" characters embossed in the plastic housing.

gold complex along the nitrocellulose membrane but, for instance, does not detect inadequate specimen volumes. FIND has developed positive controls (freeze-dried recombinant parasite antigen) which are currently being evaluated in field settings (143). Pending this, quality assurance can be done by cross-checking with microscopy (144). In addition, indicators such as invalid test results and proportions of faint and weak test lines can be monitored (112).

POC TESTING OF MALARIA AND DENGUE: THE FUTURE

Unmet Diagnostic Needs for Malaria RDTs

The decreasing burden of malaria does not imply a reduction of the numbers of RDTs used (because malaria still needs to be included in the differential diagnosis) and, to achieve universal access to malaria diagnosis, an increase of production and supply is needed.

Apart from this, there are technical challenges to RDTs. The limit of detection of commercially available immunochromatographic malaria RDTs (100 asexual parasites/µl) is too high for accurate diagnosis of malaria in pregnancy, which is a potential life-threatening infection both for the mother and the unborn child (145, 146). Likewise, in the case of pre-elimination of malaria, it is important to detect the asymptomatic carriers who act as a potential source of transmission. For this purpose, remote populations need to be screened for the presence of submicroscopic parasite densities; the product must be portable and robust and should detect parasites at a low limit of detection with only a short delay (47, 147).

The Future: Improvements in Design of Current Malaria RDTs

Potential improvements to existing malaria RDTs include the search and validation of new antigens. The *Plasmodium* antigens targeted include *Plasmodium* dihydrofolate reductase-thymidylate synthase, heme detoxification protein, and glutamate rich protein. Monoclonal antibodies designed for binding to these antigens showed promising results in affinity experiments (148). Another approach addresses the signal complex: Access Bio, one of the leading RDT manufacturers, is developing lateral flow tests which use a fluorescent signal molecule (europium) instead of colloidal gold, resulting in a lower limit of detection (up to 100× in preclinical studies). The fluorescence signal needs to be read using a specialized benchtop reader (http://www.accessbio.net/english/default2.asp).

Another interesting development is the Fyodor Urine Malaria Test (UMT), an immunochromatographic dipstick assay that detects *Plasmodium* proteins in urine of infected patients. The proteins targeted are highly immunogenic and are not cleaved by any known protease. Apart from the clear advantage—obviating the need for a finger prick—the test is also user-friendly and can be carried out with minimal training. The limit of detection is 125 parasites/µl and the product is claimed to perform equally well as blood-based malaria RDTs within the 100 to 200 parasites/µl detection limit that the WHO recommends for malaria RDTs (http://www.fyodorbio.com/products/umt/). The test is currently in clinical validation.

RDT Readers, Smart Phone, and Cloud Information Services

Fio and Holomic have developed products designed to improve RDT quality assurance and to offer built-in connectivity, making malaria surveillance programs possible.

The Fio system (http://www.fio.com/) consists of (i) a robust, environmentally resistant smart phone-based universal RDT reader, (ii) associated software that transfers images and demographic data over a mobile phone network to a central system, and (iii) a management Web portal for assessing reports and analysis tools. The system

provides geographic tracking and a module to reduce end-user errors, such as a warning in case of poor background clearing of the RDT strip. It also monitors and supports clinical practice and RDT stock management. The system has been favorably evaluated in Colombia and Tanzania. It is CE-marked for malaria and dengue RDTs and is currently deployed in 13 countries.

Holomic, which commercializes technologies of the Ozcan Laboratory at the University of California, Los Angeles, has launched a simple lightweight rapid diagnostic reader (HRDR-200) which can be attached to the existing camera unit of a cell phone. Validation and reading are performed using a smart application and allow secured transfer of RDT results and demographic and geographic data to a central server. The system generates real-time spatiotemporal health statistics. The HRDR-200 is designed for low-skilled health care workers and operates within normal cell phone operating conditions. It also supports other RDTs such as those detecting tuberculosis and human immunodeficiency virus infection (149; http://Holomic.com).

Nucleic Acid Amplification Tests: Lowering the Limit of Detection

Nucleic acid amplification tests have shown the intrinsic capability to go below the limit of detection of microscopy and RDTs; the limit of detection of PCR is approximately 1 parasite/µl. However, PCR-based assays require highly trained staff, purified nucleic acid extracts, cold chain, and expensive and power-intense thermocycler equipment, and they are prone to contamination (8, 150).

Several improvements on POC testing have been made to overcome the difficulties related to the strict requirements and high costs of molecular laboratories. They include the use of freeze-dried reagents, the development of handheld universal PCR devices (Ahram Biosystems; http://www.ahrambio.com/products_palmpcr_technology.html), the use of direct PCR (i.e., without sample preparation), as well as the detection of amplified DNA by lateral flow immune assays (PCR-NALFIA, developed by the DIAGMAL consortium) (151, 152; http://www.diagmal.eu/). Micronics is currently developing a fully automated portable real-time PCR system (PanNAT Malaria Assay) with primers, probes, and reagents incorporated in a microfluidics cartridge—with *Plasmodium* among the targeted pathogens (http://www.micronics.net/products/diagnostic-products/PanNAT).

As to the malaria diagnostic market, in 2013, Tulip Group/Bigtec Labs Joint Venture launched a lightweight benchtop portable real-time PCR platform (Truelab micro PCR platform) which uses microPCR chips that are self-sealing, thereby reducing the risk of contamination. The system is powered by a rechargeable battery pack. It integrates sample preparation, has a time to results of 45 to 60 minutes, and has a limit of detection of 2 parasites/µl of blood. The required sample volume is 100 µl of whole blood. Despite its user-friendliness, robustness, and portability, Truelab is not a handheld device fit for POC testing in view of its dimensions, its relatively long time to results, and its upper limit of operating temperature (30°C) (http://www.bigteclabs.com/productdescription.html#tabs-3). Several evaluations are under way.

Recently, it was demonstrated that blood on the strip of the RDT can be used for DNA extraction and PCR testing, allowing quality control of the results of RDTs used in the field in a reference laboratory. Successful amplification was seen with a *Plasmodium* species-specific real-time PCR after recovery of *Plasmodium* DNA from the nitrocellulose DNA-binding membrane of the RDT (153–155). Preliminary data from our laboratory also showed that dengue viral RNA can easily be recovered from NS1 RDT tests and be used for RT-PCR testing.

A Place for Isothermal DNA Amplification

Isothermal DNA amplifications are promising for field applications in malaria-endemic settings, although still in a laboratory environment (156). Compared to conventional PCR, they are less affected by the inhibitory effect of blood and may have similar accuracy when DNA is extracted using simple one-step methods such as heat treatment (157). A detailed overview of the various isothermal techniques with potential for malaria diagnosis can be found elsewhere (158). When used in conjunction with reverse transcription, this isothermal amplification can be used to amplify RNA with high efficiency for dengue diagnosis (159, 160).

Loop-mediated isothermal amplification (LAMP) employs self-recurring strand-displacement synthesis. LAMP combines simplicity of specimen processing and amplification (heating block or water bath at 65°C) with speed (30 to 60 minutes). The system is adaptable to RT-LAMP, allowing detection of the RNA of gametocytes (sexual parasite forms involved in transmission) (161), and is able to detect all *Plasmodium* species, including *P. knowlesi* (162). In nucleic acid sequence-based amplification (NASBA), a cocktail of reverse transcriptase, RNA polymerase, and RNase amplifies RNA targets. NASBA can be developed in a real-time quantitative assay (QT-NASBA) (163). QT-NASBA has also been used to determine gametocyte carriage (prevalence and density) (164). In helicase-dependent amplification (tHDA), double-stranded DNA is split by DNA helicase and coated by single-stranded DNA-binding proteins. Next, sequence-specific primers hybridize and DNA polymerases produce double-stranded DNA (165). Reaction temperature can be thermophilic (65°C, tHDA) and mesophilic (37°C, mHDA) (166).

Isothermal Amplification: Improvements

When evaluated side by side with PCR-based techniques for the diagnosis of malaria, LAMP, NASBA, and tHDA have shown comparable limits of detection and diagnostic accuracy (158). Further developments include the use of freeze-dried reagents and electricity-free heating based on exothermal chemical reactions (167). With LAMP, naked-eye detection of the end-product through turbidity is possible (168). As to availability on the diagnostic market, there is the LAMP Malaria Diagnostic Kit (Eiken Chemical and The Foundation for Innovative New Diagnostics), which has a simplified sample preparation method. The kit has been evaluated in field settings (169). In their current stage of development, isothermal methods for detection of *Plasmodium* are designed for field hospitals and mobile laboratories but have not yet been miniaturized into handheld devices, and the sample processing time is around 1 hour, thereby not fulfilling all requirements for POC testing.

Recently, a "lab on a chip" dengue RT-LAMP was developed with fluorescence-based detection of DENV2 on a paper-based device. The total diagnosis time is 100 minutes (60 minutes for the RT-LAMP reaction and 40 minutes for paper diagnosis), and the amount of specimen needed is 2 µl (170).

Approaches in Development: Hemozoin Detection and Spectroscopy

Hemozoin is a crystalline structure that results from the digestion of hemoglobin by the *Plasmodium* parasite. In *P. falciparum* malaria, its presence as a phagocytized brown pigment ("malaria pigment") in white blood cells is an indicator of severe infection, and it is one of the malaria markers used in automated hematology analyzers (28). However, one study using a benchtop nuclear magnetic resonance device to detect hemozoin in blood found limits of detection of 8,000 to 10,000 parasites/μl (171), and in another study using dark-field microscopy hemozoin could only be detected in trophozoites older than 6 hours, precluding detection of hemozoin with this method as a diagnostic marker for malaria (172). Hemozoin can further be detected through magneto-optical technology: hemozoin crystals normally are randomly oriented; when a magnetic field is applied, hemozoin crystals will align, because they contain iron from degraded hemoglobin. This alignment can be detected by a photodetector through changes in transmitted light (173). The University of Exeter and the Disease Diagnostic Group are optimizing a portable magneto-optical technology device designed for use by low-skilled health care workers (http://www.diseasediagnostic.com/about/product; 174).

In spectroscopy, wavelengths (UV light, visible light, infrared, and microwave radiations) expose molecules. Part of the light is absorbed and part is reflected; the absorption spectra are detected by a photodetector. Because each molecule has its own absorption spectrum signature, analysis of the spectra can reveal the identity of exposed molecules. Applications for malaria POC testing are in development. QuantaSpec is developing the infrared-based Spectraphone, a reagent-free image analyzing system that recognizes *Plasmodium*-infected red blood cells (http://www.quantaspec.com/). Claro Scientific is developing the SpectraWave (instrument) and SpectraNet (computer and database system) for malaria diagnostics and complete blood count analysis on Giemsa-stained slides (http://clarosci.com/).

In conclusion, RDTs targeting dengue and malaria—originally conceived for the travel medicine market—have brought reliable pathogen-based diagnosis in reach of the end user in remote and resource-limited settings. Immunochromatographic platforms housed in handheld cassettes are currently the main format for these RDTs, and ongoing research will refine their analytical and operational characteristics as well as extend their diagnostic applications. In the near future, they are expected to be complemented by field-adapted nucleic acid amplification tests, of which isothermal amplification tests are most promising in the short term.

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section IX

The Host and Host Response

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Implications of Pharmacogenetics for Antimicrobial Prescribing

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43

INTERINDIVIDUAL VARIABILITY IN RESPONSE TO ANTIMICROBIALS

Antimicrobial efficacy and toxicity are influenced by the interplay of factors related to the human host, the microbe, and the drug (1–3). A number of such factors are represented in Fig. 1 (4). Antimicrobials can be described by characteristic pharmacokinetic (PK) and pharmacodynamic (PD) profiles, which in many cases are influenced by absorption, distribution, elimination, and metabolism (ADME) genes. While some antimicrobials are eliminated largely unchanged in urine (e.g., vancomycin) or stool (e.g., atovaquone), many undergo extensive metabolism by phase I enzymes (e.g., cytochrome P450 isoforms) and/or phase II enzymes (e.g., UDP-glucuronosyltransferases). The resultant metabolites may lack antimicrobial activity, or conversely the active moiety may be generated from an inactive parent compound (i.e., prodrug). Toxic and hypersensitivity reactions may be mediated by parent compounds or their metabolites, depending on the particular drug. Antimicrobial disposition is also affected by membrane transporters in various tissues including liver, intestine, kidney, genital tract, and brain (5–7). Genetic polymorphisms that affect ADME gene expression or protein activity may be associated with interindividual differences in exposure to parent compound and/or metabolites. Some of these genotype-phenotype associations have implications for efficacy and toxicity.

Adverse drug reactions can be classified as either type A or type B (8). Type A reactions are commonly on-target reactions that are somewhat more predictable based on intrinsic pharmacologic (PK/PD) characteristics of the drug and may be influenced by pharmacogenetic and other host factors (Fig. 1). Type B reactions are off-target reactions that are largely immune mediated and may be further classified by the system of Gell and Coombs as either type I (IgE), II (antibody dependent/complement), III (immune complex), or IV (delayed, T-cell-mediated) reactions (9). Of most relevance to antimicrobials are type I or IgE-mediated reactions, which typically occur within 1 h of dosing and may be characterized by angioedema, urticaria, bronchospasm, or frank anaphylaxis, and type IV reactions (i.e., delayed hypersensitivity), which are largely mediated by T cells and are characterized by rash of varying severity and quality that may be accompanied by systemic features such as fever, malaise, or internal organ involvement (hepatitis, nephritis, pneumonitis). Non-IgE-mediated histamine release, sometimes known as red man syndrome, is also characteristic of some antibiotics such as vancomycin, caspofungin, or higher dose intravenous ciprofloxacin. Certain major histocompatibility complex (MHC) class I *HLA-B* alleles particularly predispose to severe T-cell-mediated drug hypersensitivity (10).

Although pharmacogenomic knowledge has helped to define mechanisms of antimicrobial response, translation from discovery into widespread clinical use has been limited. For some drugs, associations between human genetic polymorphisms, toxicity, and/or efficacy may be sufficiently strong and relevant, and interpretation and reporting of the test simple enough to facilitate translation into routine clinical practice. The likelihood of translation into practice, particularly for drugs whose disposition is affected by genetic polymorphisms in ADME genes, depends on a number of considerations such as the therapeutic window and treatment alternatives. However, for drugs in advanced stages of development or already FDA approved, there may be disincentives for pharmaceutical companies to pursue studies that might show that a human genetic test would be warranted for their drug, given the possibility that this added cost and complexity might cause clinicians to avoid that drug rather than obtain the genetic test.

Beyond genetic testing in clinical practice, some antimicrobials have had their clinical development halted or have been withdrawn from the market for severe toxicity, which in some cases may have been genetically determined. If pharmacogenomic knowledge very early in preclinical drug development could better anticipate potential future toxicity in humans (i.e., being able to predict whether a chemical compound is likely to be "safe" or "unsafe"), this might lead to more efficient drug discovery and design, and ultimately safer and more effective therapies.

This chapter summarizes current knowledge regarding pharmacogenetics of antimicrobial therapy. This includes human genetic factors that affect antimicrobial disposition, efficacy, and toxicity. Beyond the scope of this chapter are

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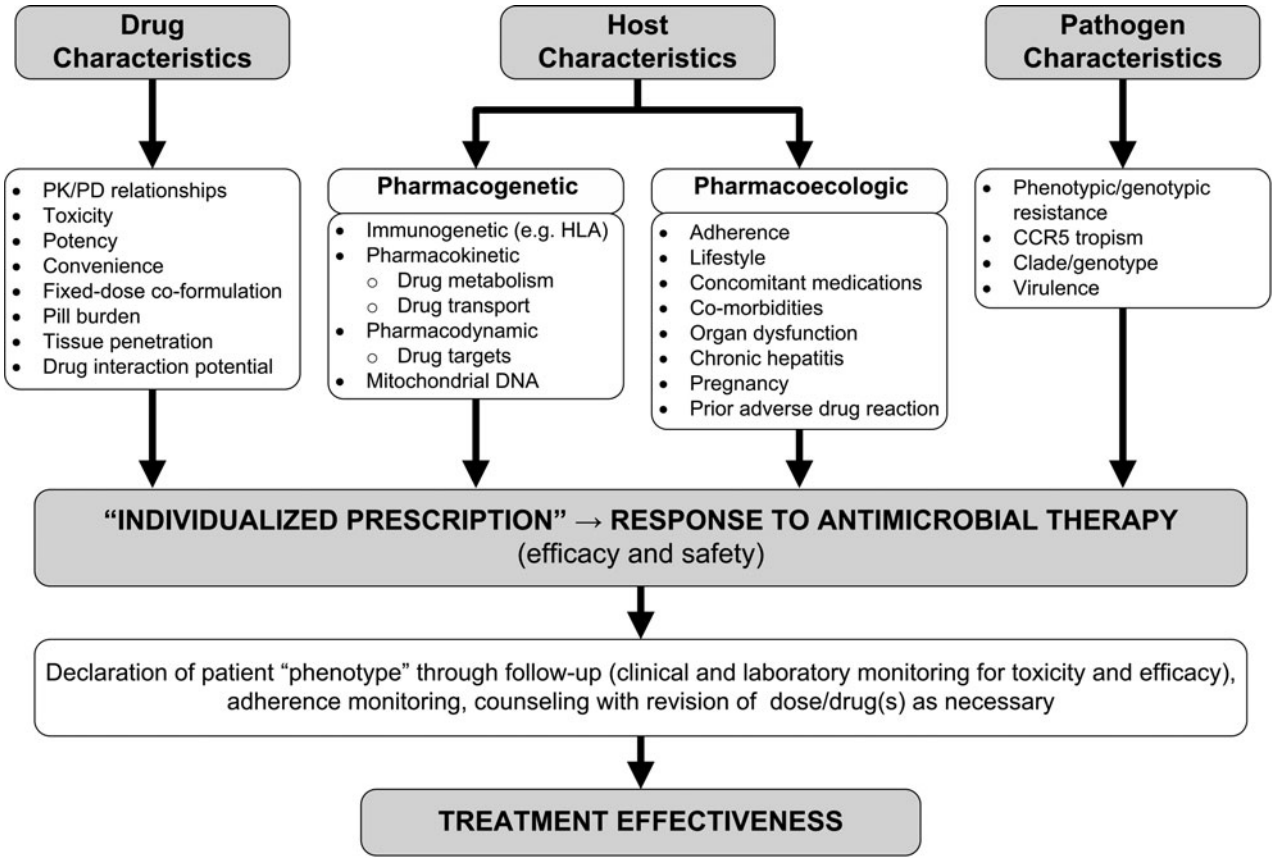

FIGURE 1 Relationships among drug, pathogen, and host factors affecting antimicrobial treatment outcome. CCR5, C-C chemokine receptor type 5; HLA, human leukocyte antigen; PD, pharmacodynamics; PK, pharmacokinetics. Modified with permission from Pavlos and Phillips (4).

microbial virulence and resistance factors, as well as immune responses to infection (Fig. 1). Here we provide details regarding pharmacogenetic associations with selected antimicrobial agents. A summary of many pharmacogenetic associations with antimicrobials is provided in Table 1, including levels of evidence for translation into clinical practice.

ASSOCIATIONS THAT PRIMARILY IMPLICATE METABOLISM AND TRANSPORT GENES

Efavirenz Pharmacokinetics and Tolerability

The nonnucleoside reverse transcriptase inhibitors (NNRTIs) efavirenz and nevirapine have been mainstays of HIV-1 therapy worldwide. Efavirenz is metabolized primarily by hepatic CYP2B6, with minor contributions by CYP2A6, CYP3A (11, 12), and direct *N*-glucuronidation by UDP-glucuronosyltransferase (UGT) 2B7 (11, 13). At least three *CYP2B6* loss-of-function polymorphisms have been convincingly associated with increased plasma efavirenz exposure, 516G→T (rs3745274) (14–19), 983T→C (rs28399499, *CYP2B6*) (19–22), and 15582C→T (rs4803419) (19) (Table 1). Different frequencies of *CYP2B6* 516G→T by race/ethnicity largely explain the greater mean plasma efavirenz trough concentrations with African ancestry than with European ancestry. The effect size on efavirenz concentrations for *CYP2B6* 983T→C is greater than for 516G→T (19), but its frequency is much less and appears to be found only with African ancestry. The effect size for *CYP2B6* 15582C→T is less than for 516G→T (19), and its frequency is high with European and Asian ancestry. Various combinations of *CYP2B6* polymorphisms yield 10 plasma efavirenz concentration strata across an approximately 10-fold range and explain approximately 35% of interindividual variability in efavirenz trough concentrations (19). The top three strata (i.e., *CYP2B6* slow metabolizer genotypes) comprise homozygosity for either 516 T/T or 983 C/C or dual heterozygosity for 516 G/T with 983 C/T. Among *CYP2B6* slow metabolizers, further increases in plasma efavirenz exposure are associated with polymorphisms in minor pathway genes *CYP2A6* (-48T→G, rs28399433) (23–25) and possibly *UGT2B7* (homozygosity for 735A→G, rs28365062) (25, 26).

Despite well-established genetic predictors of plasma efavirenz exposure, associations with central nervous system (CNS) side effects have been less consistent (14, 16, 27–35). This may relate to varying ways in which CNS side effects are defined and the fact that CNS symptoms with efavirenz lessen with repeated dosing (30). In a double-blinded, placebo-controlled study that carefully assessed CNS symptoms, efavirenz was associated with increased symptoms within the first week of treatment, but CNS symptom scores did not differ significantly between efavirenz and placebo recipients at 4 weeks and beyond (30).

Individualized efavirenz dose reduction based on *CYP2B6* genotyping could potentially decrease both side

TABLE 1 Selected pharmacogenetic associations with antimicrobial agents, listed alphabetically[a]

Drug	Phenotype	Genes	Allele	Activity	Level of evidence[b]	Selected references
Abacavir	Hypersensitivity syndrome (European, African, Asian ancestry)	*HLA*	*HLA-B*57:01*		1a	147, 150, 154
Aminoglycosides	Ototoxicity	12S RNA	1555A→G, 1494C→T, 1095T→C, ET961Cn, 961T→G, 961T→C		1b	218, 219, 222, 256
Amodiaquine	Neutropenia	*CYP1A1*	rs1048943, 2454A→G with rs4646903, 3798T→C (e.g. **2B*); rs1048943, 2454A→G (e.g., **2C*)	EM	4	85, 88
		CYP1B1	Reference allele **1;* rs10012, 142C→G with rs1056827, 355G→T (e.g. **2*)	EM	4	88
			rs10012, 142C→G, rs1056827, 355G→T with rs1056836, 4326C→G (e.g., **6*)	SM	4	88
	Malaria resistance	*CYP2C8*	rs11572080, 416G→A and rs10509681, 1196A→G (e.g., **3*)	SM	4	86, 87, 257
	Hepatotoxicity, agranulocytosis	*CYP2C8*	rs11572080, 416G→A and rs10509681, 1196A→G (e.g. **3*)	SM	4	77, 85–87
Amoxicillin-clavulanate	Liver injury, predominantly cholestatic/mixed pattern (white Europeans, North Americans)	*HLA*	*HLA-DRB1*15:01-DQB1*06:02*		1b	160, 162–164
	Liver injury	*HLA*	*HLA-A*02:01*		3	162
	Liver injury, predominant hepatocellular pattern (Spanish)	*HLA*	*HLA-A*30:02* and *HLA-B*18:01*		3	166
	Protection from liver injury (Northern Europeans)	*HLA*	*HLA-DRB1*07* and *HLA-A1*		3	163
Artesunate	Malaria treatment failure	*CYP2A6*	rs1801272, 1799T→A (e.g., **2*)	SM or null	4	77, 78, 83
	Increased artemisinin resistance	*CYP2A6*	rs5031016, 6558T→C (e.g., **7*)	SM	NA	79, 82
Atazanavir	Unconjugated hyperbilirubinemia	*UGT1A1*	**28* (rs8175317), rs887829	SM	1b	129, 134
	Drug discontinuation	*UGT1A1*	**28*, rs887829	SM	2b	130, 131
Chloroquine	Malaria resistance	*CYP2C8*	rs11572080, 416G→A and rs10509681, 1196A→G (e.g., **3*)	SM	3	257, 258
Dapsone	Hemolytic anemia	*G6PD*	Deficiency (many variants)	LOF	1b	90
	Hypersensitivity syndrome (Han Chinese)	*HLA*	*HLA-B*13:01*		1b	177, 259
Dolutegravir	Increased plasma concentrations	*UGT1A1*	rs8175317	SM	2a	136
Efavirenz	Increased plasma concentrations	*CYP2B6*	rs3745274, 516G→T (e.g., **6*); rs28399499, 983T→C (e.g., **18*); rs4803419, 15582C→T (e.g., **1C*)	SM	1b	14–22

(*Continued on next page*)

TABLE 1 Selected pharmacogenetic associations with antimicrobial agents, listed alphabetically[a] (*Continued*)

Drug	Phenotype	Genes	Allele	Activity	Level of evidence[b]	Selected references
		CYP2A6	rs28399433, -48T→G (e.g. *9); rs28399454, 5065G→A (e.g. *17); *4A to *4H	SM	1b	24–26
	Central nervous system symptoms	*CYP2B6*	rs3745274, 516G→T (e.g., *6); rs28399499, 983T→C (e.g. *18);	SM	2b	260–264
Etravirine	Increased plasma concentrations	*CYP2C19*	rs17885098, 99C→T with rs3758581, 991A→G (e.g., *17)	SM	3	135
Flucloxacillin	Liver injury (European)	*HLA*	*HLA-B*57:01* (rs2395029)		1b	168
Indinavir	Unconjugated hyperbilirubinemia	*UGT1A1*	*28, rs887829	SM	1b	265
IFN-γ	Increased HCV virologic response	*IL28B*	rs12979860 C→T; rs8099917 T→G	—	1a	178–181
Isoniazid	Hepatotoxicity	*CYP2E1*	Reference (*1A) with *CYP2E1* *1A/*1A genotype	EM	2b	49, 60
	Hepatotoxicity	*CYP2E1*	rs72559710, 1132G→A (e.g., *2) with *CYP2E1* *1A-*6-*1D haplotype; rs3813867, -1293G→C, rs2031920, -1053C→T with 7632T→A (e.g. *5A); rs3813867, -1293G→C and rs2031920, -1053C→T (e.g., *5B)	SM	3	51, 54
	Hepatotoxicity	*NAT2*	*4	SA	2b	55, 60, 62, 63, 67–69
		GSTM1	*0	Null	3	53, 54, 60
	Tuberculosis treatment failure	*NAT2*	*5	FA	2b	62
Lansoprazole omeprazole	*Helicobacter pylori* eradication	*CYP2C19*	rs4244285, 681G→A (e.g. *2)	SM	2a	144–146
Nelfinavir	Increased plasma concentrations	*CYP2C19*	rs17885098, 99C→T with rs3758581, 991A→G (e.g. *17)	SM	1b	17, 122
Nevirapine	Increased plasma concentrations	*CYP2B6*	rs3745274, 516G→T (e.g., *6); rs28399499, 983T→C (e.g., *18); rs4803419, 15582C→T (e.g., *1C)	SM	1b	20, 124, 197, 199, 200, 266
	Skin toxicity	*CYP2B6*	rs3745274, 516G→T (e.g., *6); rs28399499, 983T→C (e.g., *18)	SM	1b	267
	Hepatotoxicity if high CD4 T-cell count (European ancestry)	*HLA*	*HLA-DRB1*01*, *HLA-DRB1*01:01*		1b	267, 268
	Hepatotoxicity (White, European, South African)	*HLA*	*HLA-DRB1*01:02*		2b	203, 269
	Cutaneous phenotype, DIHS/DRESS (Italian, Japanese)	*HLA*	*HLA-Cw*8* or *HLA-Cw*8-B*14* haplotype		2b	205, 213, 267, 270, 271

	Cutaneous phenotype, DIHS/DRESS (Han Chinese, white, black, Southeast Asians)	*HLA*	*HLA-Cw*4*		1b	205, 213, 267, 270, 271
	Cutaneous phenotype, DIHS/DRESS (whites, Southeast Asians)	*HLA*	*HLA-C*04:01*		2b	205, 213, 267, 270, 271
	Cutaneous phenotype, DIHS/DRESS (Southeast Asian, Caucasian)	*HLA*	*HLA-B*35*		1b	205, 213, 267, 270, 271
	Cutaneous phenotype, DIHS/DRESS (Asian)	*HLA*	*HLA-B*35:05*		1b	205, 213, 267, 270, 271
	Cutaneous phenotype, DIHS/DRESS (Southeast Asian)	*HLA*	*HLA-B*35/Cw*4*		2b	205, 213, 267, 270, 271
	Cutaneous phenotype, DIHS/DRESS (Australian)	*HLA*	*HLA-B*35:01*		2b	205, 213, 267, 270, 271
	Cutaneous phenotype, DIHS/DRESS (Malawian)	*HLA*	*HLA-C*04:01*		3	211
Primaquine	Hemolytic anemia	*G6PD*	Deficiency (many variants)	LOF	1b	90
Proguanil, Chlorproguanil	Increased plasma concentrations	*CYP2C19*	rs17885098, 99C→T with rs3758581, 991A→G (e.g., *17)	UM	3	89
Ribavirin	Decreased anemia with HCV treatment	*ITPA*	rs1127354 A, rs7270101 C	—	1b	93–98
Sulfamethoxazole	Decreased hypersensitivity reaction in HIV-infected patients	*NAT1*	*10, *11	FA	3	75
	Hypersensitivity reactions in HIV-infected patients	*NAT2*	*6, *7, *12, *13	SA	3	74
	Hypersensitivity in HIV-infected patients	*GCLC*	rs761142T→G	—	3	76
Tenofovir	Renal tubulopathy	*ABCC2*, *ABCC10*	rs2273697 (1249G→A), rs717620 (-24T→C), rs9349256, rs2125739	—	2b	114–116
Voriconazole	Increased plasma concentrations	*CYP2C19*	rs4986893, 626G→A (e.g., *3)	SM	1b	43, 44, 46, 272, 273
	Hepatotoxicity, visual side effects	*CYP2C19*	rs17885098, 99C→T with rs3758581, 991A→G (e.g. *17)	SM	3	43–46 272, 273
	Visual side effects	*PDE6*	—	—	4	273

[a]ART, antiretroviral therapy; DILI, drug-induced liver injury; EM, extensive metabolizer; SM, slow metabolizer; UM, ultrarapid metabolizer; FA, fast acetylator; SA, slow acetylator.

[b]●●●●●●.

[c]Levels of evidence, based on PharmGKB (274). Level 1a = Annotation for a variant-drug combination in a Clinical Pharmacogenetics Implementation Consortium (CPIC) or medical society-endorsed pharmacogenomics guideline, or implemented at a Pharmacogenomics Research Network (PGRN) site, or in another major health system. Level 1b = Annotation for a variant-drug combination in which the preponderance of evidence shows an association. This association must be replicated in more than one cohort with significant *P* values and preferably with a strong effect size. Level 2a = Annotation for a variant-drug combination that qualifies for level 2b, in which the variant is within a very important pharmacogene (VIP) as defined by PharmGKB, where the functional significance is more likely known. Level 2b = Annotation for a variant-drug combination with moderate evidence of an association. This association must be replicated, but there may be some studies that do not show statistical significance, and/or the effect size may be small. Level 3 = Annotation for a variant-drug combination based on a single significant (not yet replicated) study or annotation for a variant-drug combination evaluated in multiple studies but lacking clear evidence of an association. Level 4 = Annotation based on a case report, nonsignificant study, or *in vitro*, molecular, or functional assay evidence only.

effects and drug cost. With intermediate or slow metabolizer genotypes, the daily efavirenz dose could likely decrease from the standard 600 mg to 400 or 200 mg, respectively, without compromising virologic control (36). It is reassuring that, with standard 600 mg once daily dosing, the lowest *CYP2B6* extensive metabolizer genotype stratum is not at increased risk for virologic failure with efavirenz (37). The ENCORE1, a randomized double-blinded noninferiority study, examined an alternative approach of a lower dose of efavirenz 400 mg once daily without genetic testing versus standard efavirenz 600 mg once daily (38). While this was not associated with increased virologic failure overall, it will be important to assure that such dose reduction effectively controls viral replication in patients in the lowest *CYP2B6* extensive metabolizer genotype stratum (i.e., *CYP2B6* 15582CC-516GG-983TT).

Voriconazole Plasma Exposure

Voriconazole is metabolized primarily by CYP2C19 (Table 1), and *CYP2C19* loss-of-function polymorphisms (e.g., rs4986893, *CYP2C19*3*) have been associated with higher plasma voriconazole concentrations (two and three times higher in intermediate and slow metabolizers, respectively) (85–87). Conversely, subtherapeutic plasma voriconazole concentrations have been associated with a gain-of-function allele (*CYP2C19*17*), suggesting the possible need for an increased dose in some patients (39). In *CYP2C19* slow metabolizers, secondary metabolism by CYP3A4 increases in importance, in which case coadministration of CYP3A4 inhibitors (e.g., ritonavir) may even further increase plasma voriconazole exposure (40, 41).

Hepatotoxicity can complicate therapy with voriconazole and may possibly relate to higher plasma trough concentrations (42). However, there are scant data to relate *CYP2C19* genotypes with hepatotoxicity. Two small studies showed that hepatotoxicity (predominantly cholestatic but also hepatocellular patterns) was associated with higher trough voriconazole concentrations, but there was no apparent association with *CYP2C19* genotype (43, 44). One study proposed pharmacogenetic-guided dosing of voriconazole to achieve target plasma concentrations (44), while a prospective study of 95 patients found no correlation between hepatotoxicity and levels of voriconazole or metabolites (45). Visual side effects of voriconazole have been suggested to be increased with polymorphisms in *CYP2C19* and the phosphodiesterase 6 (*PDE6*) gene (46, 47), but results have been inconsistent (45).

Isoniazid Pharmacokinetics and Hepatotoxicity

Isoniazid is a mainstay of antituberculosis therapy, and its pharmacogenetics have been most extensively studied among antituberculosis drugs (48–64). Isoniazid hepatotoxicity may affect 1 to 30% of patients, the risk being greatest with concomitant rifampin (54, 65). Onset is typically within 3 months of initiating isoniazid, with manifestations that include gastrointestinal symptoms, transaminase elevations, cholestasis, or isolated jaundice. *N*-Acetyltransferase plays an important role in isoniazid metabolism, and *NAT2* polymorphisms have been the most extensively studied.

A number of functional *NAT2* polymorphisms define acetylator status (Table 1) (54, 59), and studies have examined their relationship to hepatotoxicity (54–56, 59, 60, 62, 63). Three meta-analyses involving various ancestries suggested associations between slow acetylator genotypes and increased risk for hepatotoxicity (range of OR 1.9 to 4.7) (55, 60, 63), although one analysis showed no increased risk in intermediate acetylators compared to rapid acetylators (63). Risk appeared to be increased in all race/ethnicity groups except Caucasians, who were underrepresented (60). A study of Ethiopians with HIV-1 and tuberculosis coinfection (41 cases and 160 controls) also suggested increased risk of isoniazid hepatotoxicity with slow acetylator genotypes and possible interactions between isoniazid, rifampin, and efavirenz (65).

A randomized, controlled trial involving 172 Japanese patients with tuberculosis compared individualized isoniazid dosing based on genotype (2.5 mg/kg for slow acetylators, 5 mg/kg for intermediate acetylators, and 7.5 mg/kg for rapid acetylators) to standard dosing (5 mg/kg). There were fewer early treatment failures in patients with rapid acetylator genotypes who received higher doses of isoniazid (15% vs. 38%), and there was less hepatotoxicity in patients with slow acetylator genotypes who received lower doses of isoniazid (0% vs. 78%) (62). However, these failure rates are very high for antituberculosis therapy, which is curative in approximately 98% of drug-susceptible cases.

There are limited data to implicate polymorphisms beyond *NAT2* in isoniazid hepatotoxicity. Data regarding *CYP2E1* have been inconsistent (50, 54, 59, 60, 66). Some studies have suggested an association between *CYP2E1 *1A/*1A* and increased risk, particularly in individuals with *NAT2* slow acetylator genotypes (49, 50, 55), but this has not been replicated (52, 56) or has been found only in East Asians (60). One study associated the *CYP2E1*6* allele and **1A-*6-*1D* haplotype with increased risk of isoniazid hepatotoxicity (51), but not *CYP2E1*1C* (57).

A meta-analysis suggested an association between *GSTM1* null genotype and isoniazid hepatotoxicity (60), which may vary by ancestry (51, 53, 60), while there was no apparent association with *GSTT1* null genotype (54, 60, 61). Other genetic polymorphisms suggested to associate with isoniazid hepatotoxicity involve the tumor necrosis factor α gene in Koreans (64), mitochondrial manganese superoxide dismutase in Taiwanese (53), and lack of *HLA-DQA1*01:02* and presence of *HLA-DQB1*02:01* in Indians (48). Other than perhaps for *NAT2* polymorphisms, these findings do not have practical implications for antituberculosis therapy.

Isoniazid Peripheral Neuropathy

Data are limited regarding genetic risk for isoniazid peripheral neuropathy. Small studies suggest that *NAT2* slow acetylator genotypes may confer increased risk (54, 67, 68). In addition, in a small uncontrolled study among five Japanese patients with isoniazid peripheral neuropathy, all had *NAT2* slow acetylator genotypes based on genotyping of sural nerve biopsy specimens (69).

Trimethoprim-Sulfamethoxazole Hypersensitivity

Hypersensitivity reactions to trimethoprim-sulfamethoxazole (TMP-SMX) include generalized exanthem, drug reaction with eosinophilia and systemic symptoms/drug-induced hypersensitivity reaction (DRESS/DIHS), and Stevens-Johnson syndrome/toxic epidermal necrolysis (SJS/TEN). In HIV-infected patients with plasma viremia, mild to moderate rash affects as many as 34% of those prescribed TMP-SMX (70). Many patients with HIV-1 infection who develop hypersensitivity during treatment of *Pneumocystis jiroveci* pneumonia with high-dose TMP-SMX will later tolerate lower prophylactic doses following control of viremia, suggesting a role of immune activation. Most HIV-infected

patients who discontinue TMP-SMX for mild to moderate reactions tolerate the drug upon reintroduction (71), and desensitization may further reduce the likelihood of side effects (72).

Sulfamethoxazole (SMX) is metabolized by *N*-acetyltransferases 1 and 2 (NAT1 and NAT2), and hypersensitivity reactions are believed to be mediated by reactive hydroxylamine and nitroso metabolites (73). Among HIV-infected patients, *NAT2* slow acetylator genotypes (Table 1) have been associated with risk for SMX hypersensitivity reactions (74% in cases and 56% in controls) (74), and there is some evidence that concurrent *NAT1* gain-of-function polymorphisms may protect against SMX hypersensitivity in *NAT2* slow acetylators (75).

Glutamate-cysteine ligase is a rate-limiting enzyme in the formation of glutathione, which is important for phase II conjugation of toxic metabolites. One study of HIV-infected patients with SMX hypersensitivity (102 cases and 318 controls) suggested an association with a glutamate cysteine ligase catalytic subunit (*GCLC*) polymorphism (rs761142) (76).

Artemisinin Efficacy

Bioactivation of artesunate to the antimalarial anabolite dihydroartemisinin requires primary metabolism by CYP2A6 (77, 78), with CYP2B6, CYP1A1, and CYP1A2 playing minor roles (79). A number of *CYP2A6* polymorphisms are known to result in decreased or absent CYP2A6 enzymatic activity (80, 81), and it has been speculated that individuals with polymorphisms may be at increased risk for treatment failure with artesunate-based regimens (79, 82, 83). However, clinical studies are needed to correlate *CYP2A6* genotype with treatment outcomes. An association between *CYP2B6* polymorphisms and plasma exposure with artemisinin and its metabolites was not apparent in a study involving Cambodians and Tanzanians (84).

Amodiaquine Agranulocytosis

Amodiaquine is metabolized by CYP2C8 to the inactive, nontoxic compound *N*-desethylamodiaquine. However, with *CYP2C8* slow metabolizer genotypes, amodiaquine may be increasingly metabolized to quinoneimines (77). These quinoneimines can cause agranulocytosis and severe liver damage, which occurs in approximately 1 in 2,000 individuals prescribed amodiaquine (85). *In vivo* studies suggest that quinoneimines may also be generated extrahepatically by CYP1A1 and CYP1B1, which may contribute to agranulocytosis (77, 85). The implications of *CYP2C8* polymorphisms for amodiaquine efficacy and safety warrant further study (86–88).

Proguanil and Chlorproguanil Efficacy

The biguanides (proguanil and chlorproguanil) undergo *in vivo* biotransformation to their active antimalarial metabolites (cycloguanil and procycloguanil, respectively) by CYP2C19 and less so by CYP3A4 (77). Among Gambian adults with uncomplicated malaria, those with *CYP2C19* ultrarapid metabolizer genotypes (i.e., homozygous for *CYP2C19**17) had increased plasma exposure of these active metabolites (89). However, other studies did not find an association between *CYP2C19* polymorphisms and breakthrough parasitemia, treatment failure, *ex vivo* antimalarial activity, or mild adverse events. This lack of association may reflect compensatory activation of biguanides by CYP3A4 (77).

Other Antimalarials

Hemolysis caused by primaquine is associated with glucose-6-phosphate dehydrogenase (G6PD) deficiency, which is present in approximately 10 to 25% of sub-Saharan Africans (90). Other genetic associations with antimalarials have been reported but have not been well replicated, such as *ABCB1* polymorphisms with chloroquine neurotoxicity (77, 78), quinine neurotoxicity and mefloquine neuropsychiatric effects (91, 92), and *CYP3A5* loss-of-function genotype with quinine hydroxylation.

Ribavirin Anemia

Anemia affects 30 to 50% of patients treated for hepatitis C virus (HCV) infection with gamma interferon (IFN-γ) and ribavirin. Hemolytic anemia caused by ribavirin is more common in females, with older age, with higher ribavirin dose, and with lower baseline hemoglobin. Two polymorphisms in the inosine triphosphatase (*ITPA*) gene (rs1127354 and rs7270101) protect against anemia from ribavirin (93–98). Such protection was seen in patients with HCV genotype 2 and 3 and HIV/HCV coinfection treated with ribavirin and IFN-γ, as well as in HCV mono-infected patients treated with ribavirin, IFN-γ, and telaprevir. These protective *ITPA* polymorphisms are infrequent, and there is no recommendation for *ITPA* genotyping to inform prescribing.

Tenofovir Nephrotoxicity

Tenofovir disoproxil fumarate is a prodrug of the nucleotide reverse transcriptase inhibitor tenofovir. Although the prodrug is generally safe (99–109), some HIV-infected patients prescribed it experience declines in creatinine clearance and/or proximal tubular dysfunction with intact creatinine clearance (106, 107, 110–112). Tenofovir entry into proximal tubule cells may be mediated by solute carrier family 22 member 6 (SLC22A6, previously called organic anion transporter 1 [OAT1]) and SLC22A7 (previously called OAT2) (113), while renal tubular secretion is mediated by ATP-binding cassette, subfamily C, member 4 (ABCC4, previously called multidrug resistance protein 4 [MRP4]) (113), and possibly ABCC2 (previously called multidrug resistance protein 2 [MRP2]), although the importance of ABCC2 is uncertain (113).

Candidate gene studies have suggested genetic associations with adverse renal effects of tenofovir. A study from France associated proximal renal tubulopathy with an *ABCC2* polymorphism (1249G→A, rs2273697) (114), a study from Japan associated renal tubular dysfunction with two polymorphisms in *ABCC2* (-24T→C, rs717620, and 1249 G→A) (115), and a study from Spain associated tubular dysfunction with *ABCC2* -24T→C and two polymorphisms in *ABCC10* (rs9349256 and rs2125739) (116). Kidney tubular dysfunction included serum creatinine and/or creatinine clearance differences in some reports (114, 115) but not others (114, 115). Overall, previous reports have shown limited replication other than perhaps *ABCC2* polymorphisms (114, 115) (Table 1).

Inhibitors of HIV-1 Protease

The HIV-1 protease inhibitors are metabolized primarily by CYP3A4 (except for nelfinavir, which is metabolized by CYP2C19), and most protease inhibitors also inhibit CYP3A isoforms. The CYP3A inhibitor ritonavir (also a protease inhibitor) is now used only in lower doses as a PK

enhancer to increase plasma concentrations of concomitant protease inhibitors. Protease inhibitors are also substrates for P-glycoprotein and other drug transporters such as OATP1A2, OATP1B1, and OATP1B3. Genetic relationships with pharmacokinetics have been proposed for many protease inhibitors, but results for most have been inconsistent. The only clear associations with pharmacokinetics are between *SLCO1B1* 521 T→C and somewhat higher plasma lopinavir exposure (117–120), and between *CYP2C19* and higher plasma nelfinavir exposure (121, 122). Pharmacogenetic relationships with protease inhibitor efficacy have not been established (Table 1) (117, 123–125).

Protease inhibitors have been associated with metabolic disturbances, including ritonavir with hyperlipidemia. Genetic variants associated with hyperlipidemia in HIV-negative populations appear to be overrepresented in patients with hyperlipidemia on protease inhibitor therapy (126–128).

Atazanavir inhibits plasma bilirubin clearance by competing for binding to UGT1A1, resulting in unconjugated hyperbilirubinemia. The greater magnitude of hyperbilirubinemia with atazanavir is associated with the Gilbert polymorphism in *UGT1A1* (UGT1A1*28), which is significantly associated with reduced bilirubin-conjugating activity and unconjugated hyperbilirubinemia (129). Several studies have suggested a correlation between *UGT1A1* polymorphisms and atazanavir treatment discontinuation (130, 131). Bilirubin uptake into hepatocytes is also facilitated by OATP1B1 and OATP1B3, and it has been suggested that *SLCO1B1* polymorphisms might contribute to unconjugated hyperbilirubinemia with atazanavir (132, 133). However, in a genome-wide association study (GWAS), atazanavir-associated hyperbilirubinemia was only associated with *UGT1A1* rs887829 (which is in linkage with *UGT1A1**28), but with no polymorphisms beyond *UGT1A1* (134).

Newer Antiretroviral Agents

With the newer NNRTI etravirine, loss-of-function *CYP2C19* variants are associated with somewhat greater plasma drug exposure (135), but clear implications are lacking for etravirine prescribing. There are no data beyond this regarding the pharmacogenetics of etravirine or rilpivirine. The integrase inhibitor dolutegravir is metabolized, but UGT1A1 and the dinucleotide $(TA)_n$ repeat (rs8175317) polymorphism in the UGT1A1 promoter have been associated with an approximately 50% increase in plasma dolutegravir exposure (136). This was not judged to be clinically significant.

Proton Pump Inhibitors and *H. pylori* Eradication

Proton pump inhibitors do not have direct antimicrobial activity, but rather are used in combination with antimicrobials to treat *Helicobacter pylori* infection. By raising gastric pH, proton pump inhibitors make *H. pylori* more susceptible to concomitant antimicrobials (137). At least 20% of patients fail multidrug eradication therapy (138), likely due to bacterial resistance and host factors.

Most proton pump inhibitors are metabolized by CYP2C19 and CYP3A4. An exception is rabeprazole, which undergoes nonenzymatic metabolism (139). Plasma exposures of proton pump inhibitors reflect rates of CYP2C19 inhibition by metabolites. For example, metabolism of omeprazole and esomeprazole by CYP2C19 produces sulfones that strongly inhibit CYP2C19, leading to nonlinear increases in AUC with repeated administration (140). Loss-of-function *CYP2C19* polymorphisms are well described, and can increase plasma exposure of proton pump inhibitors, particularly when homozygous (Table 1) (141–143).

Meta-analyses suggest that the *CYP2C19* genotype may significantly affect the likelihood of *H. pylori* eradication with omeprazole, inconsistently with lansoprazole, and not with rabeprazole or esomeprazole (144–146). Choice of proton pump inhibitor and twice daily dosing may overcome the impact of *CYP2C19* genotype (146).

ASSOCIATIONS THAT PRIMARILY IMPLICATE IMMUNE RESPONSE GENES

Abacavir Hypersensitivity Syndrome

Hypersensitivity syndrome is the major treatment-limiting toxicity of the guanosine analog abacavir. This potentially life-threatening complication was well characterized during drug development and affected 5 to 8% of clinical trials participants (147, 148). In 2002, two groups independently described the association between abacavir hypersensitivity and the HLA class I allele *HLA-B*57:01* (149–151). Over the subsequent 6 years, and after overcoming many translational hurdles, the clinical utility of *HLA-B*57:01* screening to prevent immune-mediated (i.e., patch test positive) abacavir hypersensitivity was definitively shown in a randomized clinical trial (152, 153). Keys to translating *HLA-B*57:01* testing from discovery to guidelines to widespread clinical practice included the 100% negative predictive value of this test, generalizability across race/ethnicity groups, the low number of tests required to prevent a single case of hypersensitivity, and development of cost-effective, quality-assured laboratory technologies (10, 154, 155). Beyond discovery and translation of *HLA-B*57:01* screening into routine clinical practice, the mechanism underlying the specific interaction between abacavir and *HLA-B*57:01* has been explained. In 2012, three groups simultaneously published findings indicating that abacavir rapidly and noncovalently binds to *HLA-B*57:01*, altering the repertoire of peptides that bind and are immunogenic, this creating a vigorous $CD8^+$ T-cell response (156–158). Two of these groups solved the crystal structure of abacavir noncovalently bound to peptide and *HLA-B*57:01* (156, 157). This altered peptide repertoire model may more broadly apply to other drugs and drug hypersensitivity syndromes.

Amoxicillin-Clavulanate Hepatotoxicity

As many as 14% of all cases of drug-induced liver injury may be caused by amoxicillin-clavulanate (159), which affects approximately 1 in 1,000 to 10,000 of individuals prescribed this fixed-dose combination pill (160). Manifestations are heterogeneous. As many as one-half of patients present with cholestasis, but hepatocellular and mixed patterns also occur (161, 162). Biochemical abnormalities typically resolve without long-term sequelae, and fulminant hepatic failure requiring transplantation is rare. The mean age of affected individuals is approximately 60 years (161–163), and the mean time to onset after initiating amoxicillin-clavulanate is 2 weeks (161–164).

Hepatotoxicity with amoxicillin-clavulanate is likely mediated by the clavulanate component (165). While underlying mechanisms of liver injury are uncertain, immunologic reactions due to drug-hapten presentation via MHC

molecules have been proposed (160). A particular HLA haplotype (*HLA-DRB1*15:01-DQB1*06:02*) has been associated with increased risk of amoxicillin-clavulanate liver injury in individuals of European descent (160, 163, 164). This is supported by a GWAS showing an association with a MHC class II polymorphism (rs9274407) that is in linkage disequilibrium with *HLA-DRB1*15:01-DQB1*06:02* (162). Homozygosity for this haplotype may confer even higher risk (164). This haplotype, however, likely does not fully explain amoxicillin-clavulanate liver injury, which is far less frequent than would be predicted based on haplotype prevalence in northern Europeans (164). There is presently no recommendation to screen for *HLA-DRB1*15:01-DQB1*06:02* before prescribing amoxicillin-clavulanate or to avoid prescribing to individuals that are known to carry *DRB1*15:01-DQB1*06:02*.

Associations have also been suggested between amoxicillin-clavulanate liver injury and an MHC class I polymorphism (rs2523822), which is a marker for *HLA-A*02:01* (162). A protective association with *HLA-DRB1*07* has also been reported (163). Furthermore, associations of MHC class I alleles *HLA-A*30:02* and *HLA-B*18:01* with a predominantly hepatocellular pattern of amoxicillin-clavulanate liver injury have been reported in southern Europeans (166).

Flucloxacillin Hepatotoxicity

Drug-induced cholestatic hepatitis has been associated with the antistaphylococcal beta-lactam antibiotic flucloxacillin. This drug is widely prescribed in Europe and Australia, but it is not available in the United States. Liver injury affects approximately 8.5 per 100,000 individuals prescribed flucloxacillin, with onset from 1 to 45 days after initiating therapy (167). In a GWAS involving 51 cases and 282 matched controls, an MHC polymorphism (rs2395029) was convincingly associated with an 80-fold increased risk of flucloxacillin liver injury (168). This polymorphism is known to be in strong linkage disequilibrium with *HLA-B*57:01* (168).

Ex vivo studies have demonstrated the immunologic basis for *HLA-B*57:01* restricted activation of flucloxacillin-specific cytotoxic $CD8^+$ T cells, which involves a labile pharmacological interaction (169, 170). As with amoxicillin-clavulanate, many patients carry the risk allele, but very few develop hepatitis if exposed to flucloxacillin. Because nearly 14,000 individuals would have to be tested to prevent one hepatotoxicity case, routine prescreening for *HLA-B*57:01* is not recommended. However, *HLA-B*57:01* genotyping may help to implicate flucloxacillin retrospectively in individual cases of cholestasis (171).

Dapsone Hypersensitivity Syndrome

Dapsone is used to treat leprosy and to prevent malaria and *P. jiroveci* pneumonia. A coformulation of dapsone with chlorproguanil was effective for treating uncomplicated *Plasmodium falciparum* malaria (172), but was withdrawn from the market in 2008 because of a risk of severe hemolysis in patients with G6PD deficiency, which was related to the dapsone component (173–175).

Dapsone hypersensitivity syndrome is most common among Asians, and it is often associated with fever, lymphadenopathy, generalized rash, and hepatitis (176). Onset occurs a mean of 28 days after initiation of dapsone, and mortality may approach 10% (176). A GWAS involving Han Chinese patients treated for leprosy (39 hypersensitivity cases and 833 controls) strongly associated *HLA-B*13:01* (rs2844573) with increased risk for dapsone hypersensitivity syndrome (OR = 20.5, $P = 6.84 \times 10^{-25}$) (177). It is estimated that in high-risk populations undergoing dapsone-based leprosy treatment, 84 cases would have to be screened for *HLA-B*13:01* to prevent one hypersensitivity case (177).

IFN-Based Regimens and Response to Therapy for HCV

HCV affects more than 150 million people worldwide, and causes more than 350,000 deaths each year. Until recently, standard of care for HCV was combination therapy with pegylated IFN-γ and ribavirin, which is poorly tolerated, has low response rates (particularly with HCV genotype 1 disease), and requires prolonged therapy (24 to 72 weeks). Treatment is now rapidly advancing from IFN to combination regimens with novel direct acting agents that are more effective and better tolerated.

A strong association has been convincingly established between frequent polymorphisms in *IL28B* (rs12979860, rs8099917), which encodes a type 3 lambda IFN, and likelihood of response to IFN-based therapy for HCV genotype 1, as well as likelihood of spontaneous clearance of HCV without therapy (178–180) (Table 1). These polymorphisms are in a noncoding gene region, and it is believed that their effects relate to decreased basal expression of IFN-stimulated genes and greater induction of these genes upon exposure to IFN (hence more favorable treatment responses). It has also been suggested that a TT/-G polymorphism in a CpG island upstream of *IL28B* may better predict HCV clearance, perhaps by inducing IL28B and IFN-γ-inducible protein 10 (181). Lower response rates to IFN-based therapy among African Americans are due, at least in part, to decreased frequency of favorable *IL28B* genotypes. With HCV genotypes 2 and 3, overall treatment response rates are much higher than with HCV genotype 1 infections, and the *IL28B* genotype may be less predictive with these HCV genotypes.

In liver transplant recipients who become reinfected with HCV genotype 1, both donor and recipient *IL28B* genotypes have been associated with a likelihood of sustained virologic response (182–184). Data from patients coinfected with HCV and HIV-1 have also associated *IL28B* genotype with sustained virologic response to pegylated IFN and ribavirin, including previous nonresponders (185). Beyond *IL28B*, in studies from Europe and Japan an *HLA-C* genotype (C2/C2) together with *IL28B* polymorphisms increased predictive value of nonresponse from 66 to 80% (186, 187). A dinucleotide frame shift variant in rs368234815 (TT or ΔG), which generated the interferon lambda 4 protein (IFNL4), has been described and is in high linkage disequilibrium with rs12979860. The IFN-γ gene is largely inactive in human populations due to a frameshift variant. Paradoxically, rs368234815 ΔG carriers have impaired clearance of HCV and decreased response to HCV treatment (188–191). There is some evidence that *IL28B* genotypes are associated with improved efficacy and HCV virologic control with multidrug regimens that combine IFN-γ and ribavirin with new direct-acting agents, and possibly IFN-sparing regimens, suggesting an effect of *IL28B* genotype on viral kinetics (192).

There are no definitive recommendations for *IL28B* genotyping to guide HCV treatment decisions, despite this being a strong predictor of HCV response to IFN-γ-containing regimens that do not include new direct-acting agents

(193). A possible use of *IL28B* genotyping would be to predict likelihood of sustained virologic response with IFN-based regimens in patients with HCV genotype 1 and in HIV/HCV-coinfected patients with genotype 1 or 4 to possibly shorten the duration of treatment in patients with favorable genotypes. With the advent of highly effective direct acting agents and movement away from IFN-γ and ribavirin, the role of *IL28B* genotyping is unclear.

Nevirapine Hepatic and Cutaneous Adverse Events

Adverse reactions to nevirapine are primarily immune mediated, affect liver and/or skin, and occur within the first 2 months of therapy. These range from mild skin rash to severe cutaneous adverse reactions such as SJS/TEN or DRESS/DIHS. In a clinical trial from South Africa, in which participants with lower plasma HIV-1 RNA concentrations (i.e., higher $CD4^+$ T-cell counts) were stratified to receive nevirapine-containing regimens, 17% of nevirapine recipients experienced grade 3 or 4 liver toxicity, and two died of hepatic failure (194). Inactivation of nevirapine occurs primarily through hepatic CYP2B6, less so through CYP3A and other isoforms, and nevirapine induces its own metabolism (i.e., autoinduction). As with efavirenz, increased plasma nevirapine exposure has been associated with *CYP2B6* loss-of-function variants including 516G→T (16, 195–199), 983T→C (200), and 15582C→T (199). An association has been reported between nevirapine pharmacokinetics and rash, with a 50% increased likelihood of rash for every 20% decrease in plasma nevirapine clearance (201).

Genetic variants that confer increased risk for nevirapine hepatic events differ for those associated with cutaneous events without liver involvement. The first published study of an HLA association with nevirapine hypersensitivity was from Western Australia, and it implicated *HLA-DRB1*01:01* (HLA class II) and CD4 percentage ≥25% with rash-associated hepatic events in a largely Caucasian cohort (202). A relationship was later reported between *HLA-DRB1*01:02* and nevirapine-associated hepatic events (rash status unknown) in a largely black African cohort (203). In contrast, studies in Sardinia and Japan implicated *HLA-Cw*08* in hepatotoxicity (204, 205). Regarding nevirapine-associated cutaneous events, Thai studies implicated *HLA-Cw*04:01* and *HLA-B*35:05* (HLA class I) (206–208). In a large, retrospective, case-controlled pharmacogenomic study that separately considered severe cutaneous and hepatic adverse events and included cohorts of Asian, European, and African descent (209), cutaneous events were associated with *HLA-Cw*04*, especially among blacks and Asians, and with *HLA-B*35* among Asians. The *CYP2B6* loss-of-function variant 516G→T was also associated with cutaneous but not hepatic adverse events. For cutaneous nevirapine hypersensitivity reactions this suggests a possible concentration dependency, and the potential importance of noncovalent binding of the parent drug nevirapine to the implicated class I HLA allele similar to what has been reported recently for other drugs causing DRESS/SJS/TEN such as phenytoin (210). Hepatic adverse events were associated with *HLA-DRB1*01* among whites, but this allele was infrequent among blacks and rare among Asians. More recently, SJS/TEN has been associated with *HLA-C*04:01* in a Malawian cohort (211).

Immune-mediated reactions to nevirapine cannot be reliably predicted by class I or class II HLA associations, although implicated HLA alleles may share peptide-binding characteristics (212). The impact of $CD4^+$ T-cell count is expected to be greater with HLA class II-mediated hepatic events than HLA class I-mediated cutaneous events. This is supported by *in vitro* studies showing that nevirapine-specific $CD8^+$ T-cell responses and depletion of $CD8^+$ T cells more markedly abrogate nevirapine-specific IFN-γ output than $CD4^+$ T-cell depletion (213). In addition, patients on antiretroviral therapy with virologic control and high $CD4^+$ T-cell counts may not be at increased risk of toxicity when switching to nevirapine-containing regimens (214–216).

ASSOCIATIONS THAT PRIMARILY IMPLICATE MITOCHONDRIAL DNA VARIANTS

Aminoglycoside Ototoxicity

Aminoglycosides can cause bilateral sensorineural hearing loss, especially with prolonged treatment (217–221). Mitochondrial mutations that affect 12S RNA, especially 1555A→G, have been associated with particularly high risk of aminoglycoside-induced sensorineural deafness (Table 1), with additional risk factors including cumulative dose and duration of therapy (219, 221, 222). These mutations are very infrequent, and testing for mitochondrial DNA risk mutations in clinical practice before prescribing aminoglycosides is not recommended. It has been suggested that aminoglycosides be used with particular caution in patients with a family history of hearing loss.

Thymidine Analogues and Mitochondrial Toxicity

Safety and tolerability of the thymidine analogues didanosine (ddI), stavudine (d4T), zalcitabine (ddC), and zidovudine (AZT) are severely limited by various toxicities. These thymidine analogue-associated toxicities largely reflect mitochondrial injury caused by inhibition of mitochondrial DNA polymerase-gamma. Clinical manifestations include peripheral lipoatrophy (223–228), which has been associated with d4T more so than AZT, peripheral neuropathy (especially with the "D drugs" d4T, ddI, and ddC) (229–235), lactic acidosis (236–243), and metabolic disease (225, 244–247). Reported mitochondrial DNA polymorphisms (which define mitochondrial haplogroups) associated with these phenotypes are summarized (Table 2). Because of these toxicities, use of these agents is now discouraged. World Health Organization guidelines exclude d4T from first-line therapy and recommend use of AZT only when tenofovir is not an option.

Linezolid Toxicity

Linezolid is an oxazolidinone antimicrobial used to treat multiresistant Gram-positive infections. It works by binding to the 23S ribosome, preventing 30S-50S fusion in bacteria. There is evidence that major linezolid toxicities including optic and peripheral neuropathies, myelosuppression, and hyperlactatemia may be mediated through inhibition of mitochondrial protein synthesis. This suggests that mitochondrial DNA polymorphisms might predispose to these toxicities (248–252). To date, however, there are limited data to implicate mitochondrial genetic variants in linezolid toxicity (253).

FUTURE DIRECTIONS

Pharmacogenomics has significantly enhanced our understanding of the basis of host variability in antimicrobial

TABLE 2 Possible associations between mitochondrial DNA haplogroups and antiretroviral toxicities

Haplogroups	Population studied	Associated phenotypes with ART	Level of evidence[a]	Selected references
L1c	Non-Hispanic black North American	Peripheral neuropathy	3	230, 275
L3e1	Black South African	Hypertriglyceridemia	3	246
L0a2, L2a	African (Malawian)	Peripheral neuropathy	3	229
W, I, T, H, K	European (Italian) and/or non-Hispanic white North American	Lipoatrophy/lipodystrophy	3	223, 225, 226
	African (Malawian)	Lipoatrophy	3	276
H, clade HV, U	European (Spanish) and/or non-Hispanic white North American	Insulin resistance	3	244, 245
I	European and/or non-Hispanic white North American	Dyslipidemia	3	225
Clade JT, T, H, clade HV	European (Spanish)	Atherogenic risk	3	244
T	Non-Hispanic white North American	Peripheral neuropathy	3	232, 233
J, H3, U5a	Non-Hispanic white North American	Neuroretinal disorders	3	231

[a]Levels of evidence based on PharmGKB (274). Level 1a = Annotation for a variant-drug combination in a Clinical Pharmacogenetics Implementation Consortium (CPIC) or medical society-endorsed pharmacogenomics guideline, or implemented at a Pharmacogenomics Research Network (PGRN) site, or in another major health system. Level 1b = Annotation for a variant-drug combination in which the preponderance of evidence shows an association. This association must be replicated in more than one cohort with significant *P* values and preferably with a strong effect size. Level 2a = Annotation for a variant-drug combination that qualifies for level 2b, in which the variant is within a very important pharmacogene (VIP) as defined by PharmGKB, where the functional significance is more likely known. Level 2b = Annotation for a variant-drug combination with moderate evidence of an association. This association must be replicated, but there may be some studies that do not show statistical significance, and/or the effect size may be small. Level 3 = Annotation for a variant-drug combination based on a single significant (not yet replicated) study or annotation for a variant-drug combination evaluated in multiple studies but lacking clear evidence of an association. Level 4 = Annotation based on a case report, nonsignificant study, or *in vitro*, molecular, or functional assay evidence only.

response (1). The translational success of *HLA-B*57:01* screening to prevent abacavir hypersensitivity that traversed the full T1→T4 translational pathway from discovery to routine clinical use provides a real-life example that pharmacogenomics can improve drug safety, efficacy, and effectiveness. Other associations, such as *HLA-B*13:01* screening in Han Chinese to prevent dapsone hypersensitivity syndrome, have the potential to be translated into clinical practice with additional clinical data and the availability of accurate and cost-effective single allele typing for *HLA-B*13:01* (62, 177).

Major challenges still remain for many infections with a high global burden including HIV-1, tuberculosis, and malaria, for which continued study regarding the pharmacogenomics of drug efficacy and toxicity is warranted (77). In developing countries, the clinical implementation of pharmacogenetics will be far more challenging but has the potential for huge impact. For pharmacogenetics to have an impact in such countries, low cost and accessible genotyping will be required, complemented by robust evidence that genetic testing is cost effective and will improve public health for the specific application being considered.

There have been few clinical applications of pharmacogenetics to date beyond abacavir, for which the decision to prescribe is based on the simple presence or absence of the *HLA-B*57:01* risk allele, but this is almost certain to change in the coming years. Incorporation of pharmacogenomics into routine clinical care will be facilitated by increased knowledge of pharmacogenomics and its impact on drug safety, efficacy, and complex drug-drug interactions as well as by improved decision support systems for primary care physicians to help make sense out of complex data. It will be additionally driven by increased access by individuals to extensive data regarding their own personal genomes. In such an environment, discussions between providers and patients about implications of specific genetic polymorphisms in relation to the risk/benefit of antimicrobial prescribing and treatment may become routine. A hypothetical conversation might begin as follows—Provider: "I recommend that we treat your HIV infection with a regimen that includes efavirenz." Patient: "But my genotype shows that I'm a CYP2B6 slow metabolizer. Does that affect your recommendation?"

A growing number of individual clinical laboratories, as well as larger health care systems, are likely to provide and encourage pharmacogenetic testing to inform antimicrobial prescribing. Widespread availability of efficient, accurate, and cost-effective laboratory testing supported by a simple, interpretable clinical decision support will be necessary to support translation into clinical practice (255). As discussed in this chapter, several medications beyond abacavir may be candidates for pharmacogenetic testing. In some situations it will clearly be cost effective to individualize therapy based on such data, so as to reduce drug toxicity, increase efficacy, and/or optimize dosing. In addition, health care systems that promote pharmacogenetic testing may find themselves at a competitive advantage.

Future studies will be enhanced by utilization of large cohorts of precisely phenotyped individuals and high-throughput deep sequencing to further define the interplay between host, drug, and pathogen genetic signatures important to define interindividual variation in host antimicrobial response. With improved virtual and *in vitro* approaches there is likely to be enhanced ability to predict interactions between drugs and their known and unexpected targets, leading to predictive preclinical screening strategies to guide more efficient drug design and development (104, 125, 126). Furthermore, in large-scale clinical trials, genetic stratification and dosing have the potential to increase power and improve outcomes of such trials (254). Continued progress in pharmacogenomics leading

to improved antimicrobial safety and efficacy will be driven by continued acceleration of science that defines major sources of variation in antimicrobial efficacy, toxicity, and host-pathogen interactions.

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Exploiting MicroRNA (miRNA) Profiles for Diagnostics

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44

Clinical management of any disease depends upon timely, accurate, and sensitive diagnosis of the etiology of disease to determine an appropriate counter strategy. As defined by the Biomarkers Definitions Working Group, biomarkers are characteristics that are objectively measured and evaluated as indicators of normal biological, pathological, or pharmacological responses to a disease or therapeutic intervention (1, 2). An ideal biomarker should be inexpensive to detect; readily assayed; present at favorable concentrations in cells, target tissues, and/or in biological fluids; and resistant to degradation during typical storage. The biomarker should also provide insights into disease etiology, progression, and/or treatment efficacy. Biomarkers can indicate toxicity, safety, efficacy, pharmacodynamics, disease diagnosis, or prognosis following treatment or at clinical endpoints (3). Assessment of molecular biomarkers has been typically slow, expensive, and time consuming (4–6). Factors that contribute to this are the collection methods and the need for preservation, purification, and environmental stability of biological samples. Three large consortia, the NCI Early Detection Research Network (EDRN), Critical Path Predictive Safety Testing Consortium (PSTC), and the Alzheimer's Disease Neuroimaging Initiative (ADNI), are presently involved in screening thousands of biomolecules as potential biomarkers. Irrespective of their role, discovering biomarkers relies on defining their intended roles (diagnosis/prognosis, drug efficacy, sample type, assay to be used, etc.) in day-to-day clinical practice.

INTRODUCTION TO miRNAs

MicroRNAs (miRNAs) are 18- to 23-nucleotide (nt)-long small RNAs that post-transcriptionally regulate gene expression of foreign or self-genes in eukaryotes. The human genome (except the Y chromosome) encodes approximately 2,588 miRNAs, with more being discovered (7, 8), and these constitute a small but significant proportion of regulatory RNAs in the human transcriptome. MiRNAs are conserved across all eukaryotes and have been substantially expanded during evolution with only 12 miRNAs having been lost from deuterostomes to mammals (9, 10). MiRNAs are predicted to regulate approximately 60% of the human transcriptome (11, 12) and multiple normal physiological and disease processes.

miRNA genes are located on the sense/antisense strands in genic/intergenic and introns of genes as independent transcription units called mirtrons (13–15). Parent genes are typically transcribed by RNA polymerase II (POLR2A) (16, 17) and in some cases by RNA polymerase III (POLR3K) (18), producing long primary transcripts (pri-miRNAs) of variable length in the nucleus. Pri-miRNAs are processed by the microprocessor complex consisting of Drosha and DGCR8 into ~60-nt-long hairpin structured pre-miRNAs, which are then exported out of the nucleus by exportin 5 (XPO5) (19). Exported pre-miRNAs are further processed by a Dicer and Argonaute complex to produce 21- to 23-nt mature miRNA duplexes that consist of a "guide" strand and a passenger strand that is generally degraded. The guide strand nucleotides 2 to 8 represent the "seed site," which recognizes a 6- to 8-nt complementary sequence in the target gene(s), causing either a translational block or transcript decay in an RNA protein complex called an RNA-induced silencing complex (RISC) (19). Some miRNAs can also bind with perfect complementarity with the target transcript. The degree of base-pairing between the miRNA guide strand and mRNA and the composition of the RISC complex determine the downstream pathways. Perfect pairing between miRNA-mRNA and AGO2 protein in the RISC complex leads to transcript cleavage, while imperfect miRNA-mRNA binding and presence of AGO1, -3, or -4 lead to translation block. Due to the short sequence complementarity between a miRNA and its target, a single miRNA can regulate the expression of multiple genes (11, 19, 20). An overview of the miRNA biogenesis pathways is shown in Fig. 1.

Expression patterns of miRNAs vary between normal physiological processes and disease. These differences form the basis as to why miRNA profiles are being considered for diagnosis. Further, miRNA expression profiling has been used to differentiate cancerous cells. Recently, through use of a large set of cancer specimens, miRNA profiling was able to successfully differentiate the original tumor cells (21), as well as subclassify tumors (22). In addition, patterns of miRNA expression have successfully predicted clinical disease progression (23, 24). It is important to note that differential miRNA expression patterns and its diagnostic potential are not restricted to cancer diagnosis but have been used to evaluate distinct types of cardiomyopathies (25), musculoskeletal disorders (26), neurodegenerative disorders (27), autoimmune disease (28–33), and infectious

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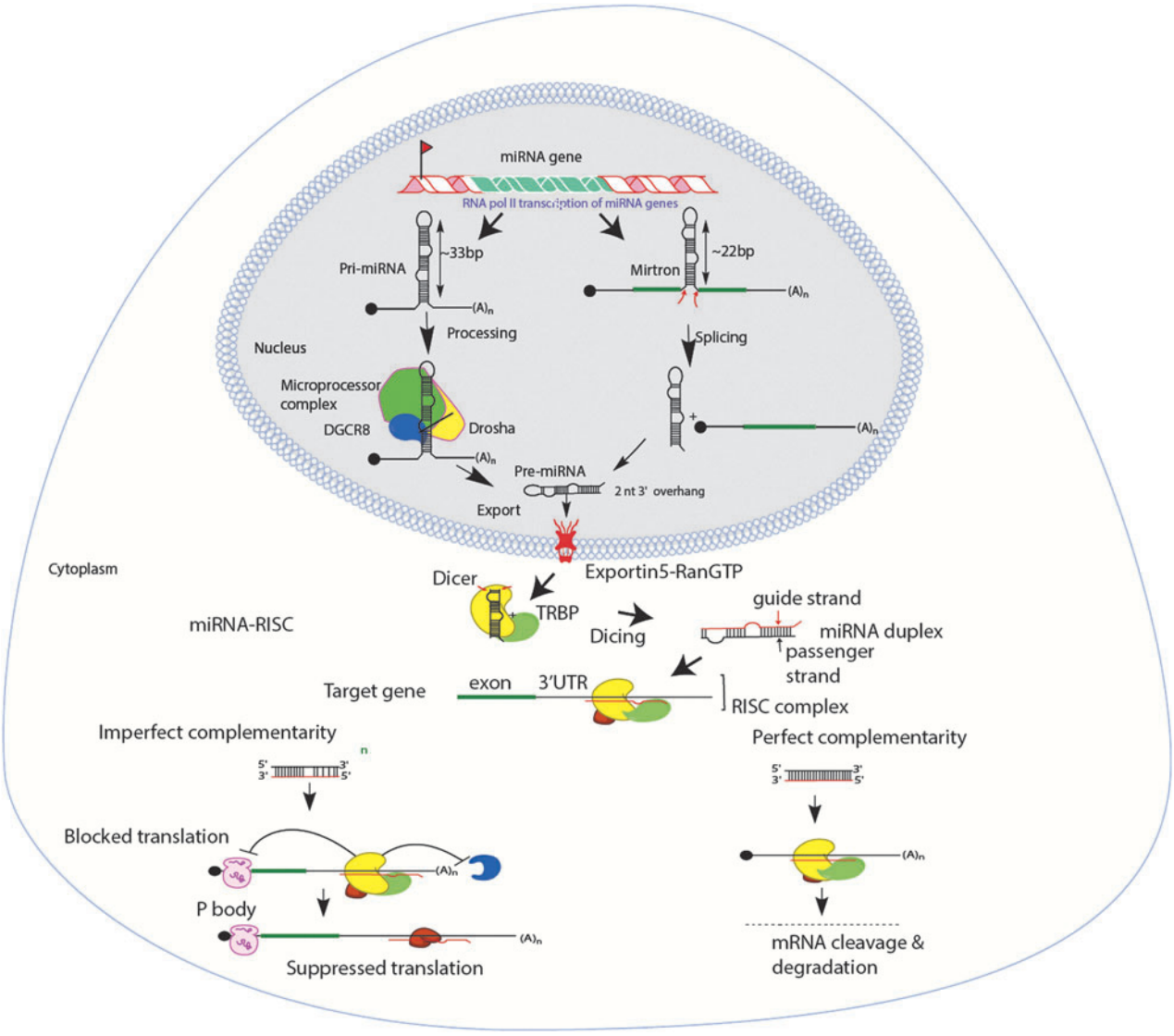


FIGURE 1 miRNA biogenesis pathways in animal cells. miRNA genes encoded in intronic/intergenic regions of the genome are transcribed by RNA polymerase II and processed by nuclear RNase III Drosha to generate pre-miRNAs that are actively exported out of the nucleus into the cytosol. Pre-miRNAs are further processed by a second RNase III Dicer to generate the mature miRNA dsRNA duplex that associates with Argonaute and other proteins to form the miRISC complex where posttranscriptional inhibition initiates.

diseases (34–40), which all drive unique miRNA profile signatures that can be used as potential biomarkers of disease progression and/or treatment.

The Human miRNA and Disease Database (HMDD) (41) is an excellent compilation of manually curated associations between miRNAs and human diseases extracted from the biomedical literature. HMDD provides relationships between known miRNAs and disease and evidence for deregulation and associated PubMed identifications that include detailed genomic annotation and information for the miRNA in question.

miRNAs AND THE IMMUNE RESPONSE

Host cells respond to microbial pathogen challenge by recognizing pathogen-associated molecular patterns (PAMPs) by using cell surface and intracellular pattern recognition receptors (PRRs). In response to a variety of biochemical moieties composing PAMP categories, host cells have developed multiple classes of PRRs that include the Toll-like receptors (TLRs), retinoic acid inducible gene (RIG-I/DDX-58)-like receptors, nucleotide oligomerization domain-like and absent in melanoma 2-like receptors (NOD2/MDA5), as well as C-like type lectin receptors and scavenger receptors. All PRR signaling cascades initiate via PAMP-PRR interaction, lead to the production of innate effector molecules (e.g., cytokines and interferons), and culminate in host antimicrobial responses that facilitate clearance of the pathogen. However, each step along this pathway is tightly regulated by miRNAs to achieve an effective temporal and spatial response, maintain homeostasis, and avoid chronic inflammatory or autoimmune diseases or malignancies. TLRs are one of the more investigated PRRs in terms of understanding of their ligands and

their regulatory and effector molecules (42–44). It is now becoming well understood that TLR ligands and pathogen infection trigger miRNA expression patterns that can be distinct among cell types, in a TLR and ligand-specific manner, suggesting that different pathways and molecular moieties are involved in the process (45–52). For example, lipopolysaccharide stimulation of TLR2 triggers miR-146a expression via NF-κβ activation, whereas miR-155 and miR-21 are induced via a MyD88/TRIF-induced Janus kinase (JAK) pathway (53). Similarly, miR-132 expression is induced by cyclic AMP response element binding protein and posttranscriptional coactivator p300 (54, 55). The deregulated miRNAs in turn regulate multiple genes and host cell pathways as a means to hone and amplify the immune response to the pathogen. Several miRNAs, such as miR-105, miR-146a, and miR-143 (56–58), negatively regulate TLR2, while miR-19a/b can upregulate TLR2 expression (59). Other miRNAs can affect TLRs; miR-223 (60) and miR-26a (61) suppress TLR3, miRNAs let-7e/-7i and miR-223 (60, 62, 63) suppress TLR4, and miR-511 positively regulates TLR4 (64).

Various adaptor and effector molecules that are recruited by TLRs upon activation are also miRNA targets. Adaptors such as myeloid differentiation factor 88 (MyD88), Toll-like receptor adaptor molecule 1 (TICAM1), Toll-interleukin 1 receptor (TIR) domain containing adaptor protein (TIRAP), kinases (IL-1R associated kinase 1 [IRAK1], Bruton agammaglobulinemia tyrosine kinase [BTK], MAPK kinases [MKKs]), TAK1-binding proteins (TABs), and inhibitor of kappa light polypeptide genes enhancer in B cells kinase beta (IKBKBs) and ubiquitin ligases (TRAFs) are targeted and governed by miRNAs upon PRR stimulation. For example, miR-146a and miR-146b are key regulators for the IRAK1/IRAK2 kinase and TRAF6 ligases (65–69). Similarly, miR-29 has been demonstrated to suppress TRAF4 expression (70). Components of the NF-κβ pathway, Fas-associated death domain protein (FADD), IKBKB, inhibitor of kappa light polypeptide genes enhancer in B cells kinase epsilon (IKBKE), receptor (TNFRSF) interacting serine-threonine kinase 1 (RIPK1) have been shown to be repressed by miR-155 (71, 72). Additionally, miR-155 regulates p38 MAPK signaling, TAB2 activity (71), and MyD88 (73) activity along with miR-149 (74) and miR-203 (75). MiR-145 has been shown to regulate a MyD88 adaptor-like protein TIRAP (76). MyD88 activity is also regulated by miR-200b/c (77) and miR-21(78). miR-346 targets BTK, a key kinase involved in TLR4, TLR7, TLR8, and TLR9 signaling pathways (79, 80).

The signaling cascades that initiate with PRR-PAMP interaction culminate in the activation and expression of cellular cytokines and antimicrobial effectors. Several studies have demonstrated that miRNAs directly or indirectly regulate transcription factors activated during TLR signaling (44, 81–88). Importantly, the NF-κβ1 transcript has been shown to be directly targeted by miR-9 (51) and miR-210 (89), while miR-329 targets NF-κβp65 (90). MiR-17-5p, miR-20a, and miR-223 target STAT3 (91, 92) and miR-155 targets CCAAT/enhancer binding protein-β (CEBP) (93), FOXP3, and E26 (94, 95), while miR-132 targets p300 (55). These are examples of some of the known mechanisms by which miRNAs govern host responses.

Because cytokine expression depends on the abundance of the cytokine transcript, the stability of the transcript, or the availability of the cellular machinery for translation, the role that miRNAs have in regulating host responses is clearly important. Further, their pattern of expression during a host response can be indicative of the state of immune activation or disease progression. MiRNAs are involved in degrading cytokine transcripts, modulating the RNA-binding proteins that stabilize the transcripts, and modulating the expression of negative regulators of cytokine expression and signaling pathways. Examples of some miRNAs that negatively regulate cytokines responses include miR-4661 suppression of alpha interferon (IFN-α) (96); miR-26a, miR-34a, miR-145, and let-7b suppression of IFN-β (87, 97); miR-125b and miR-187 suppression of tumor necrosis factor (TNF) (72, 98); miR-16, miR-365, and miR-142-3p suppression of interleukin-6 (IL-6) (87, 99, 100); miR-106a/b suppression of IL-10 (101); miR-21 suppression of IL-12 (102); and miR-29 suppression of IFN-γ (103). MiRNAs can regulate cytokine transcripts through degradation by affecting RNA-binding proteins that bind to AU-rich elements in their 3′UTRs. MiR-16 and miR-221 accelerate TNF degradation by destabilizing tristetraprolin (104), an important RNA-binding protein, while miR-579 and miR-125b reduce TNF translation by binding to tristetraprolin (72, 105).

Cytokines are generally secreted into the extracellular milieu and bind to cognate receptors to initiate autocrine and paracrine signaling modules. These signaling modules are regulated by multiple proteins including the suppressors of cytokine signaling (SOCS) family of proteins. MiR-155 has been shown to suppress SOCS1 (106), miR-146a to regulate Notch1, a positive regulator of IL-12p70 (107), and miR-98 and let-7 miRs to regulate cytokine-inducible Src homology 2 (CIS) and SOCS4 proteins (108). These studies and related ones (109, 110) are summarized in Fig. 2A to provide an overview of the role of miRNAs in governing the host innate immune response.

T cells, B cells, and antigen-presenting cells constitute core components of the adaptive immune response. Both T and B cells arise from hematopoietic stem cells (HSCs) in the bone marrow; T cells mature in the thymus, while B cells mature in the bone marrow. The process of maturation needs to be highly regulated because it commits the pluripotent HSCs into particular lineages. MiRNAs have been shown to have a critical role in T- and B-cell development. One example is the immune outcome observed in Dicer knockout mice, which have defective T-cell development, a feature that is in part regulated by miR-155 (111–113). Similarly, miR-125b is highly expressed in HSCs and has been shown to contribute to the control of the size of the HSC compartment in mice (114, 115) and to be repressed upon lineage commitment (116, 117). Additionally, miR-181a and miR-150 have been shown to exhibit dynamic changes in expression during T-cell development. MiR-181a exhibits transient up-regulation during late stages of T-cell development (118), and its inhibition impairs T-cell selection via regulation of several ERK regulatory phosphatases (119). MiR-125b regulates key cytokines that shape T-cell development including IFN-γ, IL-2 receptor beta (IL2Rβ), IL-10 receptor alpha (IL10Rα), and the IFN repressor Blimp-1 in naïve $CD4^+$ T cells. These features affect miR-125b expression, which can suppress the development of effector T cells (120), while miRNA-182 can suppress Forkhead box O1 (Foxo1) transcription factor, a negative regulator of T helper cell proliferation (121). An overview of the role that some miRNAs have in regulating the development and function of the adaptive immune response is provided in Fig. 2B.

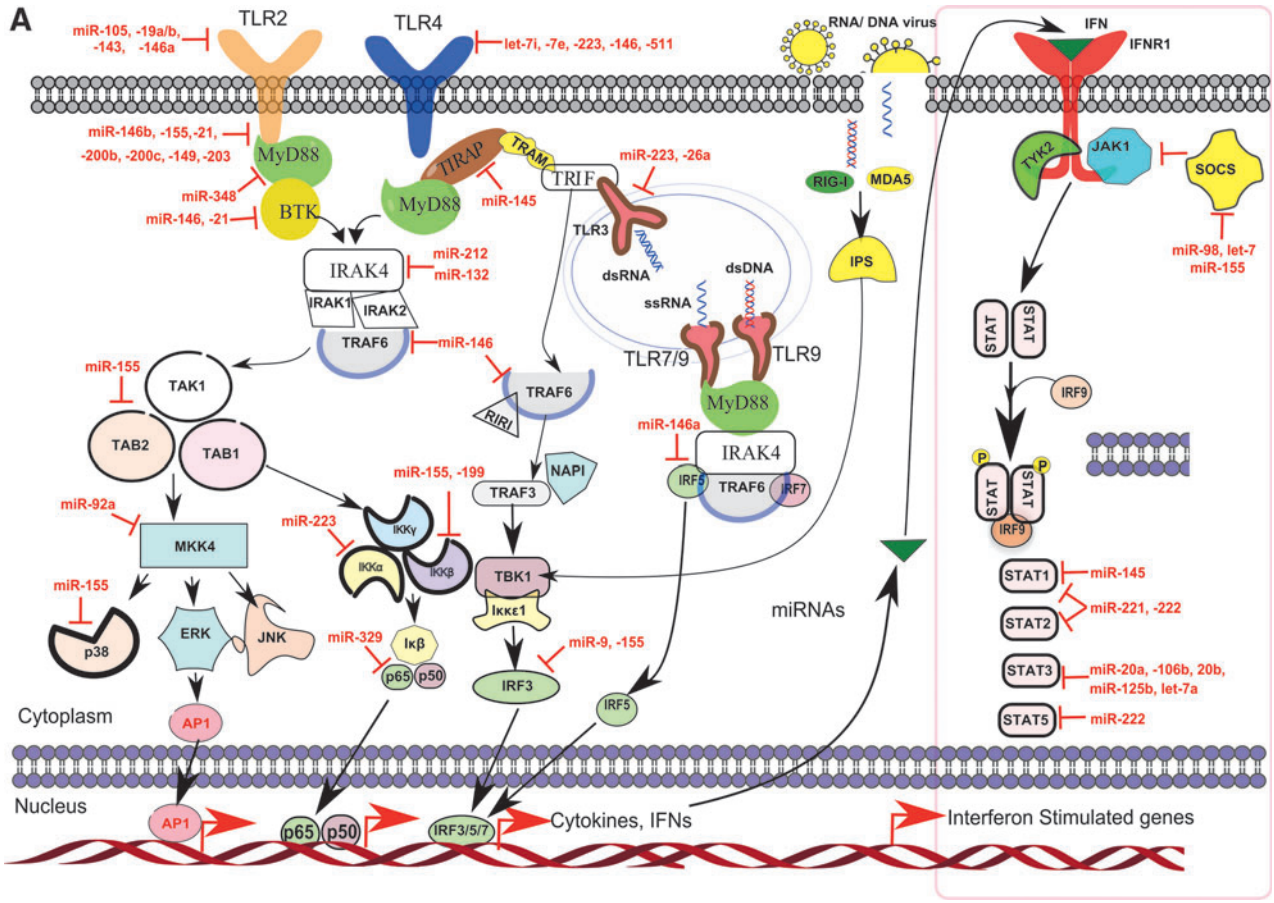


FIGURE 2 The role of miRNAs in the regulation of the host immune response. (A) Major pathways involved in the innate immune pathway are shown with major adaptor molecules. miRNAs that regulate these genes are shown in red. (B) The role of miRNAs in B- and T-cell development and function is summarized.

UTILIZING miRNA EXPRESSION PROFILES AS DIAGNOSTIC BIOMARKERS

Among the various biomolecule classes, miRNAs are very promising biomarkers owing to their stability in a variety of tissues and body fluids (122–125) and high stability within a particular sample type (126, 127). MiRNAs have been assessed in both extracellular biofluids as free entities and in exosomes. MiRNAs also associate with HDL and LDL (128) and AGO1 (129) and AGO2 proteins (130, 131). Consequently, free and exosome-associated miRNAs are present in most biofluids. The small size of miRNAs and their exosome-associated proteins makes them resistant to degradation—features that make for ideal biomarker candidates in fresh, frozen, and fixed specimens. Expression profiling of miRNAs in relevant biological specimens requires proper sample size using samples for which the origin of sample and storage and preservation techniques are documented because these variables can alter results (132–135). As in any study, a statistically controlled study design is needed with optimum sample collection time points and processing controls if the results are to be used as diagnostic biomarkers. The criteria chosen for patient selection and sample collection, storage, and analysis can introduce significant bias into the study and need to be carefully determined to obtain high-quality data (136–138). Consistent use of a validated assay across normal or diseased samples is recommended (139). It is also important to consider age, sex (140), and race (141) when profiling miRNA expression.

Biological samples are complex mixtures of various macromolecules and proteins, thus miRNA expression analysis typically necessitates purification and concentration of total RNA to small RNA fractions from these specimens. Unfortunately, contaminants in the sample, those introduced during sample processing, or operator error can alter the efficiency of the assay used for analysis and introduce biases that affect data interpretation.

DETECTING AND QUALIFYING miRNA EXPRESSION

Techniques for miRNA detection are founded on direct detection in the sample or by amplification-based methods (Table 1). Direct detection techniques rely on capture of the miRNA in solution, typically to a capture probe on a solid phase such as an array, followed by detection. These assays are sensitive and specific; however, while current technologies can detect a few miRNAs of interest they are not readily scalable for genome-wide discovery. Amplification-based techniques typically involve multiple preprocessing steps before miRNA expression can be determined, and they are thus more laborious, costly, and time consuming;

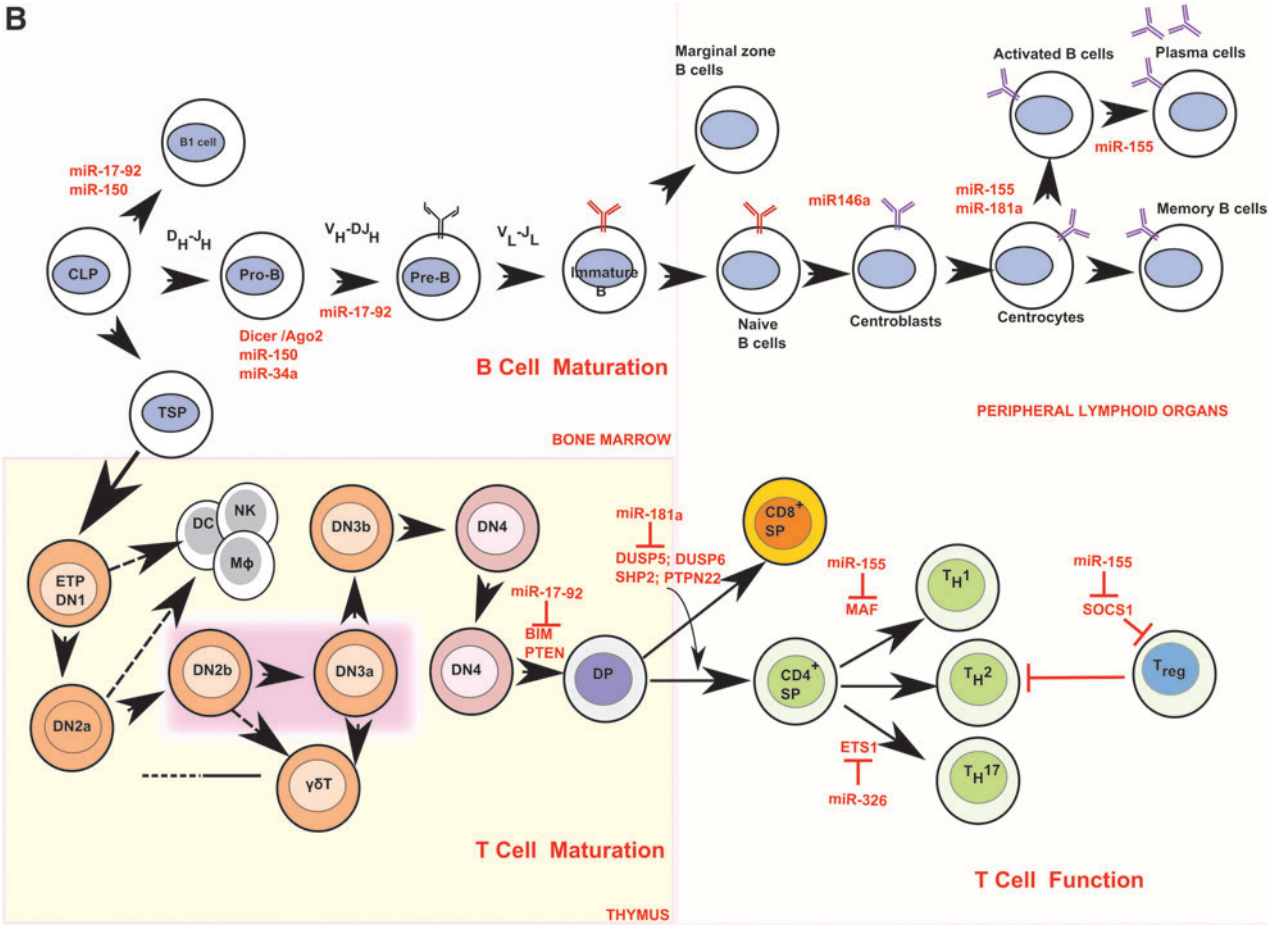


FIGURE 2 *(Continued)*

however, these methods are generally scalable and have been extensively used for genome-wide discovery and subsequent validation.

DIRECT DETECTION OF miRNAs IN SAMPLES WITHOUT AMPLIFICATION

Early efforts in miRNA analysis necessitated an RNA isolation step from the biological samples; however, recent studies have focused on alternate ways to measure miRNAs in clinical samples directly without RNA isolation or amplification steps to enable rapid detection. These techniques can be categorized into those that rely on electrochemical nanosensing for miRNA detection and quantitation, those that involve selective binding of miRNA hybrids using the p19 protein from Carnation Italian ring spot virus, or a combination of both. This was first demonstrated in a study that used an electrocatalytic nanoparticle tag strategy whereby miRNA oxidized with sodium periodate was hybridized to DNA capture probes on an electrode (142). Osmium oxide nanoparticles were then chemically ligated to the hybridized miRNAs, catalyzing oxidation of hydrazine and producing current. The technique detected miRNA concentrations as low as 80 fM (142). In a related study, modified gold-titanium microelectrode detection was performed using peptide nucleic acid capture probes that bind to miRNAs in solution (143). Captured miRNAs triggered a concentration-dependent charge along a polyaniline nanowire that was used for detection (143). Surface plasmon resonance imaging (SPRI) has also been used to detect and measure miRNA abundance directly in RNA samples (144). In SPRI, miRNA samples are first hybridized to a locked nucleic acid array and poly-adenylated using poly(A) polymerase. Bound miRNAs are detected by adsorbing poly(dT) gold nanoparticles followed by surface plasmon resonance detection. A colorimetric version of this assay has been used for detecting miRNAs (145). MiRNAs in solution are captured by biotin-labeled capture probes and subsequently hybridized to gold nanoparticles. The complex is captured on a streptavidin-coated plate, and the colorimetric signal is enhanced by silver and measured. Other techniques have exploited surface-enhanced Raman spectroscopy using gold nanoparticles (146) or silver nanorods (147–149) to measure miRNA presence or abundance in biological samples.

Alternative approaches have coupled the ability of the p19 protein from Carnation Italian ring spot virus to selectively bind to dsRNAs in a sequence-independent, but size-specific manner with nanomolar affinities and different detection technologies for miRNA detection. The p19 protein does not bind to ssRNA, rRNA, mRNA, ssDNA, or dsDNA. Among dsRNAs, the affinity of the p19 protein is maximal for 21- to 23-nt dsRNAs, and lower for 24- to 24-nt dsRNAs or dsRNAs having <19 nt (150, 151).

TABLE 1 Overview of miRNA detection techniques[a]

Technique	Method	Sen/spe/scale/cost	Amplification req.	Principle	LOD	Reference(s)
Microarray	Microarrays	V/L-M/H/H	Y	Fluorescence	pM	Multiple
Nanotechnology	Electrocatalytic nanoparticle tags (ENT)	H/H/M/H	N	Amperometry	fM	142
	Solid phase reverse immobilization (SPRI)	H/H/M/H	N	Carboxy-coated magnetic particle	aM	
	Gold nanoparticles (AuNP)	H/H/L/L	N	Probe-based colorimetry	fM	203–208
	Surface-enhanced Raman spectroscopy (SERS)	H/H/L/H	N	Raman spectra	fM	209–211
qRT-PCR based	Stem-loop qRT-PCR	H/H/L/H	Y	Fluorophore release during amplification	pM	Multiple
	SYBR Green qPCR	H/H/L/H	Y	Fluorophore binding to amplicons	fM	Multiple
	Oligo dT RT and PCR	H/M/L/L	Y	Fluorophore binding to amplicons	pM	Multiple
	Molecular beacons	M/H/M/L	N	Fluorophore excitation upon binding	nM	212–215
Amplification based	Padlock probes plus rolling-circle amplification	M/H/L/L	Y	Radioactivity incorporation	nM	216
	Bead-based flow cytometry	M/H/H/L	N	Fluorescence bead-based capture	pM	217
	Splinted ligation	L/H/M/L	N	Radioactivity incorporation	nM–μM	218
	Bioluminescence	H/M/H/L	N	Bioluminescent reporter binds to miRNA	fM	219–222
	RNA-primed array-based Klenow enzyme (RAKE)	H/H/H/M	Y	Biotin-based detection of miRNA-anti-miR complex	pM	223–225
	Invader assay	M/H/L/L	Y	Cleavase-mediated release of fluorophore from miRNA probe	nM	226, 227
	454 Pyrosequencing	L/H/H/H	Y	Sequencing	Single copy	228, 229
	SoLiD	L/H/H/H	Y	Solid-phase sequencing by synthesis	Single copy	230, 231
	Solexa	L/H/H/H	Y			
	p19 magnetic beads	H/M/L/L	N	p19 protein binds dsRNAs specifically	fM	152–154, 216

[a]Sen, sensitivity; Spe, specificity; LOD, limit of detection; L, low; M, medium; H, high; Y, yes; N, no; aM, attomolar (10^{-18} M); fM, femtomolar (10^{-15} M); pM, picomolar (10^{-12} M); nM, nanomolar (10^{-9} M); μM, micromolar (10^{-6} M).

This property has been exploited in p19 electrophoresis/electrochemical-based assays to detect miRNAs in clinical samples without RNA isolation or amplification (152–154). In the first approach, a fluorophore-labeled RNA probe-miRNA hybrid is captured by p19 protein and subjected to capillary electrophoresis in a run buffer that contains single-strand binding protein. Single-strand binding protein captures unbound probe and DNA and separates it from the p19 bound complex. This method has a linear detection range from 0.5 fM to 500 pM (152) (Fig. 3A). A complementary approach captures serum miRNAs on immobilized DNA capture probes followed by treatment with a double-strand-specific nuclease (DSN) that cleaves only capture probe-miRNA hybrids. This cleavage causes an increase in electrostatic barrier for the negatively charged ferri/ferrocynide redox couple and results in an increase in charge transfer resistance as measured by electrochemical impedance spectroscopy (155) (Fig. 3B). A trimodal electrochemical sensor has been developed that captures target miRNAs on gold nanoparticles using an immobilized miRNA-specific probes and triggers an upshift in the residual electrochemical hybridization signal (153). This upshift is dampened by the binding of p19 protein dimer to the captured miRNA (protein sensing) identifying the hybrid as a miRNA. Free target miRNA-specific probe and RNA are then added to the nanoparticles to release the substrate-bound p19 and cause a signal rebound (Fig. 3C).

The dsRNA-binding properties of the p19 protein have been recently combined with an extremely sensitive carbon nanotube field effector transistor transducer to develop a fast, facile, and low-cost nanobiosensor. Chemically assembled nanotubes functionalized with p19 show that such a nanobiosensor can bind a miRNA-probe hybrid to generate a highly sensitive specific signal (154). Alternately in-solution miRNA detection using DNA template silver nanocluster probes has been demonstrated (156). Here a DNA scaffold (12 nt long) along with a miRNA capture sequence is tethered onto silver nanoclusters, and miRNA binding mediates a change in the emission spectra multiplexed (156) (Fig. 3D).

Recently, a flow-cytometry-based miRNA detection method has been commercially introduced that utilizes biologically inert, hydrophilic porous hydrogel particles that have unique fluorescent barcodes enabling multiplexing. When total RNA is mixed with these beads, miRNAs bind to miRNA-specific probes tethered via an adaptor inside the bead. A biotin-labeled oligo complementary to the adaptor then binds and ligates to the miRNA bound downstream. Streptavidin-phycoerythrin conjugate added to the beads binds to the biotinylated miRNA and is detected by flow cytometry (157) (Fig. 3E).

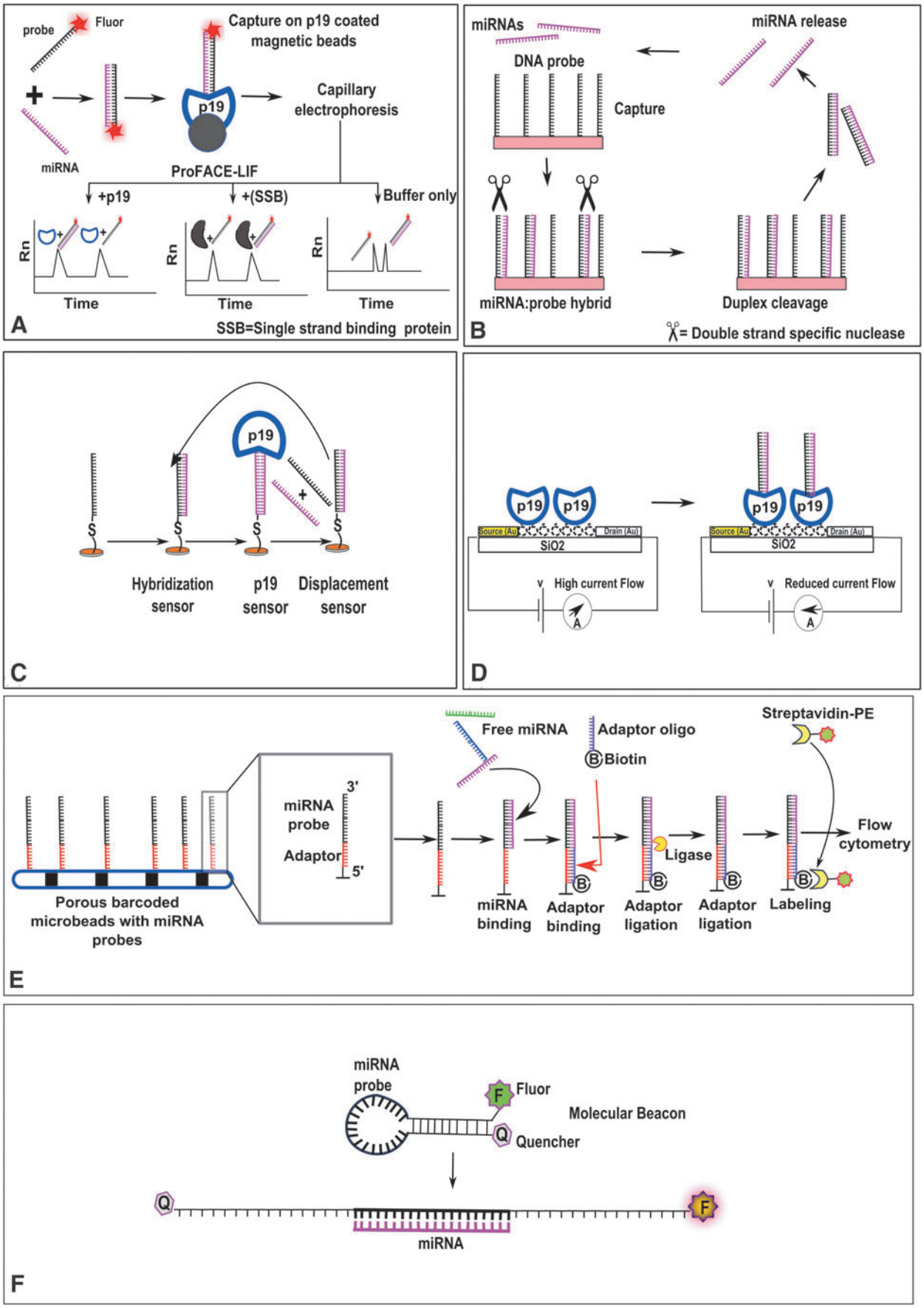
Molecular beacons are a means for detection of miRNAs. These are single-stranded probes carrying a strong quencher and a fluorophore at the two ends of the molecule. Molecular beacons are designed with stem-loop structures that normally keep the quencher and fluorophore in spatial proximity, preventing fluorescence. However, molecular beacons undergo a conformational reorganization upon recognizing a miRNA that is perfectly complementary, and this separates the quencher from the fluorophore, enabling fluorescence emission and measurement. Molecular beacons are extremely specific and sensitive and can distinguish between pri- and mature miRNAs (158) (Fig. 3F). While nanosensor-based miRNA detection techniques have been successfully used for small-scale detection, they are still not available commercially or amenable to high-throughput detection, a feature limiting their utility in screening for diagnostic miRNAs. A large number of variations around these electrochemical techniques exist and are not discussed here for brevity.

AMPLIFICATION-BASED miRNA EXPRESSION ANALYSIS

A number of factors can determine success in amplifying miRNA from clinical samples. These include the relative abundance of miRNAs relative to the other classes of RNAs in the sample, the small size of miRNA transcripts, and the availability and ease of collection of the sample. While direct detection technologies avoid amplification biases, they are also limited by the native concentration of the target miRNA and/or conditions that prevent its detection. In contrast, amplification-based detection techniques can help identify scarce miRNAs. Amplification-based detection methods can be endpoint or real-time assays. Real-time assays for miRNAs may be PCR-based or isothermal. Irrespective of the endpoint assay used to measure miRNA expression, both processes rely on successful isolation of miRNA containing total RNA/size fractionated RNA. The choice of a RNA isolation method is chiefly dictated by three main criteria: (i) the abundance of miRNAs in the sample, (ii) the abundance of potential inhibitory components in the sample, and (iii) the availability of the sample. Different specimens and body fluids vary greatly in the amount of RNA contained (159) and also exhibit variation in the number and concentrations of different miRNAs, though a small set of miRNAs seems to be shared across different body fluids (159).

Typically, total RNA isolation protocols involve either precipitation or elution. In both cases, isolation starts with a rapid lysis of the sample to release RNA from ribonucleoproteins and prevent degradation by contaminating

FIGURE 3 Overview of electrochemical and direction detection methods for miRNAs. (A) miRNAs bind to fluorophore-labeled probe in solution and are captured by paramagnetic beads coated with p19 protein which binds only dsRNAs. Bound hybrids are resolved by capillary electrophoresis alone or in the presence of buffer alone or single-strand-binding protein. Time resolved fluorescence intensity determines miRNA abundance in sample. (B) miRNAs are captured on surface-bound DNA probes and then cleaved by double-strand specific nuclease that changes the electrochemical signal on the chip in a concentration-dependent manner. (C) A triplex sensor-based approach based on hybridization; p19 binding and p19 displacement detect changes in square wave voltages in a miRNA concentration-dependent fashion. (D) miRNA binding to a p19 array on a carbon nanotube array causes reduction in current flow through the chip in a miRNA concentration-dependent manner. (E) Porous hydrogel-based microbeads carry miRNA probes which bind to target miRNAs. Biotin-adaptor oligos are attached to the hybrid followed by binding of a streptavidin-PE label and flow cytometry of the sample to detect miRNAs. (F) Molecular beacons bind to miRNA via a complementary region leading to spatial separation of the fluorophore and quencher on the beacon and production of a fluorescent signal.


probe
Fluor
miRNA
Capture on p19 coated magnetic beads
p19
Capillary electrophoresis
ProFACE-LIF
+p19
+(SSB)
Buffer only
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Time
SSB=Single strand binding protein
A
miRNAs
DNA probe
Capture
miRNA release
miRNA:probe hybrid
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= Double strand specific nuclease
B
Hybridization sensor
p19 sensor
Displacement sensor
C
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Biotin
Streptavidin-PE
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RNases typically present in biological samples or potentially introduced during sample handling. Lysis is followed by selective partitioning of the RNA from other macromolecules, including DNA, proteins, lipids, and carbohydrates. Aqueous RNA-containing fractions are then processed for RNA concentration either by salt-based precipitation or binding to charged silica matrices. Owing to their small size and lack of poly(A) tails, miRNAs bind poorly to silica columns, thus column-purified total RNA is typically devoid of the small RNA fraction, making it unsuitable for miRNA expression analysis. This limitation has recently been overcome, and a variety of commercial kits are now available that employ either solutions of different ionic concentrations and or matrices to enable selective binding of large versus small RNAs to the column followed by elution. Column-based protocols allow rapid processing of a large number of samples and result in high-quality RNA, but typically give lower or no yields in case of limiting sample volumes or dilute samples.

Solution-based RNA isolation methods (e.g., Trizol, Life Technologies) are more labor intensive, especially for a large number of samples, but give the operator more control over the RNA isolation and generally provide higher concentrations of RNA. One bottleneck typical in solution-based isolation is the need to carefully separate the aqueous RNA fraction from the organic fraction containing other biological macromolecules. Newer reagents such as RNAzol RT (MRCgene) overcome this limitation by precipitating the DNA, proteins, carbohydrates, and lipids in an easy-to-see pellet so that the RNA-containing aqueous phase can be quickly removed for precipitation. Visualization of RNA pellets can be significantly improved by incorporating co-precipitants, such as glycogen/linear acrylamide, or commercial products, such as Pellet Paint (EMD Millipore) or poly acryl carrier (MRCgene), during isolation. An overview of RNA isolation protocols is shown in Fig. 4. An A_{260}/A_{280} ratio of ≥1.8 along with $A_{260}/A_{230} \geq 1.5$ is indicative of good RNA quality but should be followed up with an assay to determine the size distribution profile or resolution by nondenaturing polyacrylamide modified agarose gel electrophoresis. Although agarose gel electrophoresis is unsuitable to resolve small RNAs, electrophoresis in a lithium metaborate buffer has been shown to give single base-pair resolution for both DNA and RNA, enabling a size distribution scan (160). MiRNA expression assays can then be performed on the isolated RNA, using either solid-phase or solution-based assays as noted previously. Solid-phase assays include Northern hybridization, microarrays, and recent hybrid techniques such as sequencing by synthesis. Solution-based assays can detect one or more miRNAs directly in the RNA population or may reverse transcribe RNA into cDNA followed by detection. Both assays are discussed in some detail below.

Northern Hybridization

Northern hybridization remains the oldest technique for determining expression of any gene. Northern hybridization relies on the sequence complementarity between the target transcript to be measured and a probe. Total/small RNA-enriched fractions are run on agarose or polyacrylamide gels along with molecular ladders, and then transferred onto nylon membranes by using either salt transfer bridges or electrophoretic transfer. A complementary ssDNA/ssRNA/oligonucleotide labeled with a radioactive or chemiluminescent tag is incubated with the target on the blot under conditions that promote binding of the probe and the target. Subsequent washes with decreasing salt concentration are used to remove nonspecifically bound probe. Blots are imaged using classical photographic film or image scanners followed by densitometric analysis for relative quantitation. Northern blots are useful for analyzing gross expression, but are labor intensive, require large amounts of RNA, are not amenable to high throughput, and do not provide accurate quantitation. As a consequence, Northern blotting is rarely used for screening miRNAs as diagnostic biomarkers and has been replaced by newer methodologies.

miRNA Microarrays

Genome-wide miRNA expression can be studied using miRNA microarrays, which are based on the principle of sequence complementarity between a miRNA probe and the miRNA in solution. Traditional oligonucleotide probes or locked nucleic acid probes that will hybridize to a single miRNA species are covalently spotted onto substrates like glass slides along with positive and negative control spots. Total or size-enriched RNA from different experimental conditions or specimens is labeled using fluorescent dyes and subsequently hybridized to the microarray slides in a hybridization buffer. The hybridization buffer composition is optimized to achieve similar hybridization conditions for miRNAs of different sequences and composition. Arrays are washed multiple times with solutions of reducing salt concentration until an optimum signal with minimal background fluorescence is obtained. Fluorescence intensity from each spot is scanned using an array scanner and converted into heat maps depicting the intensity of the signal at that probe. A variety of computational algorithms such as hierarchical clustering are then used to cluster spots that have similar intensities to determine genes that show similar expression profiles. It is a common practice to repeat arrays with dye-switched samples to confirm hybridization. Despite their widespread utility in determining miRNA expression, miRNA microarrays suffer from several drawbacks. For example, the diversity in sequence composition across different miRNAs means that with use of given ionic conditions, not all miRNAs can bind with equal stability to their probes. Further, many miRNA families contain multiple members that differ in one or a few nucleotides. Microarrays cannot typically distinguish between these members. Also, the RNA labeling and hybridization efficacy between manufacturers varies, thus a direct comparison can be misleading. Despite these drawbacks, miRNA microarrays remain the quickest way to obtain genome-wide snapshots of miRNA expression.

miRNAs from isolated total RNA or size-fractionated RNA can also be detected using padlock probes coupled to rolling circle amplification (161–163). Padlock probes are linear DNA probes with 3′ and 5′ termini that are perfectly complementary to the target miRNA and bind to miRNA in solution. Upon binding, the padlock probe is circularized by phi29 DNA polymerase, which then amplifies the bound miRNA using the miRNA as a primer for rolling circle amplification (164). Northern hybridization, microarrays, and padlock probes typically require larger amounts of RNA and are of limited utility in the case of limiting RNA or when RNA is degraded. In such cases, reverse transcription quantitative PCR (RT-qPCR)-based methods have significant advantages. RT-qPCR methods can analyze gene expression profiles from single cells, fixed tissues, and limiting amounts of biofluids; however, the features unique to miRNAs necessitate several modifications to standard RT-PCR protocols.

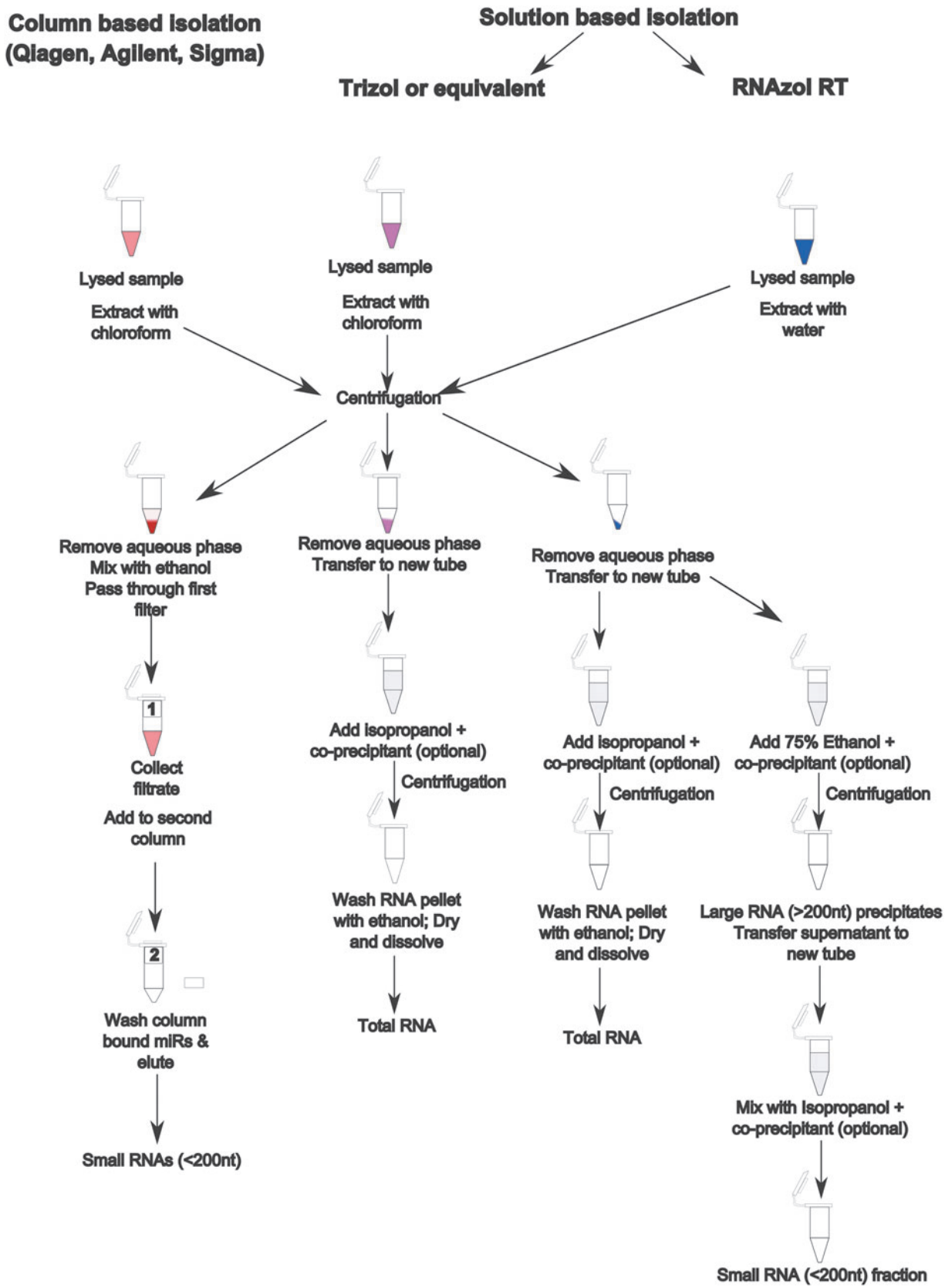


FIGURE 4 Overview of RNA isolation strategies for assessing miRNAs in cells/tissue or clinical samples. Tissues/biofluids are lysed in a lysis buffer, fractionated, and then eluted from silica columns or precipitated using a salt + alcohol combination. Size-fractionated RNA can also be isolated using modifications of these protocols.

Since miRNAs lack poly(A) tails, they typically must be polyadenylated before they can be reverse transcribed uniformly. Size-fractionated or total RNA is treated with *Escherichia coli* poly(A) polymerase in the presence of rATP to add a stretch of As (60–150) to the 3′ end of all RNAs including miRNAs followed by inactivation of the enzyme. Polyadenylated RNA may be stored at −20°C or reverse transcribed immediately. Reverse transcription of the polyadenylated RNA is primed using proprietary commercially available oligos that contain adaptor sequences for subsequent qPCR or using oligo $dT_{(16–23)}$ and a reverse transcriptase (AMLV/MMLV/recombinant) of choice. Depending on the abundance of the miRNA in the sample and detection method employed, the miRNA-first strand cDNA may be detected using SYBR Green-based qPCR/TaqMan hydrolysis probes that are commercially available. Similarly, a bead-based flow cytometry technique can be used for miRNA from tissues. In one example, 217 human miRNAs were detected from 334 cancer tissues (21). In this method, miRNAs were ligated to adaptor oligos at both ends and then reverse transcribed with oligos complementary to those adaptors. The cDNA is then PCR amplified with a biotinylated forward primer and mixed with fluorescent beads coated with streptavidin to capture the miRNA.

Real-Time qPCR/RT-qPCR

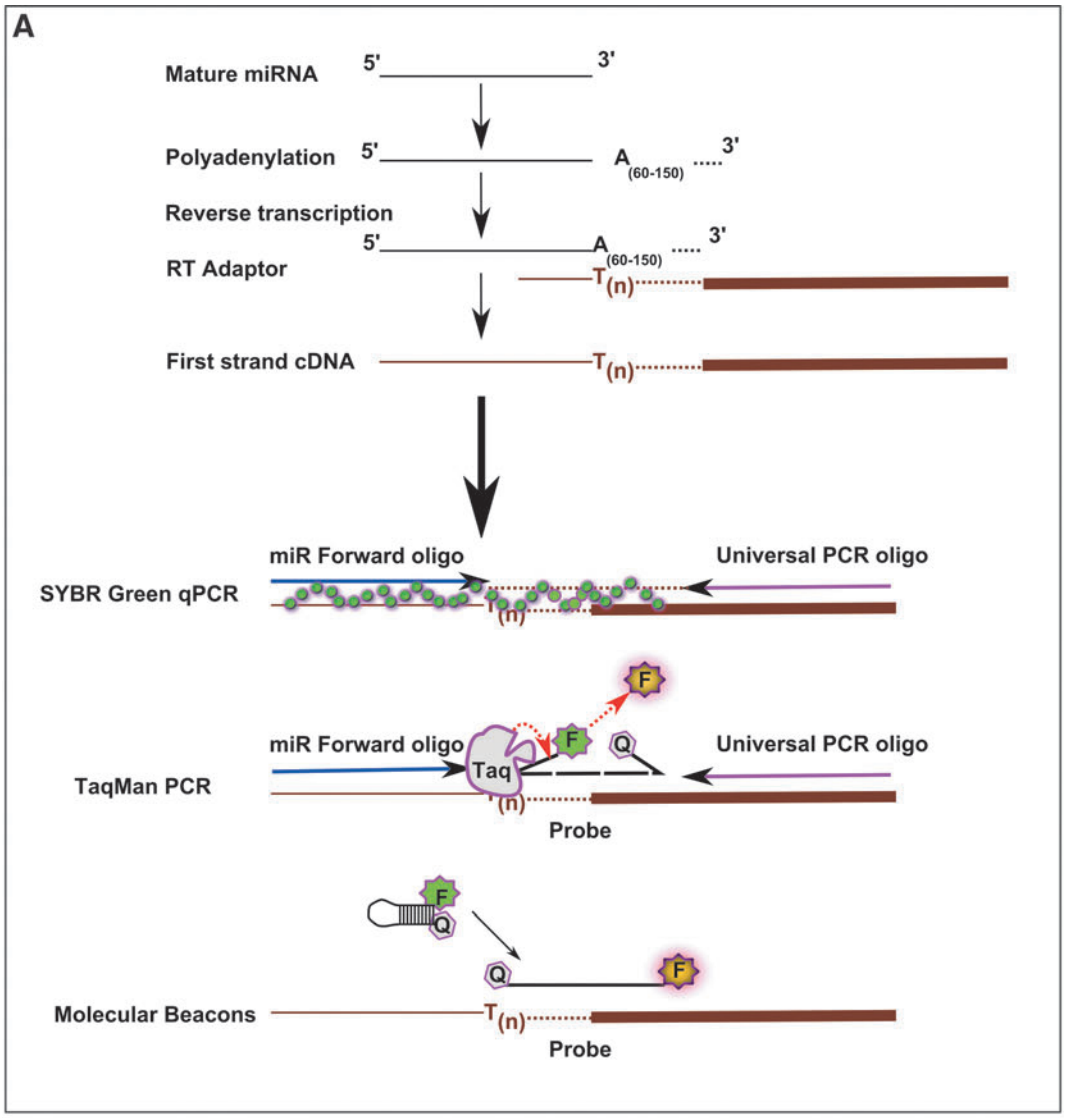
Quantitative PCR remains the most widely used method for gene expression analysis including those of miRNAs. In contrast to traditional PCR, which measures amplicon abundance as an endpoint, real-time qPCR measures the amplification of target transcripts as they are amplified during the logarithmic amplification phase of PCR, providing extremely accurate quantitation. A variety of different chemistries such as SYBR Green, TaqMan primer-probe, and Molecular Beacons and Scorpion probes are commercially available for traditional qPCR (Fig. 4). SYBR Green chemistry utilizes the property of SYBR Green (or equivalent) dyes to preferentially bind to dsDNA compared to ssDNA. SYBR Green thus binds only the dsDNA product formed at the end of each cycle, and a plot of fluorescence versus cycle number yields an amplification curve for the gene of interest. TaqMan primer-probe methods utilize the 3′-5′ exonuclease activity of *Taq* polymerase to separate a fluorophore from a quencher in the probe during amplification yielding a fluorescence signal and increasing specificity of the reaction. Molecular beacons utilize a stem-loop structure in the probe to quench the fluorophore under normal conditions and only a full complementarity with the probe triggers separation of the fluorophore and the quencher to give a fluorescent signal. Scorpion chemistry incorporates the stem-loop structure in the primer itself to bring about quenching. Real-time qPCR can be used to quantify DNA or RNA that has been reverse transcribed into cDNA relative to a reference gene (whose expression does not change under the experimental conditions being studied) or in absolute copy numbers. Commonly used reference genes include beta-actin, 18S rRNA, hypoxanthine-guanine phosphoribosyltransferase, miR-16, U6 snoRNA, miR-451, miR-142-3p, and so forth, but these must be tested individually. It is also critical that amplicon sizes of the miRNAs and the reference genes be similar to achieve identical or similar amplification efficiencies. Fold differences in gene expression relative to the reference gene are typically calculated using the ΔΔCt method (165). A variety of contaminants in biological samples can significantly affect RNA isolation and miRNA detection.

PCR primer design for miRNAs presents unique challenges. In SYBR green-based assays in which the entire miRNA sequence is used as the forward oligo, it is important to check for a compatible annealing temperature with the universal primer and a lack of hairpin structures especially in the 5′ and 3′ ends of the primer having specificity for the miRNA being studied. Since differences in qPCR chemistry, RNA isolation protocols, amount of input RNA during reverse transcription, reference genes used for analysis, and contaminants in biological samples can significantly alter qPCR results, it is now common to follow minimum information for quantitative experiments (166, 167) guidelines for reporting qPCR data. While widely used, traditional RT-qPCR-based assays for miRNA expression profiling are expensive and time consuming and have low accuracy. These disadvantages preclude exact copy number enumeration of miRNAs during a physiological process.

Digital droplet PCR overcomes these challenges by fractionating a PCR amplification into nanoliter-sized droplets, which are amplified and then subjected to rapid microfluidic analysis, enabling a rapid and precise calculation of concentrations in the sample. Droplet digital PCR was recently demonstrated to measure the absolute copy numbers of six miRNAs (miR-141, miR-375, miR-210, miR-135b, miR205, and miR-16) in pure RNA samples as well as in serum (168). An overview of real-time methods is shown in Fig. 5.

Amplification-based technologies can also use isothermal conditions for amplifying the miRNA signal (169–172). Loop-mediated isothermal amplification (LAMP) utilizes the strand displacement of DNA polymerase under isothermal conditions to obtain signal amplification from the miRNA-containing sample and a hybrid miRNA primer (171). In an alternative approach, Bi et al. (169) used a dumbbell probe that is circularized upon miRNA binding by T4 DNA ligase and then amplified isothermally by a DNA polymerase to yield concatameric copies of the miRNA-probe template. A nicking endonuclease separates these copies, which bind hemin to generate a DNAzyme that catalyzes the oxidation of a peroxide substrate 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid to generate a green product that can be measured colorimetrically (169). Ma et al. (172) modified a beacon-assisted detection amplification technique to increase its sensitivity to ~8.5 fM with a five orders of magnitude

FIGURE 5 Overview of *Taq* real-time miRNA detection techniques. Total or size-fractionated RNA is polyadenylated followed by reverse transcription using an adaptor oligo or oligo dT primer. (A) First strand cDNA synthesized can be then PCR amplified using a miRNA-specific forward oligo and a universal PCR oligo (in case of SYBR green chemistry) or a forward oligo, probe, and reverse oligo (in case of TaqMan chemistry) or using molecular beacons. (B) PCR master mix containing first-strand cDNA and miRNA-specific primers/probes is fractionated into nanoliter droplets followed by routine PCR amplification. Amplified product is then analyzed by a modified flow cytometer to detect sample populations.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Mature miRNA
5'
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Polyadenylation
A(60-150)
Reverse transcription
RT Adaptor
T(n)
First strand cDNA
SYBR Green qPCR
miR Forward oligo
Universal PCR oligo
TaqMan PCR
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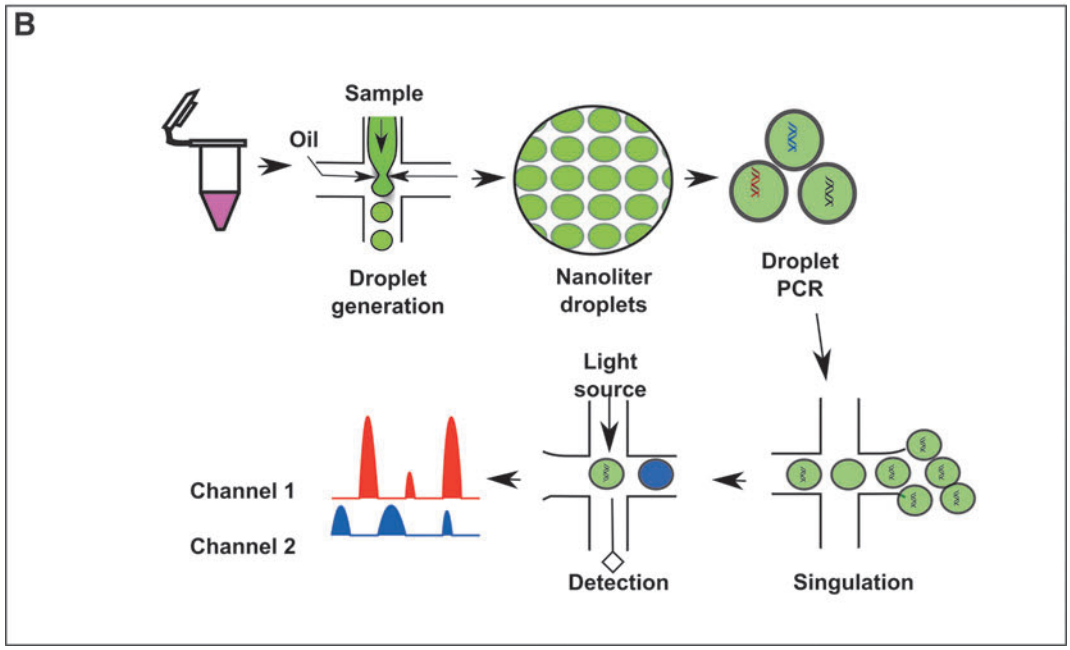
Probe
Molecular Beacons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Sample
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range. A recent study described an isothermal amplification approach that uses the Bst DNA polymerase quadratic amplification followed by lambda exonuclease-aided strand recycling and signal amplification for miRNA-molecular beacons (170). Variations of these techniques have been reported but are not discussed owing to length restrictions.

Next-Generation Sequencing Methodologies

Gene expression profiling has been revolutionized by the development of next-generation sequencing technologies. Currently, three main technologies are available commercially, specifically the Ion Torrent Personal genome machine (PGM), Pac Bio RS, and Illumina MiSeq. The Ion Torrent PGM is based on the detection of protons released during nucleotide incorporation during DNA synthesis (173). DNA fragments ligated to specific adaptors are clonally amplified using emulsion PCR with ion sphere particles which are 3-μm beads. DNA-bound beads are loaded onto silicon chips with proton-sensing wells microfabricated on them. DNA synthesis is initiated at a specific position from the ligated adaptor followed by sequential introduction of DNA bases. If a base is incorporated, protons are released and the signal is proportional to the number of bases incorporated. Ion Torrent sequencing has been used for miRNA expression analysis (174–176). Sequencing by synthesis is the approach used by Illumina MiSeq, which employs fluorescently tagged reversible terminator nucleotides to clonally amplify DNA templates immobilized on acrylamide base on the surface of a glass flow cell. In Illumina sequencing, size-fractionated small RNA is ligated to 3′ and 5′ sequencing adaptors sequentially and reverse transcribed. First-strand cDNA is then amplified using a common primer and another primer containing one of 48 index sequences. The index sequences reduce RT bias and also allow for multiplexing. PCR products are electrophoresed on a gel, and bands corresponding to miRNA products are cut and purified for library construction. Next-generation sequencing methods offer high accuracy in distinguishing miRNAs that are similar in sequence and can also identify miRNA variants, termed isomiRs, that can be expressed in a cell-specific manner and likely have functional differences that may be biologically relevant. One major drawback to this method of miRNA detection is that substantial computational support is required for data analysis, and next-generation sequencing cannot be used for absolute quantification.

Current Status of miRNAs as Diagnostic Biomarkers

Comparative miRNA expression profiling between normal and diseased states during disease progression or therapy is being extensively employed now to identify biomarkers of disease, progression, and/or treatment efficacy. While an extensive review of the miRNA biomarkers in various diseases is out of the scope of this article, we highlight recent findings on the utility of miRNAs as biomarkers for two important human pathologies viz. sepsis and HIV.

Sepsis is an unregulated pro- and/or anti-inflammatory response to systemic bacterial, viral, fungal, or parasitic infection or may also develop in the absence of any infection (sterile sepsis) in response to trauma. Pathogen-associated sepsis, which accounts for 50% of cases, is an unregulated response to PAMP recognition by host PRRs resulting in a cytokine storm, massive up-regulation of reactive oxygen species (ROS) in infected tissues, reactive nitrogen species (RNS), formation of ROS/RNS-DNA/protein/lipid adducts, deregulation of mitochondrial electron transport chains, and tissue damage (177). Traditionally, proteins such as C-reactive protein (CRP) and procalcitonin (PCT) (178) have been used as sepsis biomarkers for diagnosis and guide for antibiotic therapy. Recently, molecules such as soluble urokinase plasminogen activator (suPAR) (179), intercellular adhesion molecule 1 (ICAM1) and E-selectin (180), D dimer (181) and IL-8 mRNA (182) have been shown to have an association with sepsis severity as well. Though PCT levels are also used to guide antibiotic therapy in sepsis and other bacterial infections, a recent meta-analysis suggests a low correlation and specificity between PCT levels and sepsis/systemic inflammatory response (183). Additionally, PCT is not detected in many cases of sepsis (184). Recently, many studies have explored the utility of miRNAs as biomarkers for sepsis (185–193) (Table 2). Of these, miR-499-5p shows a gradient of increased expression from mild to severe sepsis to septic shock and with an area under curve of 0.69 can successfully distinguish among these three states using regression analysis (194).

HIV predominantly infects the $CD4^+$ population of T cells, rapidly integrates in the genome, and becomes latent. Latent HIV infection can reactivate, leading to AIDS and emergence of secondary opportunistic infections that cause severe morbidity and mortality. Progression of HIV infection is typically monitored by determining plasma viral loads and CD4 T-cell counts. However, neither of these surrogate biomarkers is reliable in patients undergoing antiretroviral therapy (ART). HIV infection predominantly causes a significant down-regulation of host miRNA expression, which correlates with the resident status of $CD4^+$ population (195) and the degree of disease progression (195). HIV actively suppresses miR-17/92 cluster expression via histone acetyltransferase Tat cofactor PCAF (196) promoting viral latency (197). This is reflected in HIV-1-infected elite controllers/suppressors (ES), individuals that maintain undetectable viral loads without ART (198). Analysis of miRNA expression in peripheral blood mononuclear cells from HIV-infected asymptomatic/symptomatic, ART naïve/symptomatic on ART, and ART failure patients showed that miR-150 and miR-146b-5p showed consistent differences between these groups and hence could be used as biomarkers for ART monitoring (199). MiRNA expression deregulated during AIDS-associated complications can also be used to monitor progression of the malady. In case of hepatitis C virus (HCV) coinfections with HIV, serum miRNA profiles show significant upregulation of miR-122, miR-22, and miR-34a. Moreover, expression of these miRNAs correlates well with liver function tests and surrogate predictors of liver injury; miR-122 was established as an independent biomarker for fibrosis in HIV patients, and miR-22 and -34a correlate with HIV/HBV and drug use markers (200). miR-21 and -122 expression has also been shown to discriminate HIV-infected from uninfected individuals, and among HIV-positive individuals, miR-222 enabled differentiation of diffuse large B-cell lymphoma and primary central nervous system lymphoma (201). Expression levels of 11 miRNAs (miR-1203, miR-1224-3p, miR-182*, miR-19b-2*, miR-204, miR-362-5p, miR-484, miR-720, miR-744*, miR-934, and miR-937) were able to discriminate between encephalitic and nonencephalitic HIV-positive individuals (202). These studies demonstrate that miRNA expression profiles can be successfully used to discriminate between healthy versus diseased individuals or between disease progression states.

TABLE 2 Summary of putative miRNA biomarkers identified to date for microbial infections

miRNA	Type of biomarker	Pathology	Detection technology	Sample type	Expression change	Reference(s)
miR-15a	Diagnosis	**Sepsis**	qRT-PCR	Serum	Induction	232
miR-16						
miR-122	Diagnosis		qRT-PCR	Serum	Repression	194
miR-499-5p						
miR-146a	Diagnosis		qRT-PCR	Serum	Repression	233
miR-223						
miR-483-5p	Prognosis		qRT-PCR	Serum	Repression	234
miR-193b*						
miR-574-5p	Prognosis		qRT-PCR	Serum	Repression	235
miR-150	Diagnosis		qRT-PCR	Serum	Repression	236–238
miR-4772	Diagnosis		Next-generation sequencing plus qRT-PCR	Blood	Induction	238
miR-574-5p	Diagnosis		Microarray plus qRT-PCR	Serum	Induction in survivors	239
miR-297	Diagnosis		Microarray plus qRT-PCR	Serum	Repression in survivors	
miR-181b	Diagnosis		qRT-PCR	Plasma	Repression in sepsis patients	240
miR-133a	Diagnosis and prognosis		qRT-PCR	Serum	Significant induction in sepsis patients	241
miR-17/92 cluster	Diagnosis	**HIV**	qRT-PCR	Serum	Induced in latent HIV infections	197
miR-150	Diagnosis		qRT-PCR	Serum	Induced in HIV-infected individuals and those on ART	199
miR-146b						
miR-122	Diagnosis		qRT-PCR	Serum	HIV/HCV coinfection	200
miR-21	Diagnosis		qRT-PCR	Serum	Induced in HIV-infected individuals	201
miR-122						
miR-1203	Diagnosis		qRT-PCR	Serum	Differential expression in encephalitic vs. nonencephalitic individuals with HIV	202
miR-1224-3p						
miR-182*						
miR-19b-2*						
miR-204						
miR-362-5p						
miR-484						
miR-720						
miR-744*						
miR-934						
miR-937						

CONCLUSIONS

It is clear that miRNAs can be biomarkers for improved diagnostics, and understanding their patterns of expression from homeostasis to disease to disease resolution offers a path forward for therapeutic treatments and personalized medicine. As diagnostic biomarkers, miRNA expression patterns can offer a better understanding of the mechanisms affecting the host and the host response to disease and can be prognostic; however, much is still to be learned about miRNA biology such as the role of miRNAs in circulation as opposed to intracellular miRNAs and those expressed by specific cells and tissues. Assays and improvement in existing assays are needed (Table 1) to provide reliable and reproducible normalized results ideally without amplification of the target miRNA, and overall, these assays need to be validated. Clinically, there is much to do to provide convincing data that support miRNA screening as a viable diagnostic tool. These studies that are needed must center on

statistical validation and statistical power, on case-control cohort studies, on healthy and diseased individuals of both sexes and all races. This is not an overwhelming task to accomplish, but a necessary one.

As cell biology, basic science, and translational medicine bridge, miRNA biomarker discovery programs must merge and drive profiling and diagnostic technologies to allow comparison of this technology to current gold standards in diagnostics. Further, functional validation of miRNAs reported to diagnostic biomarkers must be confirmed, allowing for the identification of their target genes and pathways. Given the current and recently reported studies that have addressed methods for discovery, detection, and quantitation of miRNA across a range of biological specimens and the potential of miRNAs as biomarkers of disease, there is a new field of diagnostics emerging that will undoubtedly positively impact health care and patient prognosis and treatment in the near future.

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Host Response in HIV Infection

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45

HIV is widely considered as capable of infecting all humans and of leading to relentless disease progression. However, there is considerable diversity in human susceptibility to HIV and in clinical responses. Understanding the basis of interindividual diversity to infection is important for the development of diagnostic tools and vaccine development. Today, numerous studies in genetics, genomics, and early applications of systems biology have delivered knowledge on immunity against HIV on the basis of differences in disease manifestations (Table 1). Research has also led to the identification of biomarkers, in particular in the form of pharmacogenetic applications. This chapter will discuss the knowledge acquired through new technologies and describe the genetic tests that are in use in clinics or that may progress to routine use.

GENETIC SUSCEPTIBILITY

A fundamental observation in infectious disease biology is that, given approximately equal exposure, there is individual-level heterogeneity with respect to infection acquisition and the rate of disease progression. As with many other complex traits, susceptibility to infection, and to progression after infection, is influenced by the genetic composition of the host. However, unlike many other complex traits, genetic variation in the pathogen, and the interaction between host and pathogen, also contribute to disease outcome. In the current genomic era, we have unparalleled ability to measure genetic variation in both the host and the pathogen through high-density genotyping arrays, and next-generation sequencing techniques. As discussed in detail below, genetic studies of HIV infection have focused on the individual contributions of genetic variation in the host and the virus to susceptibility to acquisition and clinical outcome. Newer studies are now focusing on the effect of joint host/virus variation on these phenotypes.

HIV Acquisition

Reduced susceptibility to HIV acquisition has been observed in multiple clinical settings including: uninfected hemophiliacs exposed to contaminated blood products, HIV seronegative individuals with multiple documented unprotected sexual exposures, and HIV serodiscordant couples, among others (reviewed in reference 1). Early genetic studies aimed at explaining this reduced susceptibility met with success by identifying the CCR5Δ32 mutation that is almost completely protective in homozygous individuals (2). However, homozygosity for this variant is rare (at ~1%) and restricted to populations with European ancestry, thus explaining only a small proportion of HIV-exposed seronegative individuals. Following this early success, several candidate gene studies in the pregenomic era reported additional associations with reduced susceptibility, none of which have been consistently replicated (3–5).

To date, there have been several genome-wide association studies (GWAS) aimed at detecting common genetic variants (i.e., those found in >5% in the population) that impact susceptibility to infection. These studies can be divided into those investigating HIV-exposed seronegative individuals (3–5) and those comparing HIV-infected individuals to the general population (6). These studies did not identify any associations beyond CCR5Δ32. Of interest, the largest of these studies, comparing 6,300 HIV-infected individuals to 7,200 population controls, initially identified the HLA region as an associated factor (6). However, the study went on to demonstrate that this association was due to the frailty bias inherent in studies of lethal diseases, where survival markers are enriched in patient samples and do not necessarily impact disease acquisition.

Although these studies have failed to identify any new associations with HIV susceptibility, they have all been limited in power either by small size or by potential phenotypic misclassification. It remains to be seen whether variants of modest effect sizes, rare variants, or other forms of genetic variation not assessed by GWAS (e.g., copy number polymorphisms and epigenetic modifications) may contribute. Larger GWAS, potentially in prospective cohorts of seronegative individuals in high prevalence areas, as well as sequencing studies, will need to be performed in order to address these issues.

Disease Progression

Similar to studies of HIV acquisition, genetic investigations of differential disease progression met with early success in identifying classical class I HLA alleles with a strong impact on outcome after infection (Table 1). The best examples of these include the association of HLA-B*57:01 and B*27:05 with elite control of viremia, and B*35:01 with increased viral load (7). In addition to these alleles, which are found predominantly in European

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TABLE 1 Knowledge gained from genomic research in HIV infection

Study/phenotype or trait	Approach	Confirmed associations
Susceptibility to infection	DNA; GWAS	*CCR5Δ32*. Studies in exposed noninfected hemophiliacs and in large HIV-infected populations failed to identify other genetic factors.
Disease progression and severity	DNA; GWAS DNA, targeted analysis	*HLA* locus, *CCR5* locus, *KIR* locus. These regions associate with viral load, disease progression, elite control study. They explain up to 15% of clinical diversity.
Disease progression and severity	Transcriptome analysis (microarrays)	The interferon response is abnormal in HIV chronic infection. Disease severity associated with elevated expression of interferon-stimulated genes.
Viral escape and adaptation	Genome-to-genome (host and viral DNA)	Human HLA diversity associated with escape and compensatory mutations across the viral genome. Some of the escape variants are associated with lower viral fitness.
Viral replication	RNA SAGE[a] and sequencing	Viral replication completes in 24 hours, accompanied with profound modifications of the cellular transcriptome.
Viral latency and response to reactivating agents	RNA sequencing	Viral latency generates minimal perturbation of the cellular transcriptome. Upon reactivation, each agent results in a transcription signature.

[a]RNA SAGE, RNA serial analysis of gene expression.

populations, investigations in non-Europeans have identified population-specific alleles of HLA class I that also impact disease (8).

Variation in the CCR5 region has also been convincingly demonstrated to impact the rate of disease progression. In addition to providing protection against acquisition when carried in homozygosity, individuals carrying one copy of the CCR5Δ32 allele have lower viral load and slower disease progression. Beyond the Δ32 allele, a variant in the promoter region of CCR5 and a missense mutation in the nearby CCR2 gene (CCR2 V64I) have also been convincingly associated with slow disease progression. However, the tight correlation between these variants due to linkage disequilibrium prevents accurate determination of which one may be causal. In further parallel studies of HIV acquisition, candidate gene studies searching for further variants that modify progression claimed a number of associations that have since failed replication.

The key contribution of the major histocompatibility complex (MHC) region (and to a lesser extent CCR5) in modifying disease progression has been underscored by GWAS, which have consistently shown these to be the strongest associations genome-wide (9, 10), with no evidence for association elsewhere in the genome. Although it is difficult to define the precise variants driving the association signal given the complexity of the linkage disequilibrium in the MHC region, key amino acid positions in HLA-B (10, 11), and an effect of HLA-C expression level (12), have been proposed as likely causal candidates. Additionally, copy number variation at the KIR locus (specifically the KIR3DL1-KIR3DS1 locus), not tagged through GWAS, has also been proposed to influence disease progression, particularly when combined with specific HLA alleles (13). This suggests that partners in the interaction with HLA are likely candidates for impacting infection outcome.

Combining the effects of the MHC and CCR5 regions has been shown to explain ~15% of the variation in viral load set point in population samples (14). What remains unknown is what proportion of the total variation in disease outcome is due to host genetic factors. New methods are now available to estimate this so-called heritability (15) from GWAS data and these are currently being applied to large patient samples. This investigation should shed light on whether further markers impacting HIV progression remain to be identified.

Viral Variation and Heritability

Unique to the study of the genetic component of infectious disease outcome is that the genome of the pathogen itself may also play a role. Owing in part to the error-prone replication machinery, HIV is highly diverse both between hosts and within a given host. After infection by a founder virus, HIV begins almost immediately to adapt to the host immune environment in order to obtain a local optimum sequence that maximizes replication capacity, while evading the host's adaptive response. Certain aspects of these adaptations have been well characterized in the context of escape mutations from class I HLA alleles and drug resistance mutations (16, 17). In a first of its kind study, Bartha et al. (18) reasoned that host proteins directly (or indirectly) interacting with the virus, and that carry functional variants, may leave a detectable imprint on the viral sequence. Such variants may not manifest their effect on the outcomes commonly investigated through genetic studies, but nonetheless may be important areas of biologic investigation. Although this genome-to-genome analysis did not discover novel associations outside of known regions, the authors did show dramatically increased power to detect MHC associations when the viral genome was considered as the outcome rather than the clinical phenotype (18). Additionally, this investigation uncovered several novel viral epitopes, as well as conserved regions in the viral genome.

Similar to host genotype, progress has been made in quantifying the relative contribution of viral genetic variation to phenotypic outcome. This is referred to as the viral component of heritability and has been investigated by comparing the similarity of set point viral load using HIV transmission pairs and/or viral sequence information. Although the studies performed to date have varied somewhat in their estimates of the viral contribution to viral load, a recent meta-analysis suggested that this was mainly due to methodological differences between studies (19). When similar methods using viral sequence information were applied across studies, the authors suggested that approximately 33% of the variation in viral load could be due to viral genetics. However, a full understanding of the

individual contributions of viral and host genetics, and their potential synergies, to disease outcome will require further study.

CELLULAR AND ORGANISMIC RESPONSES

DNA has the characteristics of stability and permanence; its analysis is generally informative long term and there is no need for retesting. However, it is not fully informative of many phenotypes either because of insufficient knowledge, or because its analysis only partially explains the trait that is investigated. Therefore, there is significant interest in studies at the levels of transcription (RNA) or translation (protein) that examine the host response in a more dynamic frame, namely time series and host-pathogen or host-immunogen responses. In particular, transcriptome and proteome analyses serve to study cellular modifications during the viral replication cycle, processes such as viral latency, and to detail the perturbations following pharmacological or immunological stimuli. These types of approaches can generate biomarkers that associate with prognosis or with treatment or vaccine responses (20). Progressively, the literature describes integrated approaches dealing with the large amounts of data generated by such studies, a field generally referred to as systems biology.

Transcriptome Analysis of Host Response

Early studies of RNA response used techniques based on microarrays: hybridization of the sample RNA to probes constituting a large or representative sample of the human transcriptome. These served to identify differentially expressed genes across different conditions, perturbations, or cellular samples although their contribution to the understanding of the biology of HIV infection and disease has been modest. Applied to clinical samples, they provided a general view of the nature of deregulation of basic cellular processes, and also of patterns of immune response. Paradoxically, the largest study (21) noted that the interferon response, as reflected in the upregulation of interferon-stimulated genes, remained abnormally elevated in chronic disease. Normalization was achieved through treatment, or in those individuals described as elite controllers, spontaneously. The pattern of interferon response in humans parallels that observed in primate models (22). Primary infection using simian immunodeficiency virus (SIV) results in a rapid, pronounced interferon response (peak) that is associated with control of the levels of viral replication (23, 24). However, while nonpathogenic models of infection (e.g., sooty mangabeys) normalize interferon response and remain disease free, pathogenic models of infection (e.g., in rhesus macaque) are characterized by persistently elevated expression of interferon-stimulated genes and progressive disease. Transcriptome analysis of HIV-infected humans with rapid disease progression is reminiscent of that of SIV-infected rhesus macaques, while the transcriptome pattern of individuals that tolerate high viral loads in plasma without progressive disease parallels the responses observed in sooty mangabeys (25).

Regulation of gene expression by microRNAs has emerged as an important avenue of investigation for factors modifying the host response to HIV. MicroRNAs are small RNA molecules (18 to 22 bp) whose main function is the post-transcriptional silencing of gene expression. Differential microRNA expression has been associated with clinical phenotypes (26, 27) and specific microRNAs have been reported as directly interacting with viral proteins (60) and contribute to latency (28). However, these results remain controversial. An in-depth analysis of microRNA expression in multiple cell lines and primary cells using deep sequencing of small RNAs suggested that the role of microRNA disregulation in HIV infection may in fact be limited (29). Genetic variation in microRNAs has also been implicated in modifying host response through interaction with a polymorphism in *HLA-C* that is strongly associated with HIV control. Specifically, Kulkarni et al. showed that increased expression of miRNA-148a, which is associated with variant rs735316, resulted in a lower expression of HLA-C alleles that carried an intact microRNA binding site in the 3′UTR (30). Downregulation of *HLA-C* expression was further shown to correlate with increased viral load in infected patients. Whether microRNA mediated regulation of transcription of other genes involved in the host response to HIV remains an active area of research.

Next-generation sequencing of RNA (RNAseq) delivers more precise, quantitative transcriptome data both of the virus and of the host. The analysis can cover the transcribed genome in depth, including the identification of short RNA and antisense transcripts. Compared with previous approaches using microarrays, RNA sequencing data are easier to normalize and more robust for comparison with transcriptome data from unrelated studies. The first studies of cellular and viral transcriptional activity in cell lines (SupT1) established that at peak of infection, 1 transcript of 143 in the cell was of viral origin (0.7%), including a small component of antisense viral transcription (31). Later studies sampled the cellular transcriptome over 24 hours after infection to time nine viral replication steps to the cellular response (32). This study observed that three-quarters of cellular expressed genes were modulated in concordance with key steps of viral replication. This profound perturbation of cellular physiology reflected an early massive cellular shutdown and progressive upregulation of the cellular machinery to complete the viral life cycle.

Increasingly, studies address the transcriptional responses in primary cells (CD4 T cells, macrophages). One of the main fields of interest is the process of HIV latency, a major obstacle to curing infection. Current strategies to eradicate HIV aim at increasing transcription of the latent provirus. Primary CD4+ T-cell models support joint host and viral RNA sequencing to investigate the characteristics of latently infected cells, the dynamics of HIV latency, and the process of reactivation induced by various stimuli (33). These models identified cells with persistent viral transcription but limited viral translation. This could be an *in vitro* correlate of the presence of cell-associated viral RNA in cells from HIV-infected individuals that are optimally controlled with antiretroviral therapy. Similarly, the response to cell activation via the engagement of the T-cell receptor led to profound transcriptome changes associated with viral production, an image that was comparable to that observed during *ex vivo* activation of latently infected cells from HIV-infected individuals (33). It is hoped that a better understanding of the cellular transcriptional landscape associated with reactivation from viral latency will support research on purging the viral reservoir (34, 35).

Most published reports focus on transcriptome analyses capturing polyadenylated, mature mRNAs. There is increasing interest in extending analysis to include the profiling of nascent transcripts, which often include nonpolyadenylated RNAs. Peng et al. (36) used total RNA and mRNAs from the same HIV-1-infected CD4+ T cells. The authors

described more differentially expressed genes by total RNAseq than by mRNAseq, suggesting a quantifiable delay between transcriptional initiation and mature mRNA production early after HIV-1 infection.

Single-cell analysis represents the new frontier in transcriptome studies. Current technologies (37) make this a reality. It is expected that single-cell work will permit a better understanding of cell-to-cell heterogeneity in response to infection, in latency, and contribute to a better description of the processes of cellular ontogenesis.

Proteome Analysis of Host Response

Transcriptome responses do not necessarily correlate with proteome representation of cellular modifications during HIV infection *in vitro* or *in vivo*. There has been significant development in mass spectrometry that now allows extensive analyses of protein responses, HIV-host interaction, and post-translational modifications (38). Proteome analyses also hold promise for biomarker discovery.

The recent literature on protein analysis in the context of HIV reflects the evolution from descriptive studies to screens that are meant to identify singular factors of relevance to the biology of infection. Navare et al. (39) assessed the host cell response on protein levels in CD4+ lymphoblastoid cells after infection with HIV using mass spectrometry global quantitation with iTRAQ (isobaric tag for relative and absolute quantification). Their analysis of differentially expressed proteins reflected the extensive changes affecting several biological pathways including protein synthesis, cell proliferation, and T-cell activation. Shetty et al. (40) used the same technology for the analysis of plasma samples from HIV-1/HCV mono- and coinfected individuals. The authors described 70 proteins that exhibited differential regulation compared to samples from noninfected individuals. The upregulated proteins are implicated in the hepatic lipid metabolism, inflammation, and acute-phase response signaling pathways. Proteome studies have also investigated the content of viral particles (http://ncifrederick.cancer.gov/research/avp/protein_db.asp); HIV, upon egressing from cells, packages cellular proteins that originate from the producer cells, either through passive incorporation or via active recruitment of cellular factors that are important for viral replication. The virus-associated host proteins reflect the proteome of the producer cell (41).

Viral entry induces cellular signaling events that can be mapped as changes in downstream phosphorylation events and the modulation of various intracellular secondary messengers. Wojcechowsky et al. (42) applied mass spectrometry-based phosphoproteomics, in combination with stable isotope labeling by amino acids in cell culture, to quantify phosphorylation sites in primary human CD4+ T cells during HIV entry. The authors found that these signaling events also modulate later stages of the virus replication cycle.

One of the most comprehensive studies in the field addressed the complexes formed between host and viral proteins. Through the use of affinity tagging and purification mass spectrometry, Jäger et al. (43) determined the physical interactions of all 18 HIV-1 proteins with host proteins in 2 human cell lines to identify 497 HIV-human protein-protein interactions. Host proteins hijacked by HIV were highly conserved across primates, defining the extent of host machinery co-opted by HIV for replication.

An additional field that combines genetics and protein evolution which is of major importance for HIV vaccine development is the analysis of pathways of development of anti-HIV neutralizing antibodies. The process by which B cells evolve through somatic hypermutation can be studied through antibody isolation from patients, B-cell next-generation sequencing, structural biology, and viral genome analysis to delineate the molecular requirements and genetic pathways that lead to the generation of high affinity neutralizing antibodies (44). These types of approaches may help define the appropriate constructs that will elicit optimal neutralizing antibodies, possibly through sequential administration of immunogens mirroring the key steps in viral evolution (45).

Overall, the large-scale approaches discussed above are converging towards protein and genetic interaction maps (interactome) that reveal the overall physical and functional landscape of biological systems (46). To date, these interaction maps have typically been generated under a single condition; however, recent studies have demonstrated the power of network mapping at very large scales in order to define the biological architecture of the response under specific conditions (46). It is expected that the important goal of identifying the biomarkers of disease will be facilitated by these novel applications.

Innate Immunity

Genome, transcriptome, and large-scale functional screens (RNA silencing and protein immunoprecipitation studies) have emphasized the role of genes of the cellular innate immunity against HIV and other viruses. On one hand, there are the canonical responses to interferon, summarized by the upregulation of several hundred interferon/stimulated genes (ISGs), and on the other hand, the discovery of several cellular restriction factors that specifically target retroviruses.

HIV-1 triggers a type I interferon antiviral response through cytosolic sensors that detect DNA intermediates

TABLE 2 Pharmacogenetic markers of interest in clinical care of HIV-infected individuals[a]

Clinical phenotype	Gene(s)	Variant IDs
ABC hypersensitivity	*HLA-B*	rs2395029/HLA-B*57
ATV hyperbilirubinemia	*UGT1A1*	rs8175347
EFV plasma levels	*CYP2A6*	rs1801272, rs5031016, rs2839943
	CYP2B6	rs3745274, rs12721655, rs35303484, rs36060847, rs35773040, rs35979566, rs28399499
NVP plasma levels	*CYP2B6*	rs1801272, rs5031016, rs2839943
ETV plasma levels	*CY2C9*	rs1057910
	CYP2C19	rs12571421
LVP plasma levels	*ABCC2*	rs717620
	CYP3A	rs6945984
	SLCO1B1	rs4149056
Response to interferon treatment in HIV-HCV coinfection	*IL28B*	rs12979860

[a]In addition to the pharmacogenetic markers included here, HIV-infected individuals can benefit from genetic prediction of risk of dyslipidemia, type 2 diabetes, and cardiovascular disease (56–58).

of reverse transcription. Major sensors identified in recent times are IFI16, cGAS, and STING. Downstream expression ISGs serve to limit virus replication at multiple stages of the virus life cycle, to shut down the host translational machinery to prevent production of progeny viruses, and to direct the adaptive immune response of ongoing pathogenic infection (47). ISGs encompass a broad selection of cellular activities, including signaling pathways, transcription factors, and effector molecules. The exact nature of their functions is largely unexplored (48). The contribution of the interferons to HIV-1 infection is complex: administration in the non-human primate model can protect from infection (49), and indeed, the founder HIV-1 isolates in human infection are relatively resistant to interferon (50). In contrast, prolonged administration of interferon in the animal model is deleterious (49), and severe human and animal infections are accompanied with marked and ultimately ineffective upregulation of ISGs (22).

Multiple specific antiretroviral factors have been identified: most prominently APOBEC3G, TRIM5alpha, BST2/tetherin, SAMHD1, and MX2. They play an important role in limiting cross-species infection. Unfortunately, human restriction factors are generally ineffective (with the exception of SAMHD1) due to the coding in the viral genome of proteins that counter those factors (e.g., Vif and APOBEC3G, Nef, and BST2), or due to a lack of specificity (e.g., Viral capsid and TRIM5alpha). Overall, the field of innate cellular defense remains of maximal scientific interest, but with limited applications for diagnostics or therapeutics.

PHARMACOGENETICS OF HIV TREATMENT

Anti-HIV treatment is prescribed lifelong. There are currently multiple drug families that can be used to establish a highly effective combination therapy (51). Personalization would be of significant interest to establish the optimal choice on the basis of efficacy and lowest likelihood of adverse effects. Despite significant progress in this field, only one application has reached the clinics. Testing for HLA-B*57 is now required before prescription of abacavir, a first-line agent, to limit the risk of a hypersensitivity reaction (52). There are however additional tests that could be routinely applied (Table 2). Of these, the best understood is the association of CYP2B6 variants with profound changes in the metabolism of efavirenz, and to a lesser extent, nevirapine (53). Loss-of-function polymorphisms of *CYP2B6* are associated with an increase in plasma drug levels, and if combined with additional variants in secondary metabolic pathways, in particular in *CYP2A6* (54), they result in compromised metabolism and are associated with severe neuropsychological toxicity and treatment discontinuation (55). Other pharmacogenetic tests could support the clinical decision, although their predictive value is smaller than for abacavir and efavirenz. In particular, HIV-infected patients can have a better prediction of risk of metabolic complications (diabetes, hyperlipidemia, cardiovascular disease) if genetic testing is included in clinical scores that use conventional demographic and epidemiological risk factors (e.g., body mass index, smoking history, family history) and type of antiretroviral medication (56–58).

Some particular actions could help bring additional pharmacogenetic markers to clinical use. First, next-generation pharmacogenetic tests would need to address existing knowledge in a single operation/multiple test array, to avoid singular tests with the associated multiplicative costs. Second, interpretation of the test would need to differentiate deterministic predictions (e.g., HLA-B*57 = hypersensitivity to abacavir) and qualitative predictions (e.g., modest increase in metabolic risk from a multimarker genetic score). Third, predictions could be refined if genetic markers were included in scores that consider other well-known risks and types of medication (e.g., cardiovascular risk considering genetic and nongenetic covariates). It is at this time difficult to predict the direction of the field, in particular because of the current emphasis in bringing whole-genome sequencing to clinical use. In this scenario, individuals would have their complete genome data stored, and singular markers would be queried as needed in the context of specific clinical questions, or upon treatment prescription.

CONCLUSIONS

Among infectious diseases, HIV is a field that has developed in sync with many of the recent technological advances in genetics, genomics, biomarker identification, and systems biology. As a result of this effort, there is a good understanding of the common genetic variants that modulate susceptibility to infection, and on the cellular and organismic responses to infection in the context of various patterns of disease. Some of the findings have translated into practical achievements. Of particular note is the identification of deletions in the HIV coreceptor (CCR5Δ32) in healthy individuals which supported the development of CCR5 antagonists as antiretroviral agents (59), and the completion of the first randomized pharmacogenetic clinical trial in medicine that led to the general use of HLA-B*57 testing (52).

It is expected that the pace of research in host-HIV interaction will foster continuous development in molecular diagnostics, and more generally, in contributing to the basic understanding of disease susceptibility and pathogenesis.

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Biomarkers of Gastrointestinal Host Responses to Microbial Infections

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46

The gastrointestinal (GI) environment is a complex ecosystem, where the host lives in complete homeostasis with its microbiota, with the two generally maintaining a delicate balance (1, 2). An immune tolerance exists between the host and its microflora and is acquired soon after birth, preventing harmful inflammation in the setting of a normal microbiota (3–5). When an infection disturbs this stability, the host response is targeted towards rebuilding the equilibrium as quickly as possible. The initial barrier between this internal environment and the outside is the intestinal epithelial cell, which not only plays a role as a physical barrier and nutrient provider, but also protects its local milieu by initiating the immune response sequence (6, 7). The microbiota is also thought to play an important role in the immune response and regulation; this has been referred to as "host-commensal mutualism" (2, 3, 8, 9). The innate response, depending on the infection, may lead to activation of pro- or anti-inflammatory signaling pathways, producing cytokines and chemokines that protect the host from this invasion. However, in some instances, this response may become deleterious to the host and exacerbate the damage.

Many products of the innate immune response are detected in the stools or serum, and may be biomarkers of intestinal microbial infections (1, 6). Recent studies have focused on biomarkers of innate system activation, such as α- and β-defensins (10–15), lactoferrin (16, 17), cathelicidins (18), and S100 proteins (19). Specific chemokines and cytokines are being investigated in greater depth, as we acquire insights into the different complex pathways activated by each infection. Biomarkers have been investigated extensively in noninfectious etiologies; especially in inflammatory bowel disease where a deeper pathophysiological understanding of the inflammatory process is taking shape (3). In inflammatory bowel disease, roles are becoming clear for defensins (20), lactoferrin and calprotectin (16, 21–27), interleukins (IL) (28–30), and M2 pyruvate kinase (31, 32) among others.

Inflammatory biomarkers are also gaining interest as biomarkers of GI infections. Fecal calprotectin levels are increased in bacterial more so than viral infections of the GI tract (33, 34), and are higher with severe disease (34); fecal lactoferrin levels are also elevated in bacterial infections and increase with severity (17). Specific cytokines such as IL-17 are triggered by multiple infections in mouse models (35–37). Long and colleagues suggest that cytokine responses may be adequate against one pathogen while inappropriate for another, as they showed variable cytokine production resulted in different outcomes with different *Escherichia coli* strains (38). Depending on the host and the pathogen, cytokine expression can be harmful, or at the least, used by the pathogen to create its own niche and exacerbate the disease.

Detecting specific infections through analysis, the host response will likely gain utility as a diagnostic tool (39). For example, several studies have identified distinct gene-expression profiles with different viral respiratory pathogens (40–42). A better understanding of the specific individual's host response to microbial infections may explain the broad spectrum of symptoms encountered and provide insight into why the same infection can be completely harmless in one individual, and cause severe symptoms or death in another.

In this chapter we will discuss biomarkers of the host response to the major GI pathogens known to cause human diseases and for which we have some basic understanding of the pathophysiology of the infections. Table 1 summarizes what is known thus far about agents of gastroenteritis and biomarkers released by the host in response to these agents.

BACTERIAL INFECTIONS

Although there are thousands of bacterial species in the normal intestinal habitat, there are relatively few bacterial pathogens associated with GI disease. Some bacteria, such as *Shigella* and *Salmonella*, directly invade the host epithelium, using specific virulence factors and leaving a trail of inflammation in their wake. These organisms have evolved to avoid the host inflammatory response and may in fact benefit from induction of innate responses. Others, such as *Clostridium difficile* and Shiga toxin-producing *E. coli*, do not directly invade the host but instead attack from a distance using secreted exotoxins. There is growing evidence that *C. difficile* may also benefit from the innate inflammatory response.

C. difficile

C. difficile is a spore-forming anaerobic bacterium implicated in antibiotic-associated diarrhea. It is one of the most common causes of hospital-acquired infections, and

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TABLE 1 Agents of gastroenteritis and biomarkers described in their pathogenesis[a]

Pathogen	Potential biomarkers
C. difficile	Fecal IL-8, fecal CXCL-5[b]
	MK-2, p38 pathway[b]
	Serum CRP, procalcitonin, fecal lactoferrin[b]
	RANTES, IL-23[b]
	CCR1, MIP-1α
	IL-1β, IL-8, IL-12, IL-18, IFN-γ, TNF-α, leptin
	IL-6, IL-10, IL-22
Shiga toxins (Stx): *S. dysenteriae* serotype 1 and STEC	TNF-α, IL-1, IL-6, IL-8, IL-12, IL-10
	MCP-1, MIP1α, RANTES, CXCL-1, CXCL-2, CXCL-3, CXCL-5
	p38 MAPKs, MK2
Shigella	Fecal lactoferrin, IL-1β, IL-6, IL-8, IL-18, TNF-γ[b]
	IL-10, TGF-β, IL-17A, IL-22
	IFN-γ, IL-1, IL-3, IL-4 (down-regulated)
Salmonella	IL-1α, IL-1β, IL-12, IL-18, IFN-γ, TNF-α, CXC, C5a
	IL-17, IL-22, IL-23
	Fecal calprotein and lactoferrin, IL-12, IFN-γ[b]
Diarrheagenic *E. coli*	Fecal TNF-α, IL-6, IL-4, IL-10[b]
	Fecal lactoferrin, IL-8, IL-1β[b]
Campylobacter	IL-1β, IL-6, IL-8, IL-10, IL-12, IFN-γ, TNF-α
	MIP-1α, MIP-1β, RANTES
	Fecal calprotein[b]
	Serum IL-8[b]
Yersinia enterocolitica	TNF-α, IL-12, IL-18
	TNFRp55, IL-6, NF-κB
Vibrio cholera	IFN-γ, IL-2, IL-4
	MIP-2, NOS2, IL-6
Helicobacter pylori	IL-12, IL-23, IL-18, TLR4
	IL-1β, IL-6, TNF-α, IL-8
Rotavirus	Fecal and serum IFN-α and IFN-β[b]
	Serum IL-6, IL-10, IFN-γ, IL-18[b]
	IL-8, GM-CSF, MIP-1β, IP-10, GRO-α, RANTES
	CCL15, CCL5, CXCL10, CXCL11
Cryptosporidium parvum	IL-8, IL-15, IFN-α, IFN-β
	IFN-γ, IL-18, IL-12, TNF-α
	GRO-α, RANTES, IP-10, MIG, lymphotactin, MIP-1β
	IL-4, IL-15
	CXCL-10
Giardia lamblia	IL-4, IFN-γ, IL-5, IL-6, TNF-α, MCP-1[b]
	CCL2, CCL20, CXCL1, CXCL2, CXCL3
Entamoeba histolytica	IL-1β, IL-8
	Serum TNF-α[b]
Helminths	IL-4, IL-5, IL-9, IL-10, IL-13, IL-21, IL-25, IL-33
	IFN-γ, IL-12, IL-18, IL-27
	[b]Serum CCL11, CCL17 (TARC)
	[b]IL-5, IFN-γ

[a]CXCL, C-X-C motif ligand; CCl, C-C motif ligand; CCR, C-C motif receptor; CRP, C-reactive protein; EAEC, enteroaggregative *E. coli*; EPEC, enteropathogenic *E. coli*; ETEC, enterotoxigenic *E. coli*; IL, interleukin; GRO, growth-related oncogene; IFN, interferon; IP, inducible protein; MCP, monocyte chemoattractant protein; MIG, monokine induced by IFN-γ; MIP, macrophage inflammatory protein; MK-2, mitogen-activated protein kinase (MAPK)-activated protein kinase; NF-κB, nuclear factor kappa-light-chain-enhancer of activated B cells; NOS, nitric oxide synthase; RANTES, regulated on activation, normal T-cell expressed and secreted; STEC, Shiga toxin-producing *E. coli*; TLR, Toll-like receptor; TNF, tumor necrosis factor; TNFR, tumor necrosis factor receptor.

[b]Found in studies in humans.

now also an important cause of community-acquired diarrhea. Its morbidity and mortality have continued to increase over the past decade, and a new epidemic strain has emerged (BI/NAP1/027) which has been linked with severe disease (43–46). The spectrum of illness caused by C. *difficile* is broad, and includes asymptomatic carriage, mild diarrhea, severe colitis, and toxic megacolon. Specific host responses to this pathogen may explain this broad range of symptoms, including the observation that up to 70% of infants, and 15% of adults, may be asymptomatically colonized with toxigenic strains of C. *difficile* (47–49). Alteration of the host/microbiome symbiosis is especially important with this infection, as disease traditionally occurs in people exposed to antibiotics, and re-establishing this balance with fecal transplantation successfully treats refractory cases (50–54). Excellent reviews addressing the immune responses to C. *difficile* have been published recently (55–57).

C. *difficile* secretes toxins, specifically TcdA and TcdB, known to disrupt the actin cytoskeleton and induce the release of inflammatory mediators (55, 57, 58). This leads to fluid accumulation, edema, increased mucosal permeability, and neutrophil recruitment (55). *In vitro* and mouse models of C. *difficile* infection (CDI) show that toxin exposure induces inflammation with elevation of multiple inflammatory mediators such as IL-1β, IL-8, IL-12, IL-18, interferon γ (IFN-γ), tumor necrosis factor α (TNF-α), and leptin (55, 59–63). Some studies suggest that the inflammation is mediated by CCR1 (C-C motif receptor 1), MIP1α (macrophage inflammatory protein 1α, and RANTES/CCL5 (regulated on activation, normal T-cell expressed and secreted/CCL5) (64). The p38-dependent activation of mitogen-activated protein kinase (MAPK)-activated protein kinase (MK2 kinase), known to regulate IL-8 production, appears to be an early driver of the neutrophilic inflammatory response in CDI (65–67). It is believed that this overwhelming inflammatory process may incite injury and be responsible for the severe complications seen with CDI, as elevated inflammatory responses and neutrophil infiltration of the GI tract have been associated with worse clinical outcomes (68–70). There is evidence that the host attempts to modulate this inflammation. For example, surface layer proteins may play a role in immunomodulation via IL-1β, IL-6, IL-10, and IL-22 (60). The eukaryotic initiation factor 2α (eIF2α) phosphorylation and IL-22-pSTAT3-RegIIIγ axis are pathways that may help contain, and even possibly counteract, the epithelial damage caused by C. *difficile* (63).

Host biomarkers of inflammation such as serum procalcitonin, C-reactive protein and fecal lactoferrin are elevated in patients with CDI (71–76). Many human studies have implicated elevated fecal IL-8, a chemokine also known as neutrophil chemotactic factor for its robust neutrophil chemoattractant qualities, mainly produced by macrophages and epithelial cells, in severe cases of CDI (68, 69, 77–79). C-X-C motif ligand (CXCL)-5 (also known as epithelial-derived neutrophil-activating peptide) is also associated with severe CDI cases (68, 69). CXCL-5 is produced following epithelial cell stimulation with IL-1 and TNF-α, and is known to have chemotactic and activating functions on neutrophils, mainly during acute inflammatory responses. PMK2 and p38 have also been shown to be associated with CDI in humans (65, 69). Multiple additional fecal and systemic biomarkers have been investigated (70, 79), and many more are expected to be revealed with the use of multiplex polymerase chain reactions (PCR). Some biomarkers show potential promise: RANTES (CCL5) may be associated with CDI more often than with other causes of diarrhea (70), and IL-23 may be a major player in CDI and its severity (79, 80).

Despite the increasing knowledge on the host response to C. *difficile*, many questions remain unanswered, especially regarding the wide spectrum of disease encountered, and why individuals colonized with toxigenic strains do not develop disease. Bacterial and viral coinfections with CDI are common (81, 82) and may complicate the picture. Gaining more insight into the pathophysiologic inflammatory process in CDI may lead to new therapeutic approaches and preventative measures in CDI.

Shiga Toxins: *S. dysenteriae* Serotype 1 and Shiga Toxin-Producing *E. coli*

Shiga toxin is an exotoxin produced by *Shigella dysenteriae* serotype 1. Shiga toxin-producing *E. coli* (STEC), such as *E. coli* O157:H7, produce structurally related toxins called Stx1 and Stx2. Stx can cause severe hemorrhagic colitis but can also induce extraintestinal manifestations such as neurologic complications and hemorrhagic uremic syndrome manifested by renal failure and thrombocytopenia (83, 84). It is important to note that *Shigella* is a human-adapted pathogen, and studies on its inflammatory pathogenesis are limited due to the lack of animal models (85). No human studies investigating serum or fecal biomarkers with *S. dysenteriae* or STEC are yet available. Animal studies have mostly investigated the role of Stx and lipopolysaccharides (LPS) on the GI tract and kidneys, rather than the bacteria as a whole.

In vitro and animal studies show that STEC can form attaching and effacing lesions on intestinal epithelial cells (86–88). It can then suppress the inflammatory process during the colonization phase by suppressing nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) activity, possibly through p65 inhibition (89), and induce lower mRNA levels of IL-8, IL-6, and IL-1α (90). This is especially important in cattle where colonization is of significant epidemiologic importance.

Multiple studies have focused on the renal damage caused by Stx in the setting of hemorrhagic uremic syndrome. During acute infection, Stxs induce a significant influx of inflammatory cells through induction of multiple cytokines such as TNF-α, IL-1, IL-6, and IL-8 (85, 91–94), monocyte chemoattractant protein 1 (MCP-1), MIP1α and RANTES (95, 96), CXCL1 (GRO-α), CXCL2 (GRO-β), CXCL3 (GRO-γ), and CXCL5 (ENA-78) (97). In one study, exposure to Stx resulted in stabilization of IL-8 transcripts, with possible prolonged IL-8 production, and increased neutrophil infiltration into the intestinal lumen (98, 99). Jeong et al. showed that piglets infected with *Shigella dysenteriae* serotype 1 had high levels of proinflammatory cytokines in their feces and gut segments: IL-8 and IL-12 levels increased initially, followed by IL-1β, IL-6, IL-8, and TNF-α, IL-10 increasing later in the course (100).

Stxs are potent inhibitors of host protein synthesis. They act as an *N*-glycosidase and cleave off a single adenine of the 60S ribosomal subunit (85). The resulting ribotoxic stress response leads to the activation of multiple MAPKs signaling pathways such as p38 MAPKs, MK2, JNKs (c-Jun N-terminal kinases), and ZAK (85). Thorpe et al. suggested the activation of at least one member of the MAPK family in the p38/RK cascade (99). This has been further demonstrated over the past decade with

numerous studies investigating the role of p38 pathway in Stx-induced disease (94, 101–103). Murata et al. suggested that macrophage stimulation activates the tyrosine kinase c-YES, increasing the expression of tissue factor. They postulated that activation of the IKKβ/proteasome/NF-κB/Rel and MEK/ERK2/Egr-1 pathways via activation of PI3-kinase may worsen disease outcome during infections with *E. coli* O157 (104). Stx can also enhance the phosphorylation of eIF4E and eIF4E-binding proteins (4EBP1) through a p38 MAPK/Mnk1/eIF4E signaling pathway involved in maintaining host cell translation (105). Despite all these investigations, and the presumed role of inflammation in disease pathogenesis, there are still no therapies available targeting these pathways.

Shigella

Shigella comprises four species: *S. dysenteriae*, *S. flexneri*, *S. boydii*, and *S. sonnei*. We have discussed specifics to *S. dysenteriae* serotype 1 in the previous paragraph. These bacteria are highly adapted human pathogens that can cause severe inflammatory colitis or dysentery, with as few as 10 to 100 bacteria required for transmission (106). As mentioned above, there is no perfect animal model to study *Shigella* as mice are not susceptible. A macaque monkey model, rabbit ligated ileal loop, and a new guinea pig model have been used to replicate human disease (107, 108). Excellent detailed reviews on *Shigella*'s virulence factors and host interaction are available (107–109).

When *Shigella* reaches the colon, it enters the M cells that overlay the lymphoid follicles from the apical side of the intestinal epithelium. The bacterium may then invade resident macrophages, activating caspase-1 and inducing the production and secretion of IL-1β and IL-18, resulting in macrophage death through pyroptosis (110–112). Upon release from the killed macrophage, the bacteria then invade and spread cell-to-cell from the enterocytes' basolateral surface (109). Epithelial cell invasion activates the NOD1-RICK pathway, NF-κB, and MAPK pathways, with the production of multiple proinflammatory chemokines, cytokines, and antimicrobial peptides; most importantly IL-8 (113, 114). This inflammatory response leads to the massive recruitment of neutrophils with ensuing edema, erythema, and production of a mucopurulent exudate, leading to abscess formation and mucosal hemorrhages. Pore et al. describe that *Shigella's* major outer membrane protein induces the nuclear translocation of NF-κB, activates p38 MAP kinase, and enhances the production of different cytokines and chemokines such as ILp70, TNF-α, IL-6, MIP-1α, MIP-1β, and RANTES (115). Studies suggest that IL-1β and IL-8 elevation in shigellosis may be deleterious to the host, as it promotes neutrophil influx and may facilitate bacterial invasion without increasing bacterial clearance (116–119).

Human studies are again sparse with *Shigella* infections. Raqib et al. analyzed plasma and fecal samples from patients with shigellosis and detected high levels of IL-1β, IL-6, IL-8, and TNF-α. The fecal levels detected were far higher than the serum samples, emphasizing the importance of investigating the local inflammatory response (120). Greenberg et al. found that patients with *Shigella* had higher fecal lactoferrin, IL-8, and IL-1β than those with *Salmonella* or traveler's diarrhea due to *E. coli* (121), suggesting a significantly more pronounced inflammatory process.

Shigella has developed strategies to evade killing by the host immune response. It is capable of evading autophagy (107, 109, 122). It is thought that LPS glucosylation may promote this evasion (123). Additionally, an optimal level of inflammation is important for *Shigella* pathogenesis and the organism appears to titrate the host inflammatory response. An overt inflammatory response may be detrimental to the bacterial survival whereas a weak response may be insufficient for its dissemination (107). The *Shigella* proteins ShiA and OspG have been identified as effectors involved in the down-regulation of inflammation (124, 125). *Shigella* has been shown to inhibit the production of some antimicrobial peptides such as LL31 and human β-defensin-1 (HDB1) (126). It promotes production of anti-inflammatory mediators such as IL-10 and TGF-β that impair the development of an efficient Th1-type immunity (127, 128), thus escaping the IFN-γ-mediated immune response which is essential to clearance by innate immunity (129–132). *Shigella flexneri* was found to prime predominantly IL-17A and IL-22 producing Th17 cells (133). Fecal IFN-γ levels were found to be depressed in the acute phase of shigellosis, progressively increasing during the convalescent phase (120). In addition, a selective down-regulation of the receptors for IFN-γ, TNF-α, IL-1, IL-3, IL-4, and TGFβ was observed at the onset of the disease, with a gradual reappearance during the convalescent stage, suggesting dissociation in the immune regulation between cytokine production and cytokine receptor expression (95).

Shigella has mastered the art of manipulating the host immune system. Gaining better understanding of its effectors and interactions with the host may open new opportunities to develop strategies for prevention and treatment against *Shigella* and other organisms that have similar survival strategies.

Salmonella

Salmonella enterica subspecies contains over 2,000 genetically similar serovars. *S. enterica* serovar Typhi (*S. typhi*) and *S. paratyphi* A cause typhoid fever, while nontyphoidal *Salmonella* (NTS) organisms, on which we will focus our review, cause a wide spectrum of disease extending from asymptomatic colonization to gastroenteritis, bacteremia, and focal infections (134).

Salmonella enterica serovar Typhimurium has been studied extensively over the past years as we gained significant understanding of its pathophysiology. *Salmonella* Typhimurium has the ability to penetrate the intestinal epithelium and survive within macrophages (135). *Salmonella* Typhimurium is a facultative intracellular organism which elicits a robust acute intestinal inflammatory response to which it has adapted, and which allows the organism to outcompete the existing microbiota, thus taking advantage of the immune response and turning it to its favor (2, 98, 136–139).

The early innate response to *Salmonella* Typhimurium involves secretion of proinflammatory mediators such as IL-1α, IL-1β, IL-12, IL-18, IL-23, IFN-γ, and TNF-α (140–142). CXC chemokines and C5a are also released from epithelial cells. These initial events induce macrophage activation, neutrophil recruitment, and the release of antimicrobials into the intestinal lumen, targeting all phases of the bacterial growth: intracellular, extracellular, and luminal (140).

Two main pathways are then initiated (Fig. 1): IFN-γ and IL-23 pathways. IL-18 activated by caspase 1, along with IL-12, stimulates T cells to amplify the inflammatory responses induced by *Salmonella* through the production of IFN-γ (143–148). A dramatic increase in IL-17 and IL-22 expression, thought to be orchestrated by IL-23, is observed in animal models within a few hours after *Salmonella* infection (35, 149–151). IL-17 stimulates

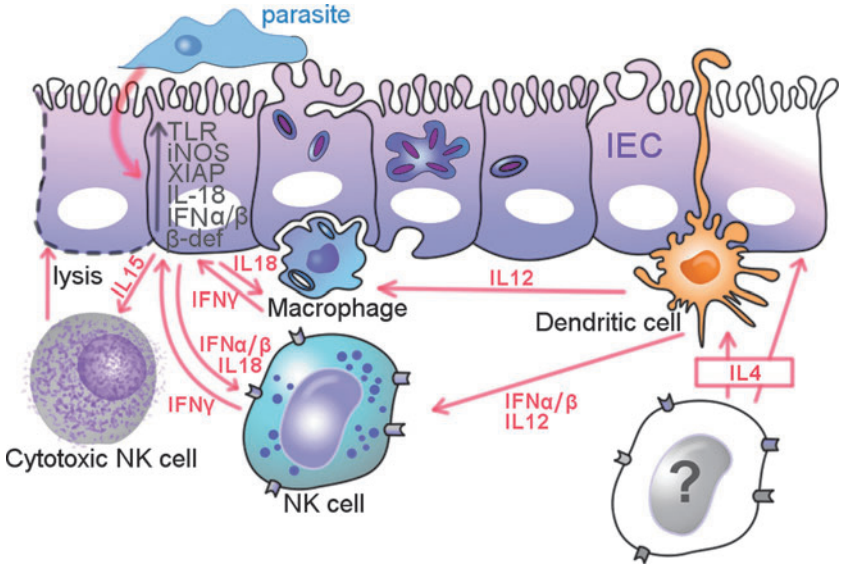

FIGURE 1 Antibacterial inflammatory responses against *S*. Typhimurium infection. Inflammatory cytokine production was triggered upon detection of *S*. Typhimurium by mononuclear cells, epithelial cells, and complements which leads to production of antibacterial responses by macrophage activation, neutrophil recruitment, and epithelial release of antimicrobials. (Adapted from reference 140.)

epithelial cells to secrete CXC chemokines and the bone marrow to produce granulocyte colony-stimulating factor (G-CSF), resulting in enhanced neutrophil recruitment (135, 152). Neutrophils are known to help prevent dissemination to extraintestinal sites, as neutropenia is a risk factor for bacteremia; however, they may potentially be harmful to the host as they contribute to the damage caused in the GI tract (135). IL-22 induces the production of antimicrobial proteins and peptides such as mucin, nitric oxide, lipocalin-2, calprotectin, and RegIIIγ (135, 153). Studies suggest that anti-inflammatory cytokines such as IL-4 and IL-10 may inhibit the host defense against *Salmonella* and be detrimental to the host (142). Human studies have found elevated fecal levels of calprotectin (34) and lactoferrin (17) with *Salmonella*. IL-12p40 and IFN-γR1 deficiencies have been associated with severe NTS infections (154, 155).

Although IL-17 and IL-22 are of significant importance to NTS, they do not seem to play an important role in the pathogenesis of typhoid fever (156), unlike IL-1β, IL-6, IL-18, and IFN-γ, which are major effectors in the disease process (157–159). It is postulated that an uncontrolled activation of the innate immune response in typhoid fever can lead to detrimental systemic inflammation, intravascular coagulation, sepsis, and death (156). Microarrays and transcriptional profiling of peripheral blood to investigate the host response to typhoid fever have been developed, revealing a distinct signature during the infection, treatment, and convalescence phases (160). Performing such extensive profiling of the host immune response to infection is a promising technology that may be a future component of infectious diseases diagnostics.

Diarrheagenic *E. coli* (except STEC)

At least five pathotypes of diarrheagenic *E. coli* strains have been identified. We have previously discussed STEC, also known as enterohemorrhagic *E. coli* (EHEC) or verotoxin-producing *E. coli* (VTEC). Enteropathogenic *E. coli* (EPEC) causes watery diarrhea almost exclusively in children younger than 2 years of age. Enterotoxigenic *E. coli* (ETEC) is known for causing traveler's diarrhea with watery stools and abdominal cramps. Enteroaggregative *E. coli* (EAEC) causes watery diarrhea in developed and developing countries (161). The diarrhea caused by enteroinvasive *E. coli* (EIEC) is similar to the one caused by *Shigella* species. The immune response is thought to be similar to the one described with *Shigella* (described previously).

Studies on EPEC have focused on attaching/effacing lesions. After initial adherence to the intestinal epithelial cells, EPEC injects secreted effector proteins (Esp) directly into host cells through a Type III secretion system (T3SS), which then modulate host inflammation (162–164). EPEC is thought to inhibit RNase-L in a T3SS-dependent manner providing a mechanism by which EPEC evades IFN-induced antibacterial activities (165). Hardwidge et al. identified unique proteins that were expressed differentially in the presence of TTSS-delivered EPEC effector proteins. For example, they found increased talin 2, gelsolin, cofilin, and gelatin, and decreased integrin-linked kinase 2 and calcium-transport ATPase (166).

Studies in EAEC have found elevated IL-8 levels through TLR-5-dependent p38 MAP kinase (167). Elevated levels of fecal lactoferrin, IL-8, and IL1-β have been seen in ETEC and EAEC (121, 168). IL-10 has been found to increase durations of both asymptomatic and symptomatic ETEC infections (38, 169).

Long et al. examined different *E coli* species and the immune response they induce in the GI tract (38). He found that high levels of TNF-α, IL-6, and IL-4 were associated with decreased durations of EPEC infection, while increased TNF-α, IL-6, and IFN-γ were associated with increased durations of ETEC infection, thereby stressing on the importance of different cytokine profiling with different pathogens.

Campylobacter

Campylobacter is one of the most common causes of diarrheal illness in developed countries (170). Most human

illness is caused by *Campylobacter jejuni* and *Campylobacter coli*. These two species grow best at 37 to 42°C and commonly live in the intestinal tract of birds and mammals, including food production animals and pets, without causing clinical signs (171).

C. jejuni is a unique pathogen able to execute *N*-linked glycosylation of more than 30 proteins related to colonization, adherence, and invasion. The only toxin of *C. jejuni*, cytolethal distending toxin (CdtA,B,C), is important for cell cycle control and induction of host cell apoptosis and is considered a major cause of pathogenicity. Very recently, outer membrane vesicles (OMVs) were suggested to play an important role in colonization and delivery of virulence factors into host cells (172). Molecular bases of virulence mechanisms of *C. jejuni* are not fully elucidated yet.

Live and heat-killed *C. jejuni* are capable of inducing maturation and cytokine production in dendritic cells (173). This leads to activation of NF-κB and the other cytokines, including IL-1β, IL-6, IL-8, IL-10, IL-12, INF-γ, and TNF-α (173). Interestingly, purified *C. jejuni* lipooligosaccharides play an important role in the elevation of cytokine production by dendritic cells and in the initiation of a Th1 adaptive immune response (174). Asymptomatic infection by *C. jejuni* is common among children in the developing world, and acute infections are frequently followed by periods of asymptomatic shedding (175) which may be due, at least in part, to the ability of this pathogen to avoid the host immune response. TLRs such as TLR-2 and TLR-5 also play minor roles in *C. jejuni* enteritis, indicating that *C. jejuni* flagellin and lipoproteins are not crucial for activation of the immune system (176). Stahl et al. showed that TLR-4 signaling underlies the majority of the enteritis, whereas TLR-2 signaling had a protective role, promoting mucosal integrity (177, 178). Chemokines, including CC families (MIP-1α, MIP-1β, RANTES) and CXC families (growth-related oncogene α [GRO-α], IP-10, and monokine induced by gamma interferon [MIG]), are upregulated in *Campylobacter*-infected dendritic cells (179). Edwards et al. suggested a significant role of IFNγ, IL-22, and IL-17A in establishing host antimicrobial immunity during the acute and effectors phase of infection (180). The signaling pathways phosphatidylinositol 3-kinase (PI3K/Akt) and mitogen-activated protein (MAP) kinases ERK and p38 were found to be involved in *C. jejuni*-induced IL-8 and IL-10 expression (181). High concentrations of fecal calprotein have been described in patients with *C. jejuni* infection and seem to correlate with severe disease (34, 177).

Yersinia

Genus *Yersinia* has 17 known *Yersinia* species, of which only *Y. enterocolitica*, *Y. pseudotuberculosis*, and *Y. pestis* are human pathogens (182). *Y. enterocolitica* and *Y. pseudotuberculosis* cause self-limiting gastroenteritis (which we will focus on here) while *Y. pestis* causes radically different diseases (bubonic, septicemic, or pneumonic plague) (183). Because of its ability to grow and thrive in cold weather, *Y. enterocolitica* infection has higher incidence in temperate and cold countries.

The common clinical symptoms associated with *Y. enterocolitica* infections are diarrhea, abdominal pain, fever, and sometimes vomiting, which predominates in young children and is often self-limiting.

In humans, the virulence of *Y. enterocolitica* is controlled by the presence of chromosomal high pathogenicity island (HPI) and 70-kb plasmid, pYV (184, 185). Complete virulence of *Y. enterocolitica* is dependent on plasmid pYV, which encodes a type-III secretion system that confers antiphagocytic properties to *Yersinia* (186). However, although pYV is necessary for infection, it is not sufficient for disease. Several additional virulence factors have been identified on the *Yersinia* chromosomes, including genes important for the establishment of infection and survival within the mammalian host (187), including genes within HPI (*ybtQ*, *ybtP*, *irp1*, *irp2*, and *psn*) that are involved in the yersiniabactin system which are negatively regulated by the iron-responsive regulator Fur (188, 189). A small conserved RNA chaperone protein, Hfq, is also known to be required for full virulence of *Y. enterocolitica* (190).

Y. enterocolitica pathogenesis is still not completely understood, but several possible mechanisms have been suggested. The primary pathogenic event of *Y. enterocolitica* is to adapt and colonize the human intestinal tract at a temperature of 37°C and to penetrate the intestinal wall, which is thought to be controlled by 70-kb virulence plasmid (pYV/pCD) genes, absent in avirulent strains. Several genes on the pYV plasmid are expressed only at 37°C but not at 25°C (191). *Y. enterocolitica* virulence factors include invasin (Inv), 103-kDa outer membrane proteins, and other surface proteins such as Ail, PsaA, and YadA which may help in invading epithelial cells (192, 193). Upon *Y. enterocolitica* infection, there is almost no measurable host response in the first 36 to 48 hours. *Yersinia* outer proteins (Yops) secreted by type III secretion systems (T3SS) are likely responsible for the initial inhibition of phagocytic functions. This initial quiet phase is followed by an influx of activated phagocytes at the site of infection, inducing an acute inflammatory process and producing cytokines (194).

Animal studies have shown that $CD4^+$ and $CD8^+$ T cells are required for control of *Y. enterocolitica* infection (195), as are IFN-γ-mediated Th1 immune responses, including macrophage production of TNF-α, IL-12, and IL-18 (196). Inhibition of T-cell proliferation and dendritic cell functions by Yops are primary mechanisms by which *Y. enterocolitica* evades both innate and adaptive immune responses (197). Tumor necrosis factor receptor p55 (TNFRp55) modulates macrophage functions in response to *Y. enterocolitica* stimulation through mechanisms involving nitric oxide, IL-6, and NF-κB pathways, suggesting an essential regulatory role of TNF via TNFRp55 signaling (198). Increased inflammation associated with the redirected host cell death could initially benefit *Y. enterocolitica* but later contribute to a generalized beneficial immune response and eventual clearance of bacteria (199).

Vibrio

Vibrio is a genus of Gram-negative bacteria possessing a curved-rod shape. Several species of *Vibrio* are pathogens, including *V. cholerae*, *V. parahaemolyticus*, and *V. vulnificus*. *V. cholerae* is one of the major bacterial pathogens responsible for the devastating diarrheal disease cholera.

Studies of *V. cholerae* mutants demonstrate that cholera disease requires the production of virulence determinants: toxin-coregulated pilus (TCP), which is controlled by the *tcp* operon and promotes small bowel colonization (200, 201), and cholera toxin (CT), which is encoded by the *ctx* operon and elicits a secretory response by small bowel epithelial cells. Expression of both *tcp* and *ctx* operons is regulated by the ToxR regulon (202), a multicomponent hierarchy of transcription factors that integrates physical and chemical signals, cell density, and the physiological state of the organism.

The immune response is not thought to contribute to the pathology of the infection, as the *Vibrio* toxin and the bacteria's multiple virulence factors seem to play dominant roles. Indeed, cholera is a noninflammatory diarrhea. However, innate immunity certainly seems to be important in controlling the infection. *In vitro* studies show that heat shock protein purified from *V. cholerae* O139 causes elevation of INF-γ followed by IL-2 and IL-4 expression (203). A dose-dependent decrease in IL-8 and TNF-α production was observed in cells subjected to *V. cholerae*, demonstrating its suppressive action on the production of proinflammatory cytokines in epithelial cells (204). In a mouse model, at the peak of infection, neutrophil recruitment accompanied by induction of KC, macrophage inflammatory protein 2 (MIP-2), nitric oxide synthase (NOS)-2, interleukin-6 (IL-6), and IL-17α has been described (205).

Helicobacter pylori

Helicobacter pylori is a Gram-negative bacterium colonizing the mucus layer of the gastric epithelium of more than 50% of the world's population, with higher incidences in developing countries. Infected individuals can be asymptomatic carriers or can go on to develop gastritis, peptic ulcer disease, or gastric cancers (206, 207). In the last decade, several studies have been performed to investigate the role of *H. pylori* in shifting the susceptibility to gastroenteritis in children (208). Some studies suggest increased risk of chronic diarrhea in children with *H. pylori* (209, 210) while others suggest no connection (211). Differential bacterial pathogenicity and host susceptibility may explain this diversity. Depending on the anatomical site of colonization, *H. pylori* may decrease gastric acid secretion in some people, thereby potentially reducing the effectiveness of the gastric acid barrier to intestinal pathogens, and increasing it in others (212).

A tremendous amount of effort has been invested in elucidating the interaction between *H. pylori* virulence factors, their association with the host, and consequences in pathogenesis or disease outcome. *H. pylori* has a high level of genetic diversity which helps in adaptation in the host colonization, persistence, and disease outcome (213). The *H. pylori* genome has ~30 *hop* gene paralogous encoding outer membrane proteins (OMP) which are important for bacterial adherence (214). Cytotoxin VacA is also a very important virulence factor in pathogenesis of gastric carcinoma and peptic ulceration. This toxin can induce multiple cellular activities, including cell vacuolation, membrane channel formation, disruption of endosomal/lysosomal function, apoptosis, and immunomodulation (215).

H. pylori has evolved complex strategies to maintain a mild inflammation of the gastric epithelium while limiting the extent of immune effector activity. Portal-Celhay et al. provided an excellent review of the host immune responses to *H. pylori* colonization (216). When compared with lipopolysaccharides (LPS) from *Enterobacteriaceae*, *H. pylori* LPS is 1,000-fold less active and only weakly activates macrophages (217). However, *H. pylori*'s attachment to epithelial cells produces an elevation of inflammatory cytokines such as IL-1, IL-6, TNF-α and mostly IL-8 (218, 219). The proinflammatory cytokine IL-1β is associated with gastric cancer, and IL-1β polymorphisms correlates with increased cancer risk in *H. pylori*-infected humans (220–222). *H. pylori* contributes to Th1 polarization, which is linked to the development of peptic ulcers, by stimulating both IL-12 and IL-23 secretions from neutrophils and monocytes (223). This Th1-predominant immune response is associated with elevated levels of IL-12, IL-18, and TNF-α. The severity of gastritis associated with *H. pylori* infection has been correlated with mucosal expression of the TNF-α subunit CD68 and IFN-γ (224). Hofman et al. observed an up-regulation in receptors involved in bacterial recognition, signal transduction, inflammation and immune response, proteolysis, apoptosis, and cell proliferation in antral biopsies from infected patients compared to uninfected individuals (225). *H. pylori* infection decreases the expression of let-7b, increasing the production of TLR4, NF-κB, COX-2, and Cyclin D1, thus contributing to the initiation of the immune response and the inflammation of the gastric mucosa (226).

VIRAL INFECTIONS

Viruses interact with the host at all stages of their replication, from the time they enter the cell, to their transcription, translation, synthesis, and packaging, to cell exit. These interactions are essential for the virus to multiply, but also enable the host to recognize the presence of a foreign material and to put in place defense mechanisms. Viruses have, however, developed strategies of their own to evade and/or manipulate the host immune response in their favor.

Rotavirus, a double-stranded RNA virus, continues to be the main cause of diarrhea and dehydration in children worldwide (227). Two vaccines have been available since 2006 and have been effective in decreasing disease severity in developed countries (228); however, this infection continues to cause severe disease and death in developing countries.

The innate immune system is a critical first line of defense against this infection (229). When rotavirus is recognized in the infected epithelial cells through retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5), IFN-α and IFN-β are induced (229). Rotavirus is able to modulate the innate immune system signaling and cell proliferation pathways. Its nonstructural protein 1 (NSP1) has the ability to bind interferon regulatory factors (IRF) 3, 5, and 7 and mediate their degradation by the proteasome, thus limiting the expression of IFN-α/β in epithelial cells (230–235). Despite this ability, significant amounts of IFN-α/β are still found in stool (236) and serum (237) samples of children with rotavirus. Animal models interrogating the function of IFN-α/β in disease pathophysiology show minimal effect. Although mice with impaired IFN-α/β responses have more viral replication than wild-type (238, 239), they had similar disease severity and viral replication (240). In fact, evidence suggests that IFN-γ may be more important in controlling the infection (241, 242).

In vitro, rotavirus was shown to induce IL-8 through NF-κB (243) and IκB kinase (IKK) activation (244). Other cytokines and chemokines have been detected *in vitro* and in mouse models such as GM-CSF, MIP-1β, IP-10, GRO-α, and RANTES (243, 245, 246). JNK and p38 activation may be important for optimal rotavirus replication (247). It is interesting to note that NSP1 can inhibit activation of NF-κB, modulating the immune response once again (230, 234, 248). Recent studies have highlighted the differences in host responses, especially within the IFN system, after infection with different strains of rotavirus (230, 249). Bagchi et al. analyzed the total gene expression profile of cells infected with different strains of rotavirus. Despite some strain-specific differences, they found that the

IFN-inducible genes (viperin, IL-18, TAP1, IFN, alpha inducible protein 6, GBP1, GBP2, and OAS1), as well as some cytokines (CCL15, CCL5, CXCL10, and CXCL11) were up-regulated significantly by all strains. They also describe that all strains induced down-regulation of anti-apoptotic genes and up-regulation of proapoptotic genes such as MX1 and STAT1 (250).

Translational studies are still rare. Children with rotavirus diarrhea have elevation in their serum IL-6, IL-10, and IFN-γ (251). Plasma IFN-γ levels were higher in children who developed persistent diarrhea due to rotavirus compared to those whose diarrhea resolved (252). Gao et al. found that serum and stool levels of IL-18 and IFN-γ were increased and associated with severity in children with rotavirus enteritis (253). Fecal calprotectin and lactoferrin are usually not significantly increased in viral gastroenteritis (17, 34).

As more viral etiologies of gastroenteritis are being detected by specific diagnostic techniques, we expect to see a significant improvement in our knowledge of viral/host pathogen interactions and their contribution to gastrointestinal disease.

PARASITIC INFECTIONS

Multiple protozoan parasites, including *Cryptosporidium parvum*, *Giardia lamblia*, and *Entamoeba histolytica*, can adhere to and multiply within enterocytes. The control of these infections relies on the production of multiple cytokines that activate the host defensive mechanisms to limit invasion and survival of the parasite.

Cryptosporidium parvum

C. *parvum* is an intracellular protozoan parasite that primarily infects the epithelial cells of the small intestine. Infection typically leads to a self-limiting watery diarrhea in immunocompetent individuals. In patients with an impaired immune system, especially $CD4^+$ T-cell deficiency such as AIDS, it can cause a chronic and potentially life-threatening infection (254). The infection is typically confined to the epithelial cells and does not cause invasion.

C. *parvum* infection is characterized by neutrophils, macrophages, dendritic cells, and T lymphocytes recruitment into the epithelial surface and the underlying lamina propria (255–257). The initial contact of the parasite with the intestinal epithelial cells leads to apoptosis (258–260), and increased expression of nitric oxide, prostaglandins, and β-defensins (261–265), in addition to proinflammatory cytokines such as IL-8, IL-15, IL-18, GRO-α, and IFN-α/β (254, 266–268). Chemokines meant to recruit dendritic cells (257) and prompt NK cells, macrophages, and cytotoxic NK cells to produce IFN-γ are secreted (254). IFN-γ plays a dominant role in host immune response to infection (269–274). In addition to its involvement during the innate immune response, it is also a key player during the Th1-mediated adaptive immune response. IFN-γ inducible protein 10 (IP-10), monokine induced by IFN-γ (MIG), i-TAC, lymphotactin, MIP-1β, and RANTES are strongly up-regulated in C. *parvum* infection (275). The role of TNF-α is uncertain as its expression has been increased in C. *parvum* infections (275, 276), but TNF-α deficient mice control the infection as effectively as wild-type mice (277). IL-12 has been implicated in the IFN-γ pathway as well, although its role in C. *parvum* infection is not completely understood (278–280). IL-18 appears to be involved in the regulation of the Th1/Th2 responses, and works synergistically with IL-12 to activate macrophages to produce IFN-γ (281–283). IL-15 has a role in activating the NK cell-mediated pathway (284), possibly through an IFN-independent mechanism (285). IL-4 also seems to work synergistically with IFN-γ in the immune defense against C. *parvum*, and may promote dendritic cell activation (286). Refer to Fig. 2, adapted from McDonald et al., for a visual summary of the pathways discussed.

A recent study suggested that let-7i may play a role in the regulation of NF-κB-mediated epithelial innate immune response (287). CXCL10 is highly up-regulated in intestinal epithelial cells of AIDS patients with active cryptosporidiosis and may play an important role as well (288). TGF-β, an anti-inflammatory cytokine secreted by epithelial cells, plays an important role in regulating and repairing damage following C. *parvum* infection (276, 289, 290).

C. *parvum* attempts to evade the host response through multiple mechanisms. It prevents epithelial cell apoptosis (291, 292) through activation of NF-κB (293), it increases expression of osteoprotegerin, thus modulating the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) (294), and it causes depletion of STAT1α and dysregulation of the IFN-γ pathway (295).

Giardia lamblia (*G. duodenalis* or *G. intestinalis*)

G. *lamblia*, a flagellated, binucleated protozoan parasite, is one of the most common causes of diarrhea worldwide. Infection can be completely asymptomatic, or can cause acute or chronic diarrhea, with malabsorption and failure to thrive. Most infections are self-limiting, although reinfection is common. *Giardia* infection in humans typically causes no mucosal inflammation, and the defense mechanisms are mostly local, through IgA or local effectors (296–298). Many G. *lamblia* strains are unable to colonize adult mice. Studies have therefore used G. *muris*, an imperfect model as it may be resistant to immune mechanisms capable of killing G. *lamblia* (299, 300).

B cells and antibodies play a major role in eradicating *Giardia* infections, and individuals with immunoglobulin deficiencies have severe disease (301). However, *Giardia* has the ability to undergo extensive variation of its surface antigens, variant-specific surface proteins (VSPs), making the antibody response ineffective in some cases (302–304). Studies on the local effectors for *Giardia* are controversial. Nitric oxide may inhibit proliferation of G. *lamblia* trophozoites *in vitro* (305, 306), although studies have shown that it may be ineffective at killing the parasite (307). *In vitro*, G. *lamblia* is sensitive to cryptdins 2 and 3, defensins, and indolicidin (308–311). Mucin may inhibit *Giardia* trophozoite attachment *in vitro* (312), although some evidence shows it may protect the parasite from destruction (313). The microbiota has been postulated to play a role as well (309, 314, 315).

G. *lamblia* can inhibit the innate immune response to minimize inflammatory reaction (316, 317). IL-8 and other proinflammatory cytokines do not appear to play a role, unlike other infections of the GI tract (296). On the other hand, mast cells are essential, and IL-6 production appears to be necessary in early control of the infection (318–320), as is TNF-α production (321). G. *lamblia* live parasites and extracts were found to activate dendritic cells, with secretion of small amounts of IL-6 and TNF-α, but no IL-12 secretion and enhanced IL-10 production (322). Although mice lacking IL-4 or IFN-γ are able to control infections (323), higher levels of these cytokines are found in infected cells and humans (38, 324). Performing gene

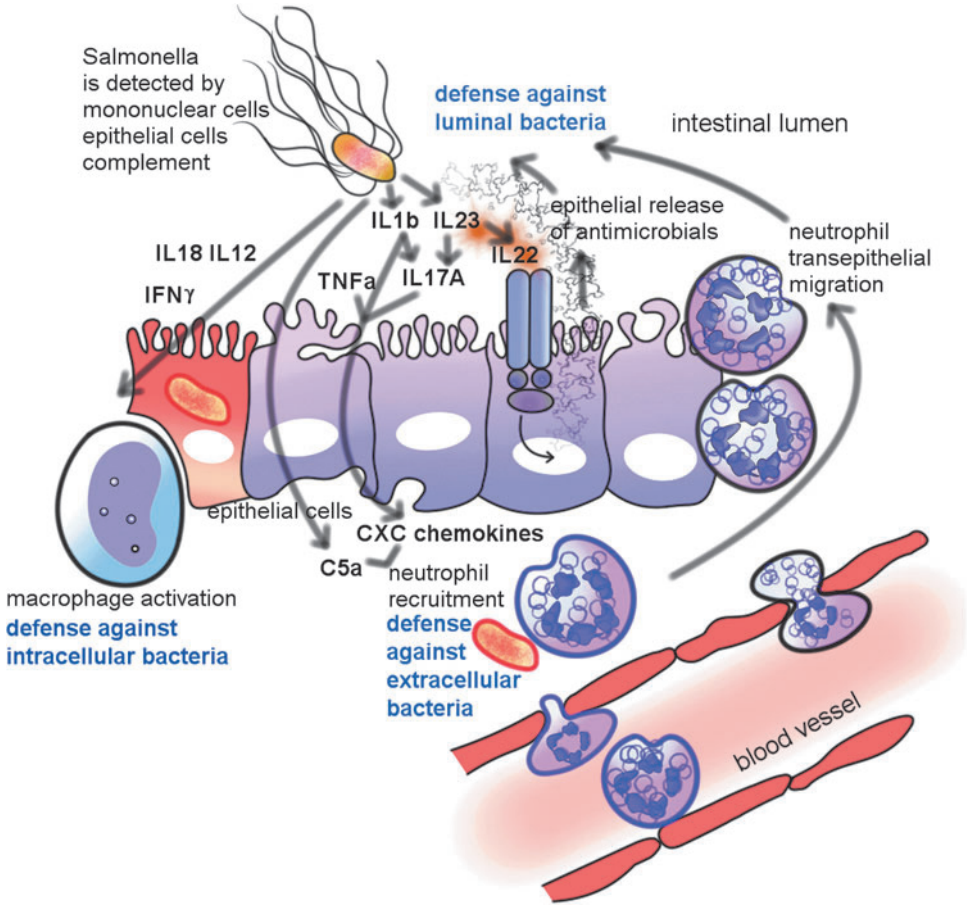

FIGURE 2 Innate immune responses during C. *parvum* infection. Parasite infection to intestinal epithelial cells (IEC) leads to production of various antiparasite molecules which help to maintain epithelial integrity. Infected IECs increased production of inflammatory cytokines such as IL-15, IL-18, and INF-α/β which help in establishing immune effector mechanisms, such as IFN-γ production by NK cells and macrophages and cytotoxic NK cell activity. Dendritic cells produce cytokines (e.g., type I IFN and IL-12) that activate NK cells and IL-4 from an unknown cell may promote dendritic cell maturation. (Adapted from reference 254.)

expression analyses, Röxstrom-Lindquist et al. found a novel chemokine profile (CCL2, CCL20, CXCL1, CXCL2, and CXCL3) to be induced by G. *lamblia*, but not by other pathogens (325).

Human studies show that infected individuals have high serum levels of IL-5, IL-6, and IFN-γ (326), as well as TNF-α (327). Long et al. investigated fecal biomarkers in multiple infectious diarrhea agents and found that high levels of MCP-1, IFN-γ, IL-4, and IL-5 were associated with longer diarrheal symptoms in G. *lamblia*, while increased IL-8 levels were associated with shorter durations (38).

Entamoeba histolytica (Amebiasis)

E. histolytica is an invasive enteric protozoan parasite that can cause dysentery, colitis, and liver abscesses. It is acquired when infective cysts are ingested through contaminated food or water. Trophozoites are released into the terminal ileum and migrate to the colon where they colonize the mucus layer (328). Amebic cysts are resistant to stomach acid and excyst in the lumen of the intestine. Trophozoites then attach to the surface via Gal/GalNAc lectin. Goblet cells and submucosal glands secrete mucin to inhibit attachment (329, 330); however, amebae produce cysteine proteases that can disrupt this mucus layer, overcoming this first hurdle (331).

Upon invasion, intestinal epithelial cells release proinflammatory cytokines, such as IL-1β, and IL-8, to recruit immune cells (332, 333). It has been shown that amebic trophozoites activate the transcription factor NF-κB in human intestinal epithelial cells, initiating this inflammatory response with the resultant damage to the intestinal tissue (332, 334, 335). Prostaglandins secreted by the trophozoites (336) may trigger IL-8 production as well (337). The parasite thus turns the host inflammatory machinery on to induce damage. IFN-γ is secreted by lymphocytes and TNF-α by activated macrophages (338, 339). High blood TNF-α levels are associated with increased risk of first, and recurrent, *E. histolytica*-related diarrheal episodes in children (340). IFN-γ and TNF-α are present for a transient period of time, and their levels decline as tissue damage progresses in liver abscess, as opposed to IL-1β and IL-8 levels which persist throughout the infection (341). TNF-α stimulates neutrophils and macrophages to release reactive oxygen species and nitric oxide, which can kill the parasite

but also contribute to tissue destruction (342, 343). Amebae possess many virulence factors that help them evade the immune system (329). Not only can they phagocytose neutrophils, they can limit nitric oxide production (344), and their peroxiredoxin protects them from neutrophil reactive oxygen defenses (345). The anti-inflammatory cytokine IL-10 may play a role in resistance to amebiasis (346), as could leptin signaling through STAT3 and SHP2/ERK pathways (347–349).

Helminths

Helminths comprise a variety of parasitic worms, including intestinal and filarial nematodes, cestodes, and trematodes. About 2 billion people are infected with intestinal helminths worldwide, mostly in poverty-stricken developing countries. Soil-transmitted helminths, mainly trichuriasis, ascariasis, and hookworm, cause significant morbidities (350). Many studies have investigated *Trichuris muris* in mice models to replicate the helminth disease seen in humans. Klementowicz et al. wrote an excellent review on host responses to *Trichuris muris* (351).

Although there are many individual variations between helminths, they share some common host response features. They are known to induce a significant Th2 response with elevation of IL-4, IL-5, IL-9, IL-13, IL-21, and IL-25 (IL-17E) (352, 353). This coincides with the recruitment of eosinophils, mast cells, basophils, and macrophages (354). Resistance to these infections may be carried out by IL-4 and IL-13 (355, 356), and IL-25 and IL-33 through induction of the innate lymphoid cells (357, 358). IL-9 is important in the acute phase of parasite immunity (359), and IL-10 plays a role in the polarization of Th2 response (360). In addition to the role played by Th2, development of an inappropriate Th1 response can lead to chronic infection. High levels of IFN-γ, IL-12, and IL-18 were observed in a mouse model of *Trichuris muris* infection, and resistance has been conferred when IFN-γ or IL-12 was depleted (351). IL-27 and WSX-1 interact in the early stages of infection to trigger Th1 responses in susceptible animals (351). An ineffective parasite expulsion, and a more severe GI inflammation, have been associated with increased production of proinflammatory cytokines such as IL-12/23, IFN-γ, and IL-17A (361). IL-6 limits Th2 response, modifies the Treg-cell phenotype, and promotes host susceptibility following helminth infection (362). In addition to lymphocyte responses, goblet cells and their products, mucins and resistin-like molecule β (RELMβ), are important for effective parasite clearance from the GI tract (363, 364). A delicate balance between epithelial cells, goblet cells, basophils, mast cells, eosinophils, and Th1 and Th2 is thus essential for development of immunity against intestinal helminths.

Few studies investigating biomarkers during human helminth infections have been published. Geiger et al. described serum CCL11 (eotaxin-1) and CCL17 (TARC) to be serological indicators of multiple helminth infections in humans (365). Lertanekawattana et al. examined human duodenal mucosa cytokines and found a high IL-5/IFN-γ ratio in noneosinophilic, helminth-infected patients compared to noneosinophilic, uninfected patients; and a high IgE receptor type I (Fc epsilon RI)/mast-cell tryptase ratio in eosinophilic, helminth-infected patients compared to eosinophilic, uninfected patients (366). More human studies addressing host/pathogen interplay may help develop new diagnostic tests and therapeutic strategies, impacting billions of people worldwide.

CONCLUSION

Gastrointestinal infections remain a major scourge, each year causing more than 2 million childhood deaths worldwide. Accurate diagnosis is critical to treat infections and as described in this chapter, a deeper understanding of the host response to distinct pathogens may guide the development of future diagnostic and therapeutic modalities. Furthermore, it is becoming evident that several pathogens take advantage of the innate host response to suppress competing microbes and to facilitate their expansion. Finally, intestinal inflammation accounts for much of the clinical disease during intestinal infection with a variety of organisms. Elucidating which features of the inflammatory response are beneficial and which are predominantly harmful may lead to targeted suppression of the inflammatory response as a means to decrease injury and shorten the duration of intestinal illness.

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section X

Information Technology

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Point-of-Care Medical Device Connectivity: Developing World Landscape

JEFF BAKER

47

The past 20 years have witnessed enormous technological improvements in the development of simple and accurate point-of-care (POC) diagnostic tests for detection and management of infectious diseases. At the same time, many stakeholders are now demanding real-time access to this accurate and robust diagnostic information, especially in low- and moderate-income (LMI) countries with resource-limited settings. New technologies and drugs for early diagnosis, continuous monitoring, and treatment of HIV infection have improved the prognosis of infection and the quality of life among individuals living with HIV. In 2009, 5 years after the availability of antiretroviral therapy (ART) was expanded in sub-Saharan Africa, drug treatments alone reduced mortality due to HIV infection and acquired immunodeficiency syndrome by 20%. However, in the same year only one-third of the 15 million people in LMI countries who were in need of ART received it. In these areas, a lack of resources, be they infrastructure, financial, or human, can hamper access to care for the resident populace. These deficits result in delayed treatment, poor patient follow-up, and poor adherence to ART, all of which contribute to the high rate of transmission and mortality. Therefore, substantial efforts have been taken to build low-cost and POC HIV diagnostic and monitoring solutions that effectively make early diagnosis and treatments available to even the most resource-constrained communities (1).

In LMI countries, huge strides have been made in the identification, care, and treatment of patients with life-threatening chronic infectious diseases such as HIV. Additionally, more attention is now being dedicated to closing the gaps in care, such as the deployment of innovative POC diagnostics. However, better drugs, more effective treatments, and deployment of the critical accompanying diagnostics at the POC in of themselves will not be enough to turn the tide in the war on HIV. Ensuring that the critical patient data are available where and when they are actionable will greatly help achieve this. Deploying an accompanying medical device connectivity (MDC) solution can help ensure that critical patient data are available where and when they are needed.

The purpose of this chapter is to describe the concepts behind MDC, to describe the challenges and opportunities ahead, and to provide some specific examples of MDC currently in use. This is a rapidly evolving field, such that obsolescence is always an imminent threat. However, several technology-independent common themes are emerging that are worth summarizing here.

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DEFINITION OF CONNECTIVITY

With regard to patient care, there is no universally accepted definition of the term "diagnostic connectivity." The *Webster's Dictionary* definition is as follows: "The quality, state, or capability of being connective or connected (connectivity of a surface); especially: the ability to connect to or communicate with another computer or computer system" (www.merriam-webster.com/).

For the purpose of this chapter we will refer to MDC as the application of medical device technology and process engineering to improve patient flow, increase patient safety, and improve patient outcomes. Fundamentally, MDC is used to remotely control, configure, and monitor patient diagnostic data. It also captures device operational metrics, surveillance data, and other key information. These features result in the timely creation of effective surveillance data and enable remote deployment of screening/diagnosis, patient care, and any instrumentation for health testing at the POC.

MDC is composed of, but is not limited to, the following elements:

1. eHealth
2. mHealth
3. Health information exchange (HIE)
4. Public health surveillance

eHealth and mHealth

Global applications of mHealth and eHealth tools are rapidly expanding, and this trend is anticipated to continue to accelerate in the foreseeable future. This expansion will create many opportunities for the improvement of patient outcomes. When effectively implemented, integration of these applications facilitates improved data analysis, data sharing, and data interactions between systems. Increased data utilization and sharing will enhance public health surveillance, monitoring, and evaluation. This, in turn, will augment routine health care interventions and result in improved outcomes. Integration also promotes a coordinated strategy for information, communication, and technology that can increase system efficiency and reduce costs for an implementing agency, country, and/or program.

eHealth

The World Health Organization (WHO) defines eHealth as the use of information and communication technologies for health. Examples include treating patients, conducting research, educating the health care workforce, tracking diseases, and monitoring public health (http://www.who.int/topics/ehealth/en/). Generally, this term can be interchangeable with "health informatics," which covers electronic and digital processes; essentially, eHealth is the exploitation of various Internet assets in health care.

eHealth can encompass a range of services or systems:

- Electronic health records: enabling the communication of patient data between different health care professionals
- ePrescribing: access to prescribing options
- Telemedicine: physical and psychological treatments at a distance
- Health knowledge management: best practice guidelines, epidemiological tracking, etc.

Privacy issues related to patient data and medical records are, of course, of paramount importance; widespread acceptance of eHealth tools will likely depend on the implementation of successful data security protocols.

Over the past few years many LMI countries, such as the United Republic of Tanzania and the Republic of South Africa, have developed extensive eHealth policies. In many cases these policies are foundational, or in essence are created to help guide the country's entry into and deployment of eHealth (e.g., Tanzania National eHealth Strategy [2]). Other countries have advanced their eHealth initiatives to a state in which they have identified challenges to implementation and developed a series of framework documents to support their strategies. The Department of Health of the Republic of South Africa identified a serious lack of interoperability between heterogeneous systems as a key obstacle to implementation and developed the "Normative Standards Framework" to address this and plan a path forward (3).

mHealth

The term "mHealth" refers to the use of mobile devices in health care delivery, including:

- Collection and aggregation of patient-level health data
- Delivery of health care information to practitioners, researchers, and patients
- Provision of real-time monitoring of patient vital signs (such as heart rate, temperature, and pulse oximetry data)
- Direct provision of care (via mobile telemedicine)

In response to the proliferation of mobile phones and their networks in LMI countries, there has been an equal increase in mobile data collection, data delivery, and mobile access to data. As a result, a large number of projects are currently under way or have been completed that focus on providing mHealth solutions for public health initiatives. Many of these projects are very innovative and have demonstrated positive results. However, for the most part, they have remained relatively small and have not scaled up.

HIE

Electronic HIE enables clinicians, other health care professionals, and patients to electronically access and securely share a patient's medical information. In theory, this improves the quality, the speed, the safety, and the cost of patient care. An effective HIE system can also link the patients' medical information, the electronic medical record data, to the public health surveillance data.

Public Health Surveillance Data

Public health surveillance is the ongoing systematic collection, analysis, and interpretation of data, closely integrated with the timely dissemination of these data to those responsible for preventing and controlling disease and injury (4). The WHO defines public health surveillance data as the continuous, systematic collection, analysis, and interpretation of health-related data needed for the planning, implementation, and evaluation of public health practice. Such surveillance can:

- Serve as an early warning system for impending public health emergencies
- Document the impact of an intervention or track progress toward specified goals
- Monitor and clarify the epidemiology of health problems, to allow priorities to be set and to inform public health policy and strategies (http://www.who.int/topics/public_health_surveillance/en/)

The collection, aggregation, and reporting of public health surveillance data is an essential aspect of public health.

Increases in international attention to health and health care delivery have been accompanied by demands for statistics that accurately track health progress, evaluate the impact of health care programs and policies, and increase accountability at the country, region, and global levels. Despite the targeted efforts by programs and countries, the ability to respond to this demand is constrained by limited data availability, reliability of data, and quality of data.

CURRENT LANDSCAPE OF POC MDC

In most aspects, POC MDC in LMI countries is in its infancy. The vast majority of the projects in this arena never advance beyond the pilot and/or demonstration stages. Very few have achieved scalability. Those projects which have started to scale up are generally industry/manufacturer-led projects executed in response to market demands.

A complete MDC solution will require many elements and components. Most manufacturers are focused on their particular product and deliver their connectivity solution to address a market demand. Diagnostic manufacturers generally do not have the universal focus required to deliver a broad medical diagnostic connectivity solution.

Key Barriers and Issues

- Interoperability:
  - o Universal standards: Due to the variety of systems throughout the world, there is a huge diversity of standards for communicating data between devices and systems. Any new system must be capable of communicating with both local and domestic eHealth systems, as well as reporting to and incorporating data from other international surveillance systems.
  - o Data-field standardization: Currently, the data captured and the data parameters are neither uniform nor consistent.

  - Organizational interoperability: This is concerned with the definition of business goals, modeling of business processes, and organizational collaboration issues. It refers to the ability of organizations to effectively communicate and transfer meaningful data and information (3).
  - Syntactical interoperability: This is concerned with data formats and message formats. Messages transferred by the communication protocols must have a well-defined syntax and coding but also carry data or content at the same time (3).
  - Technical interoperability: This addresses the technical matters of connecting systems and services through interfaces and protocols. Applying appropriate software engineering techniques and methodologies does this. This is usually associated with the hardware/software components and the systems and platforms which enable machine-to-machine communication (3).
  - Semantic interoperability: This involves the meaning of content, focused on the human rather than machine interpretation of the content. It refers to a common understanding between people of the meaning of the content (information) being exchanged (3).
- Data storage location:The location where the data are physically stored needs to be carefully considered to accommodate various country-specific regulations. Some countries allow their data to be stored in "off-country cloud-based" data centers. Other do not.
- Integration: Integration is the process of physically or functionally linking multiple subsystems or disparate data to create a combined system or unified solution. Types of integration include:
  - Data integration: This is the combination or exchange of data from multiple sources into a tool or platform that uses them for transactional or analytical purposes.
  - Process integration: This concerns the understanding, management, and coordination of individual processes among multiple systems to achieve complex objectives.
  - User experience integration: This simplifies the user experience, enabling user interaction with multiple back-end systems or data sources in a consistent user interface.
  - Infrastructure integration (entity aggregation): This combines the infrastructures used by multiple systems into a common asset. Infrastructure integration is typically needed when a critical mass of deployments exposes inherent inefficiencies and excess infrastructure.
- Data ownership: One critical issue is accounting for data ownership. In many countries, the ministry of health (MoH) or equivalent agency considers itself the owner of any data generated within its country. This includes data created by any organizations operating within that country (e.g., universities, nongovernmental organizations, etc.)
- Data privacy/confidentiality: Data privacy and patient confidentiality need to be ensured in any large-scale MDC program. Currently, most MDC projects do not collect patient-specific data. They typically focus on supply chain management and instrument operational metrics. To advance beyond the simple capture of instrument operational metrics requires the deployment of unique patient identification (UPI). UPI systems are nonexistent in many LMI countries.
- Scalability: The vast majority of the projects in this field never advance beyond the pilot and/or demonstration stages ("pilot-itis") (The GSID Diagnosis and Reporting System, Global Solutions for Infectious Diseases, personal communication, I. Francis, Ian.Francis@gsid.com). Very few have achieved scalability. Any new MDC system which seeks to scale past the pilot phase level will need to expand its reach (geographical reach, participant reach, etc.).
- Vendor neutrality: It is critical to deploy a platform which is vendor neutral and unbiased. Equally of interest is the utilization of potentially open-source connectivity and reporting components.
- Sustainability: The resource challenges of sustaining a large MDC system are one reason that most projects have not scaled past the pilot stage. A large MDC system requires significant up-front resources and assets, but to succeed, it needs a source of ongoing capital to support continuing operations.
- Language: For success, multiple languages may be required. However, for reasons of expediency and workflow, one common alphabet may be required. This can pose a challenge across global regions (English, Russian, Chinese, etc.).
- Funding/resourcing: The costs associated with MDC consist of up-front costs such as hardware and modem acquisition, as well as recurring costs such as mobile data push charges and so on. This level of resourcing requires sustainability.
- Simplifying potentially complex architecture: An MDC system needs to be enabled to accept data feeds from numerous sources and entities (different diagnostics, different therapeutic areas, different patient care plans and care outcome inputs, etc.).
- Centralized and decentralized systems: An MDC system needs to accommodate both centralized and decentralized health delivery systems. It also needs to be flexible enough to accommodate hybrids of both.
- UPI: For an MDC system to be successful, it must have a reliable UPI system.

This list is not intended as a compendium of all the necessary elements for the successful deployment of a POC MDC solution in LMI countries. However, it is an attempt to capture the key topics and issues.

Obstacles to Integration

Considerable investments of time, money, human resources, and physical infrastructure are required to integrate systems. Prior to making such a commitment, the parties involved should address a number of questions related to the requirements needed to achieve predetermined goals. The discussion frequently begins with high-level questions and becomes more specific and implementation-focused as the conversation continues. Objections to integration are commonly raised at more specific, technical levels; however, these challenges often stem from higher-order questions that remain unresolved or unaddressed. For this reason, it is important to resolve such first- or second-order questions before addressing the specific and technical concerns. We present five general steps that provide a roadmap to successfully navigate the major obstacles to integration (5).

The hierarchically organized questions include:

1. Is there any value to be derived from the integration?
2. Are there organizational incentives and alignment to invest in integration?
3. What is the regulatory, contractual, and policy framework for data sharing?
4. Is integration feasible at the semantic (data meaning) level?
5. Is integration feasible at the technological level?

Resolving Obstacles and Basic Prerequisites of a Solution

While the obstacles to integration present a challenge, pragmatic approaches exist to mitigate the obstacles at each stage of the process. A brief breakdown of each approach is offered below.

A. Goals and objectives of integration
   1. Data sets pertaining to national and international indicators can be useful tools for achieving priority health outcomes.
   2. Systems integration workshops can help implementers tackle common problems to achieve common goals.

B. Organizational incentives and alignment to integrate
   1. Funders can mandate, or at a minimum strongly suggest, that participants share data that can be used in other programs as part of their agreements.
   2. Reduce the risk and cost of integration by providing a value-added health information system, where the "default" option is to contribute and utilize data from a pre-existing integrated system.
   3. Ensure that costs of integration are explicitly budgeted as a value-added activity and not expected to be absorbed in overhead.

C. Data-sharing framework
   1. Ensure that the participant country has a data-sharing framework in place.
   2. The funder should have clear policy guidelines and contractual language that define the obligations of implementers who will share data with the funder and the participant country.

D. Semantic feasibility
   1. Maintain up-to-date data dictionaries and metadata associated with information systems.
   2. Include quality assurance efforts as part of any project that produces useful data to validate assumptions about its quality.

E. Technological feasibility
   1. Use interoperability profiles of standards and interoperability specifications to reduce the cost and complexity of implementing standards.
   2. Integrate first and standardize later. Successfully integrate systems before solving the generic technological challenges; this will increase efficiency and ensure that the standardization process is better informed (5).

The creation of a successful connectivity solution will require some basic prerequisites, such as:

A. Technology characteristics
   1. Global availability
   2. Strong ecosystem of component suppliers
   3. Established test and certification processes
   4. Strong interoperability based on standards
   5. Market tested with documented performance

B. Cost effectiveness
   1. Low total cost of ownership
   2. Ultra low power/battery sensitive
   3. Multiple suppliers to select from
   4. Use of off-the-shelf components
   5. Avoidance of traditional high costs associated with cellular roaming

C. User experience
   1. Superior user experience
      a. Zero-touch installation
      b. Simple ongoing usage
   2. Seamless fit into the existing operator workflows
   3. Strong power performance
   4. No health risks
   5. No out-of-pocket expenses for operator

MOBILE PHONE NETWORK CONNECTIONS

Mobile phones are increasingly ubiquitous in LMI countries and now account for four in every five connections worldwide. As Elsie Kanza, of the World Economic Forum, recently said, "Regardless of social class, almost everyone [in Africa] has a mobile phone, or two or three. Even in remote villages, mobile phones have replaced the bicycle or radio as prized assets" (6). Therefore, mobile phone networks are probably the most accessible and reliable vehicles for the transmission of data in LMI countries.

Today most mobile phone network operators have resilient architecture and networks. However, there are various device considerations which need to be taken into account to achieve improved connection reliability:

- Most connectivity projects to date simply embed a global system for mobile communication module and direct the device to open a point-to-point protocol session.
- Generally, pay-as-you-go SIMs (subscriber identity modules) are used for development/testing of systems which require minimal data transmission.
- The devices generally default connection reliability to the network and rely on the network strengths and weaknesses.
- Most mobile phone network operators identify the application requirements and establish criticality of network reliability early in the project.
- Most map the application's interaction with the network at the firmware level through the use of a "smart connection management" approach. This should be done at the product development stage.
- Most understand the capabilities of the network and prioritize how the device should interact with the network, e.g., General Packet Radio Services (GPRS), Short Message Service (SMS), Unstructured Supplementary Service Data (USSD), etc.
- 2G/3G is sufficient for meeting the performance needs, but the cost of connectivity must be considered.
  - o 2G networks will be decommissioned in the future, but the impact can be minimized with careful planning and foresight.
  - o The longevity of 2G availability needs to be monitored and will impact sourcing efforts.

IDEAL FEATURES OF A NEW SYSTEM

As stated before, the vast majority of medical diagnostic connectivity projects in this space (LMI countries) never advance beyond the pilot and/or demonstration stages. Very few have achieved scalability. Those which have are generally industry/manufacturer-led projects executed in response to market demands. These typically are only focused on capturing and pushing the operational metrics and ensuring the supply chain management of specific POC medical diagnostics.

To advance to a more inclusive connectivity solution, which will directly impact and improve patient care, a UPI system will be necessary. As stated before, UPI systems are not universally available in LMI countries. Once a UPI system is deployed, you can then advocate for a convergence of HIEs with analytics driven from eHealth (The GSID Diagnosis and Reporting System, Global Solutions for Infectious Diseases, personal communication, I. Francis, Ian.Francis@gsid.com).

THE FUTURE CONNECTED ECOSYSTEM

The future medical diagnostic connectivity ecosystem should strive to capture all the data feeds which either directly or indirectly impact patient care. Further goals are to couple these data feeds with electronic patient management systems, treatment decision engines, supply chain asset management systems, and a host of other supportive resources available to the care team and/or directly to the patient and the patient's caregivers. The system should enable government health care planners, private and public funding organizations, implementing partners, clinicians, patient care teams, and industry/manufacturers to seamlessly access either patient identified or de-identified data depending upon the need and the rules established for data access. Points to consider include:

- The medical diagnostic connectivity solution needs to be comprehensive, which means incorporating all diagnostics, patient care plans, treatment outcomes and electronic surveillance.
- The solution needs to focus on workflow first (operator and consumer workflows).
- The solution must be flexible to accommodate a wide range of support from simple interventions (e.g., malaria care) to more complex interventions (e.g., cancer care).
- The solution must synchronize to all stakeholder time horizons.

Lateral Flow Test (LFT) Readers

The past 20 years have witnessed enormous technological improvements in the development of POC diagnostic tests. At the same time, many stakeholders in LMI countries are now demanding real-time access to this diagnostic information. This demand is not limited to instrument-based diagnostics. It also includes a high level of interest in data, which includes "digital footprints" for non-instrument-based diagnostics.

One such POC diagnostic improvement, which has been largely deployed in the LMI countries, has been the lateral flow test (LFT). However, real-time access to LFT results has been elusive. The concept of testing body fluids with rapid diagnostic tests dates back thousands of years. The development of the LFTs we are familiar with today can be tracked back to the 1950s. Although the technology may be considered old according to modern standards, it is by no means outdated.

Although it is difficult to estimate the number of LFTs manufactured each year, global revenue for medical/clinical LFTs was estimated at about $3.0 million in 2010. This is expected to grow to about $4.2 million by 2015. So although the technology has been around for many years, the market demand continues to grow, for many reasons:

- The results are obtained quickly.
- LFTs generally have a long shelf life.
- They are usually inexpensive.

However, LFTs are not without their drawbacks. The interpretation of LFT results can be quite subjective. The results are visually read and as such rely on operator interpretation and experience. Also, the results are generally limited to a defined detection window, so enduring test results are difficult to obtain. Furthermore, in their current format, it is not possible to seamlessly enter LFT results into a patient electronic medical record.

LFTs continue to offer significant clinical advantages, but the technology may be in need of a retrofit. LFT readers may provide that retrofit. LFT readers can mitigate the drawbacks of LFTs while expanding the utility of this incredible technology. Today, LFT readers generally fall into two different categories: instrument based and smartphone apps.

LFT Readers: Instrument Based

Several diagnostic manufacturers provide fully quantitative assay results from LFTs through the use of handheld diagnostic devices known as lateral flow readers. By utilizing unique wavelengths of light for illumination in conjunction with either CMOS (complementary metal-oxide semiconductor) or CCD (charge coupled device) detection technology, a signal-rich image can be produced from the actual test lines. By means of image-processing algorithms specifically designed for a particular test type and medium, line intensities can then be correlated with analyte concentrations. Alternative nonoptical techniques are also able to report quantitative assays results.

One key driver supporting the use of LFT readers is the removal of operator interpretation of test results. By removing the operator "subjectivity of read," you can remove one of the critical drawbacks of LFTs. Time to obtain the test result is another key driver. With LFT readers, tests can take as little as a few minutes to get results. However, there is a tradeoff between time and sensitivity. It may take more time to get results from the more sensitive tests.

From a development perspective, an instrument-based LFT reader can require a significant investment in both development time and resources. In an attempt to minimize the number of different LFT readers, many manufacturers are attempting to standardize their LFT formats so one reader can be used for a variety of different tests. Also, there is significant pressure to deliver these LFT readers at very low prices to consumers. Development of an LFT reader also requires a significant amount of cooperation between the instrument developer and the various LFT manufacturers. Each LFT has unique performance characteristics, which comprise the results of the test, such as signal intensities, ghost lines, etc. Further, LFTs are not static. Raw material suppliers often change with manufacturers. When

a change occurs, the performance characteristics can also change. The LFT reader is required to account for all of these.

Most instrument-based readers are designed for use in high-income countries and are generally considered to be expensive. The reason for the high costs is that volumes for these instrument-based readers are generally low, since most readers are designed for use with a single test or a group of tests from a single company.

LFT Readers: Smartphone Applications

The high mobile phone penetration and rapidly growing telecommunications infrastructure throughout the world represent an unprecedented opportunity for reading and transferring POC diagnostic data. From 2005 through 2010 mobile phone subscriptions grew 70%, and they continue to climb. In 2013 there were 6.8 billion mobile phone subscriptions. Hence, exploiting the existing mobile phone infrastructure to monitor health conditions will accelerate the efforts toward medical diagnostic connectivity. The use of smartphone cameras has been suggested for diagnostic applications in infectious diseases, dermatology, microscopy, ophthalmology, and paper-based microfluidic devices (The GSID Diagnosis and Reporting System, Global Solutions for Infectious Diseases, personal communication, I. Francis, Ian.Francis@gsid.com).

Generally, smartphone cameras have standardization challenges. In optical analysis of colorimetric assays, the integration of color-balancing functions of the camera phones is optimized for photography in bright ambient light. Many companies are developing smartphone application algorithms with interphone repeatability for both Android and iOS operating systems. The app can transform a smartphone into an LFT reader.

Smartphones deploying such an application have the potential to serve as low-cost POC LFT readers. They also have the potential to read many different LFT test types (HIV, malaria, etc.) and configurations (dipsticks, cartridges, etc.).

Some basic mobile device requirements are the following:

- Currently supporting platforms from 2.3.6 to 4.1.2, API levels 10 to 16 (Android)
- Android platform 2.3 or newer
- 2G, 3G, or Wi-Fi
- Touch screen, at least 480 × 800 pixels and 16M colors
- At least 4 GB storage and 768 MB RAM
- At least a dual-core 800 MHz processor
- Camera preview resolution 800 × 480 pixels or more, 20 fps or more
- Autofocus, minimum focus distance 10 cm or less
- LED flash
- Accelerometer
- GPS (if location data are to be used)

Some basic data capture parameters are as follows:

- Test result(s)
- Test location
- Time of scan
- User/operator who performed the scan
- Critical patient data, including:
  - Patient ID
  - Patient name
  - Patient date of birth
  - Patient gender
- Enduring scanned image of the test

GxALERT: POC MDC EXAMPLE

Prior to the availability of Xpert MTB/RIF (a product of Cepheid), multidrug-resistant tuberculosis (MTB) diagnostic methods required weeks to deliver a definitive result. Delayed result reporting led to patients being left untreated or placed on ineffective therapies. If untreated, patients may continue to spread MTB to others in the community, increasing the disease burden. Xpert MTB/RIF provides a 2-hour detection of MTB and rifampin-resistance mutations.

While Xpert MTB/RIF represents a huge leap forward in the detection of MTB, early on, the test results were still physically transcribed into paper ledgers. This manual transcription resulted in significantly delayed initiation of effective treatment. The MoHs generally had inadequate tools for forecasting multidrug-resistant drug (MDR) needs, MDR ward beds, and poor guidance on site selection and the number of instruments they required.

SystemOne (a technology strategy firm) and Abt Associates (a public policy consulting firm) co-invented GxAlert, a software system and platform for networking Cepheid's Xpert MTB/RIF machines. By doing so, they were able to push real-time test results to a secure cloud database. Once in the database, these results are connected into case management systems, patient records, and other critical information systems. The data are also formatted and pushed out as SMS text alerts to patients, doctors, and treatment centers, as email reports, and as Web dashboards. This enables GxAlert to use whichever method is appropriate for each actor in the health system to ensure maximum adoption of the data created by Xpert MTB/RIF.

GxAlert is a set of open-source software that operates in a cloud database, or it can be installed on a national MoH server. It uses USB modems, existing Wi-Fi, or Ethernet connections to transmit the data. It has been implemented with closed user groups. It consists of configuration documentation to direct Xpert MTB/RIF to send duplicates of test results over the "best available" network (3G, 2G, SMS text, Wi-Fi, Ethernet) to free the data and get discreet messages, alerts, and reports to the 10 major consumers of diagnostic data in the health system:

1. Patients
2. Referring clinicians
3. Receiving institutions/MDR wards
4. Lab techs/lab managers
5. MoH supervisors
6. Device manufacturers
7. Funding partners
8. Project managers
9. Pharmacy/pharmaceutical suppliers
10. Logistics management units in the MoHs

GxAlert was created as a result of an unmet need voiced to Abt Associates by one of their largest clients, the Nigerian MoH. SystemOne was brought in due to their technological expertise in the area of systems design. GxAlert represents a very innovative and novel approach to a significant gap in patient care.

GxAlert originated because there was no commercial solution available on the market at that time. Device manufacturers generally did not offer a viable solution that delivered all of what the market demanded. At each step in development, GxAlert added features only when demanded by the clients and only when driven by specific use cases/users. It is a great example of a POC medical device connectivity solution designed and delivered to improve patient outcomes.

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WHONET: Software for Surveillance of Infecting Microbes and Their Resistance to Antimicrobial Agents

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48

INTRODUCTION

WHONET is a free informatics tool developed and supported by the WHO Collaborating Centre for Surveillance of Antimicrobial Resistance in Boston for surveillance of bacterial infections and their resistance to antimicrobial agents. Since its inception in the 1960s as a set of mainframe-based routines, through the latest Web incarnation, WHONET has had two major objectives. The first is to analyze the reports of microbiology laboratories to delineate the specific problems with infections seen in health centers and the communities they serve. The aim is to provide infection control, antimicrobial stewardship committees, and public health professionals with the data they need to develop appropriate interventions to stem the spread of antimicrobial resistance.

WHONET's second objective is to enable multiple laboratories to merge their reports into multicenter, national, or global files to track the spread of antimicrobial-resistant organisms and support collaborative efforts to manage and contain them. With modern advances in Web-based information technology, WHONET supports the WHO Global Action Plan for Containment of Antimicrobial Resistance to build a global collaborative for antimicrobial resistance surveillance.

Microbial Infections

A small fraction of the many types of bacterial species in the world, i.e., the pathogens, cause infections that can sicken both people and animals. Pathogens vary, however, in how often they infect the hosts that carry them. For example, many humans carry *Neisseria meningitidis* asymptomatically in their upper respiratory tract, but only a smaller percentage go on to develop pneumonia, sepsis, or meningitis with this species. However, the related species *Neisseria gonorrhoeae* often infects the mucosal membranes of the genitals, throat, and rectum of those who acquire the organism, and it is the rare individual who is asymptomatic. Thus, both are pathogens, but relatively few *N. meningitidis* organisms cause infections, in contrast to *N. gonorrhoeae*, which typically causes symptomatic infection.

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Bacterial organisms recovered from a body site of a patient are considered to cause infection when the patient has clinical evidence of an infection consistent with that type of pathogen. The presence of symptoms motivates the patient to see a health care provider, who often takes cultures from inflamed body sites and sends them to a microbiology laboratory for culture and antimicrobial susceptibility testing. The microbiology laboratory in turn isolates the organisms in that sample and determines their species identity and, ultimately, their susceptibility to antimicrobial agents. This information is typically transmitted to the heath care provider to support the selection of therapeutic agents. However, these data have greater value beyond the individual patient, which is where WHONET often enters the picture.

Analyzing and Reporting Data

The generation of data for bacterial identification and antimicrobial susceptibility testing from hundreds if not thousands of microbiology laboratories globally from a wide range of infections has enormous potential for surveillance of infectious diseases. Fortunately, the data in these reports are standardized, at least to a certain degree, globally with fields for patient identification, patient location, date when the specimen was taken, type of organism identified, and the antimicrobial susceptibility data (i.e., MIC or zone of inhibition sizes) for disk diffusion testing and the interpretation of the results (i.e., susceptible, intermediate, or resistant). Such reports are a resource that may be overlooked because they are unique to microbiology laboratories. Other clinical laboratories, such as for chemistry or hematology, report analytes, such as serum sodium or hematocrit, that are unique to each patient and unrelated between patients. In contrast, a microbiology laboratory identifies organisms that may have epidemiologic significance, because they have spread from one patient to another patient, and thus laboratory reports have value beyond the individual patient because of the aggregate information that exists in a hospital or even a region.

Worldwide, data accumulating in the reports of all microbiology laboratories can track the global spread of pathogens, and WHONET can analyze and report those data. Bacterial pathogens in particular have long been known to spread in both local and global epidemics. For the past 80

years, global spread of antimicrobial resistance has been occurring in parallel with the dissemination of antimicrobial resistance elements within and among bacterial species. The information needed to interrelate and manage these parallel epidemics or genetic elements and strains is contained within the files of the world's microbiology laboratories.

The World's Microbiology Laboratories

Nearly all microbiology laboratories produce and store reports of pathogens and antimicrobial susceptibility data, but the laboratories are irregularly distributed throughout the world. In well-resourced countries, data on pathogens and resistance profiles are reported routinely for most infections, while in countries with fewer resources, fewer patients have access to diagnostic services, and thus there are fewer cultures and less data available for analysis. In many places in the world almost no data are available on pathogens and resistance. This could be expressed for a country or region in terms of the total number of infections reported by its laboratories as a fraction of its population, which could be a useful health care quality index and would vary by orders of magnitude across the world.

The fewer patient-care microbiology laboratories a country has relative to its total population, the more important each becomes for monitoring that country's public health. The prevalence of different types of pathogens and of their resistance to various antimicrobial agents differs between countries and needs to be sampled as broadly as possible in each country as a guide to its use of antimicrobials. The reported pathogens of the few infected patients able to be tested in a limited-resource country need to be summarized and made available to help inform treatment of all those who are not tested.

This need for locally relevant data on resistance trends may be overlooked by public health agencies with limited resources already stretched by responding to traditional public health problems, yet effective response requires reliable data from any laboratory, public or private, that can provide it. It is possible that much of the mortality and morbidity attributable to antimicrobial resistance comes from places where febrile patients get antimicrobial agents that are chosen by caregivers lacking information or informed experience and are often choosing from a very limited list of available drugs. A core reason for the development of WHONET was to give to caregivers or policymakers a free software tool that could access and analyze whatever information was available to assist the health care provider with antimicrobial selection and resistance containment interventions (1–3).

WHONET DEVELOPMENT: 1966 THROUGH PRESENT

The initiative that has become WHONET has evolved as both laboratory sciences and informatics technologies have advanced over the past half century. Following a 4-month fellowship in 1962 in Clinical Microbiology under Dr. John C. Sherris at the University of Washington, Dr. Thomas O'Brien, as director of the Peter Bent Brigham microbiology laboratory, introduced into clinical practice disk diffusion-based antimicrobial susceptibility testing following recommendations published in 1966 by the University of Washington collaborators Bauer, Kirby, Sherris, and Turck (4). These recommendations were subsequently supported by an international collaborative study headed by Ericsson and Sherris (5).

Systematic data collection and information management was a challenge at that time. For the greater part of the 20th century, the collection and management of health data were accomplished tediously through manual data collation, review, and tabulation. Graphic presentation of results required specialized skills and equipment available only to government agencies and large enterprises, and opportunities for productive sharing of data collections were limited. But through collaboration with the aviation firm and defense contractor Avco Corporation, computerized surveillance of microbiology test results was initiated in March 1966. This effort utilized computer keypunch cards of microbial reports analyzed by FORTRAN routines on a time-shared IBM 360 mainframe computer. The addition of a computer-driven plotter permitted the graphical display of disk diffusion frequency distributions, as seen in Fig. 1 (6).

These early demonstrations of the feasibility and value of monitoring available laboratory findings prompted a contract in the 1970s from the U.S. Food and Drug Administration to survey such results and to evaluate their comparability, initially in nine other U.S. medical centers and then in a foreign medical center. Data were collected

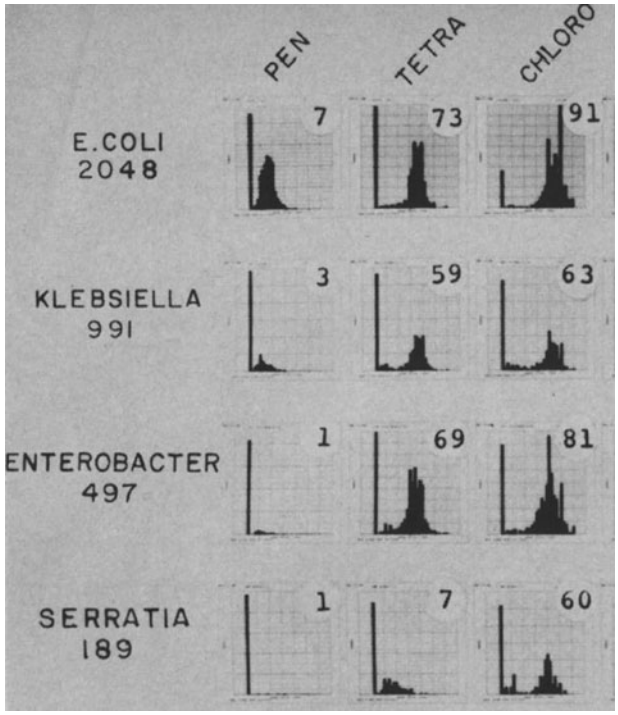

FIGURE 1 Frequency distributions of inhibition zones of *Escherichia coli*, *Klebsiella* spp., *Enterobacter* spp., and *Serratia* spp. isolates from 1966 through 1969 around disks of penicillin, tetracycline, and chloramphenicol (6). Isolates toward the right of each graph are susceptible, isolates to the far left possess high-level resistance, and isolates in the middle exhibit moderate or intermediate levels of resistance. Circled numbers represent the percent susceptible to the indicated antimicrobial. A comparison of the four species indicates that isolate subpopulations with similar levels of resistance exist across all four species but in very different proportions. (Reprinted from reference 6 with permission.)

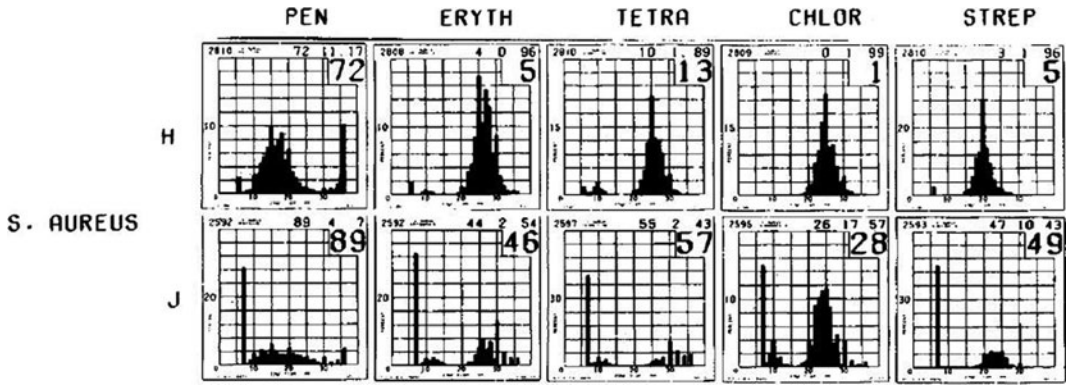

FIGURE 2 Frequency distributions of inhibition zones of *Staphylococcus aureus* from 1973 to 1974 around disks of penicillin, erythromycin, tetracycline, chloramphenicol, and streptomycin (9). Boxed numbers represent the percent resistance (in contrast to Fig. 1) to the indicated antimicrobial. Histograms in the first row are from a health care facility in the United States and with the exception of penicillin exhibit very low rates of resistance. Histograms in the second row are from a facility in a European country with remarkably high rates of resistance, in approximately half or more of the tested isolates for four of the five antimicrobials shown. (Reprinted from reference 9 with permission.)

from collaborating centers initially on handwritten report forms and subsequently on optical scan sheets mailed to Boston for processing and analysis. The results from the foreign center were strikingly different from those of the U.S. centers, suggesting that antimicrobial resistance was spreading irregularly through the world and needed to be tracked by laboratories worldwide (highlighted in Fig. 2) (7–12). Significant differences were also noted between resistance proportions in animal and human populations (13, 14).

The availability of personal computers (PCs) in the early 1980s offered new opportunities for international collaboration. The purchase for several foreign laboratories of IBM PCs as soon as they were introduced in 1982 allowed them to enter and analyze their own data and also to share computer diskettes with the Brigham and Women's Hospital in Boston for global data overviews. The software was written in GW-BASIC, and local customization of the tools was required to support the different patient location lists and antimicrobial test panels used at each site. A WHO workshop on surveillance of antimicrobial resistance was held in 1982 (15), and in 1985 in recognition of the accomplishments of the Boston group in international collaborations (16–21), WHO designated the center a WHO Collaborating Centre for the Surveillance of Antimicrobial Resistance (22).

WHONET

Significant reorganization and integration of the existing code base was initiated in 1989, and the name WHONET was given to the set of integrated functions. The first major advance was the introduction of a universal data file structure based on standardized required and optional data fields. Standardizing the data structure made it no longer necessary to maintain distinct code bases for each participating facility, thereby simplifying software dissemination, data management, and integrated data analysis. Compatible data files from any facility around the world using WHONET could be easily shared and analyzed together (23–27).

In parallel to the introduction of this universal data file standard, a new configuration module within WHONET was created that permitted laboratories to define their own sets of antimicrobial agents, antimicrobial test panels, antimicrobial interpretative criteria, patient locations, optional data fields, and microbiological alerts. For the first time, this permitted the widespread dissemination and independent use of WHONET by local and national data managers without reliance on the Boston WHO Center for software customization and maintenance. For priority national and international collaborations, optional data modules were added to facilitate participation in specific projects, including WHONET-Argentina, NORM-Vet (Veterinary component of the Norwegian Surveillance System for Antimicrobial Drug Resistance), EARSS (European Antimicrobial Resistance Surveillance System), and its successor EARS-Net (European Antimicrobial Resistance Surveillance Network).

Another significant advance during this period was the merging of the data entry and distinct data analysis modules (isolate listings, histogram, scatterplots, and resistance profiles) into a single code base in Microsoft QuickBASIC, permitting the reuse of common code among all modules. In the mid- to late 1990s, WHONET was migrated to the mouse-friendly VisualBasic for DOS (VB-DOS), Windows-compatible Microsoft Visual Basic 4, and subsequently to Microsoft Visual Basic 6.

BacLink

In the early 1990s, a growing number of laboratories, notably in high-resource countries, were acquiring mainframe and PC-based laboratory information systems to support local clinical reporting, specimen processing, and archival needs. Many were simplistic, maintained with simple desktop software such as Lotus 1-2-3, Microsoft Excel, Paradox, dBASE, FoxPro, and Microsoft Access. Others were more sophisticated, with local informatics technology (IT) teams responsible for system development and support.

In one respect, these local IT solutions were an obstacle to WHONET adoption: it is unreasonable to expect that laboratory staff should manually enter the same set of microbiological test findings into both a local information system and then again into WHONET. Such time-consuming and error-prone double-entry of test results is demotivating to staff and not sustainable in the long term.

In other respects, these local IT systems were an excellent opportunity for expanding WHONET use. Facilities had the resources and staff for local language-appropriate solutions customized for local reporting and specimen workflow needs. Yet these local systems typically had minimal capabilities for data analysis and offered no support for data sharing with other centers. If our group could develop tools for the capture and standardization to WHONET structure of disparate microbiology data stores, facilities would have the capabilities of their local systems customized for routine day-to-day needs with the added power of WHONET's analytical capabilities and opportunities for data sharing with other facilities with otherwise incompatible systems.

From 1989 through 1993, such data translation routines were customized from a common template for each new information store that we encountered in our major partners. While this was of great value to these specific institutions, such an approach would not have been sustainable in the long term for a large number of partners because this facility-specific customization could only be accomplished through specialized programming by the WHO Center in Boston.

By 1993, the data translation template had evolved into a generic set of routines which were able to standardize to the WHONET file structure from the most common data feed variations encountered in practice, including delimited text files, commercial database systems, and common laboratory instruments such as Vitek and Microscan. The addition of a user interface and local configuration files to the data translation template allowed the release of BacLink in 1994 using the mouse-friendly VB-DOS as the programming language. In subsequent years, BacLink was migrated to the Windows-compatible Microsoft Visual Basic 4 and Microsoft Visual Basic 6.

For local software developers and data managers, BacLink saved time. There was no need for local programmers to learn the intricacies of WHONET data fields, formats, and coding systems. Of even greater significance is that BacLink provided a user-friendly interface for configuring data file imports by facility staff without programming expertise, such as microbiologists, infection control staff, and epidemiologists. Thus, nonexperts without an advanced IT background now had a tool that allowed them to capture and standardize disparate data sources into simple and shareable WHONET files.

WHONET and the Web

The Web offers new opportunities for data management strategies, multicenter collaborations, and widespread secure or public access to queryable databases. The first version of WHONET for the Web was distributed as WHONET 2012, developed in Microsoft Visual Studio 2008 with Microsoft Visual Basic.NET and ASP.NET Web pages, with a special initial focus on the reporting needs of the Centers for Disease Control and Prevention (CDC) National Healthcare Safety Network (NHSN) initiative.

WHONET 2014 supports user-defined roles and secure logins, facility configuration, data entry and editing, and the queryable tabular and graphics display of WHONET analysis results. Expanding beyond the typical laboratory data feeds of older versions of WHONET, WHONET 2014 also supports the import of patient admission/discharge/transfer data to facilitate the tracking of patient movements into and within health care facilities.

Critical benefits of centralized Web-based data storage and management include:

- Centralized, secure data configuration, management, storage, backups, and virus protection by professional IT staff, often not available at the peripheral level
- Real-time national data collection and data feedback to participating facilities
- Real-time analyses, alerts, and action for outbreaks and other findings/strains of public health concern
- Simplified training of laboratory staff, who would still require training in data entry, analysis, and interpretation, but not in file management

WHONET USE

From a base of fewer than a dozen regular international collaborators in the 1970s and 1980s, WHONET use has expanded to support surveillance of resistance in over 2,300 hospital, public health, food, and veterinary laboratories in over 110 countries throughout the world (Fig. 3).

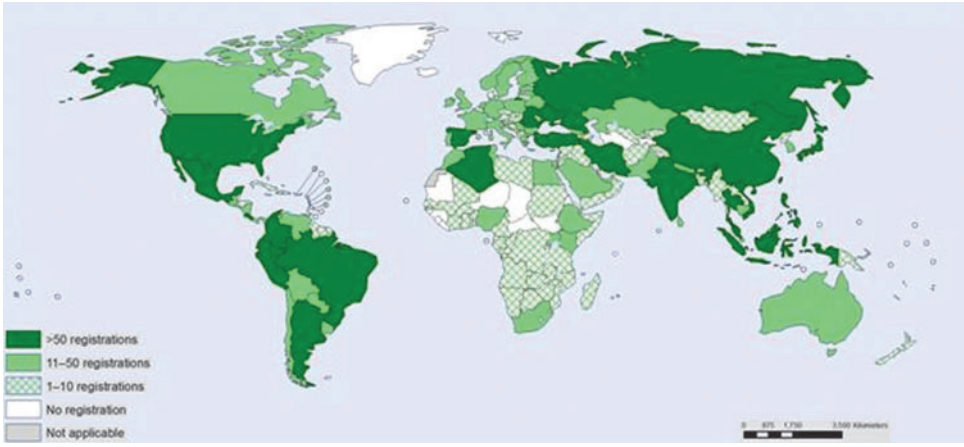

FIGURE 3 The number of individuals by country registered on the WHONET website as of July 2013. The total number of registrations worldwide at that time was approximately 5,800. Not all registered individuals are WHONET users, and not all WHONET users are registered on the WHONET website, but the map reflects well the relative use of WHONET by country worldwide.

From an initial focus on individual hospital-based laboratories, WHONET activities gradually shifted in the 1990s to subnational and national networks collaborating with or coordinated by ministries of health, reflecting a greater appreciation by national authorities of the health and economic burden attributable to antimicrobial resistance.

A new level of international collaboration was achieved in the late 1990s by the establishment of two regional initiatives for surveillance of antimicrobial resistance, both relying to a large degree on WHONET for data management: the Latin American Network for Surveillance and Monitoring of Antimicrobial Resistance (ReLAVRA, at present with over 600 laboratories in 14 countries) (28) and the European Antimicrobial Resistance Surveillance System (EARSS) (29) and its successor EARS-Net (30), with over 900 laboratories in 30 countries. A notable surveillance network with global scope is the Armed Forces Health Science Center–Global Emerging Infections Surveillance initiative (AFHSC-GEIS) network run by the U.S. Navy and Marine Corps Public Health Center, with nightly downloads into WHONET results from over 700 Army, Navy, Air Force, Marine, and Coast Guard laboratories worldwide (31).

At the hospital level, primary software users are microbiology laboratory staff, infection control practitioners, pharmacists, and infectious disease clinical specialists. Primary software uses at this level include identification of quality control deficiencies, clinical decision support and therapy guidelines, notification of possible outbreaks and new resistance threats, temporal trends in resistance emergence, characterization of resistance mechanisms, and clinical research.

At the national and regional levels, laboratory staff and clinicians are joined by public health epidemiologists, research scientists, and dedicated surveillance data managers. At this level, priority focus areas are laboratory capacity-building through targeted feedback reports to participating facilities, national standard treatment guidelines, benchmarking comparisons of facilities by resistance rates and infection patterns, and monitoring of the geographic distribution and spread of important pathogens, including the detection of multifacility pathogen outbreaks. References for national and regional publications and national reports on the software use are available on the WHONET website (www.whonet.org).

Looking to the future, there are new opportunities for regional and global collaborations in the surveillance of antimicrobial resistance. We are within reach of a truly global system for collaborative surveillance of antimicrobial resistance (33). Two of the six WHO regions (Pan American Health Organization [PAHO] [28] and WHO Regional Office for Europe [EURO] [29, 30]) have long-standing programs for regional surveillance of antimicrobial resistance, and three additional WHO regions (WHO Regional Office for the Eastern Mediterranean [EMRO] [34], WHO Regional Office for South-East Asia [SEARO] [35], and WHO Regional Office for the Western Pacific [WPRO] [36]) made significant progress in this direction in 2014. The WHO Regional Office for Africa (AFRO) is working to build the needed local and national capacity to support clinical diagnostic, research, and surveillance activities (37).

In the area of external quality assurance, two WHO-affiliated external quality assurance programs (38, 39) have closed, but five of six WHO regions—PAHO (28), EURO (29, 30), EMRO (40), AFRO (41), and the Pacific Island states of WPRO (pptc.org.nz)—have maintained long-standing external quality assurance programs for routine bacterial identification and antimicrobial susceptibility testing. The remaining two WHO Regions (SEARO and non–Pacific Island Member States of WPRO) are in the process of establishing such programs.

In May 2014, the World Health Assembly passed a resolution (42) calling for a Global Action Plan on containment of antimicrobial resistance to be developed by WHO and presented at the 2015 World Health Assembly meeting, including an action plan for surveillance of antimicrobial resistance. Important components of this strategy will be (i) building on the significant national and regional accomplishments already achieved and coordinating these into a global platform for surveillance collaboration and (ii) strengthening laboratory, epidemiological, and IT capacity and activities in WHO member states where these are currently lacking. Because WHONET is recommended for use in a number of WHO materials (43–46), our center will actively support the WHO Global Action Plan through training, technical support, strategic guidance, and software tools for Web-based data entry and collaboration.

WHONET MODULES

The daily routine results generated by microbiology laboratories around the world constitute a rich, yet largely untapped, window into complex and evolving microbial populations worldwide. The underlying mission of our group has been the provision and support of information management tools for capturing, sharing, and understanding these test results to support patient management strategies and public health interventions.

To achieve this mission, these tools must be reliable, secure, user-friendly, configurable, and relevant to the evolving expectations and needs of diverse software users. Since the mid-1960s, we have endeavored to accomplish this, incorporating new possibilities permitted by ongoing advances in information technology. WHONET can be downloaded free of charge from the WHONET website, www.whonet.org. Core WHONET functional modules are laboratory configuration, data entry and data file management, and data analysis.

Laboratory Configuration Module

The data management needs of microbiology laboratories around the world are similar in many respects but differ in others. The WHONET laboratory configuration module permits the flexible customization of the software for the greatest suitability for each laboratory.

- Laboratory identification: The user indicates the country, laboratory name, and laboratory code.
- Antimicrobials: Antimicrobials tested by the laboratory, susceptibility test methods (disk diffusion, MIC determination, and Etest), organism group-specific test panels, and selective reporting rules. WHONET supports the antimicrobial interpretive breakpoints published by the U.S. Clinical and Laboratory Standards Institute (CLSI), the European Committee on Antimicrobial Susceptibility Testing (EUCAST), the Comité de l'Antibiogramme de la Société Française de Microbiologie (CA-SFM), and the British Society of Chemistry (BSAC), among others. Users can manually edit interpretive criteria as needed for local applications.

- Patient care areas and sample collection locations: Software users enter in a locally relevant list of hospital and outpatient locations. For veterinary, food, and environmental laboratories, this list may also include farms, markets, water sources, and other relevant sample collection areas. In addition to specific local location descriptors, users can indicate a medical department (such as medicine or surgery) and location type (such as intensive care unit, farm, or restaurant) to facilitate data sharing and standardized analysis.
- Data fields: When the user selects "New laboratory," WHONET creates a laboratory configuration populated with a standard list of patient, location, specimen, and microbiological data fields commonly requested by routine clinical laboratories. The user is free to customize this list by removing unneeded fields or incorporating additional data elements (patient demographic or clinical details, specialized microbiological testing, research findings, veterinary and food product information, user-defined fields, EARS-Net, WHONET-Argentina, etc.) selected from a number of optional data modules.
- Microbiological alerts: WHONET includes a list of over 180 predefined microbiological alert rules about important, unlikely, and infrequent test results to aid laboratory staff in identifying strains of public health concern that merit local or national action, such as confirmation, investigation, and containment. Alerts of unlikely or infrequent resistance phenotypes, such as vancomycin-resistant *Staphylococcus aureus*, may also suggest deficiencies in laboratory test performance or reagent quality. In addition to the predefined WHONET rules, software users can also define new rules of local or national priority.

Data File Management and Clinical Reporting Module

WHONET includes a number of functions related to the creation, editing, and manipulation of data files, including the following.

- Data entry and editing: After configuring a new laboratory, the software user begins entering sample details and test results through the WHONET data entry module. WHONET provides immediate feedback to data entry staff personnel on important strain phenotypes or possible testing errors. Facilities with a local laboratory information management system typically do not use the WHONET data entry module, but rather import their data into WHONET periodically (daily, monthly, end-of-project, etc.) using the BacLink software described below.
- Combine or export data files: This function serves two purposes that can be used together.
  - o Combine separate WHONET files into a single, larger file, for example, merging 12 monthly files into a single yearly file or files from 20 separate WHONET facilities into a single national database. Though combining files is not required by the WHONET analysis modules, which can integrate thousands of separate files into a single analysis, some users find that combining files facilitates data management.
  - o Export WHONET files to standard file formats defined by a number of projects in which WHONET users participate including EARSS, EARS-Net, Global Foodborne Infections Network (GFN), and CDC-defined Electronic Laboratory Reporting of notifiable results.
- Encrypt data files: To facilitate collaborations with partners, WHONET permits the creation of data file copies in which confidential information such as patient names, date of birth, and other patient-identifiable information is removed.
- Clinical reporting: Many laboratories without a comprehensive laboratory information system use WHONET to generate configurable reports with patient sample results to be shared with clinicians.

Regarding antimicrobial susceptibility test results, WHONET permits the entry of either quantitative test results (disk diffusion zone diameters and MIC values) or qualitative test interpretations (resistant "R," intermediate "I," or susceptible "S"). For reasons elaborated below, the WHONET Center strongly recommends the entry of quantitative findings, which facilitates systematic assessment of laboratory test performance and enriches the epidemiological and public health value of data analyses. The scientific value of data resources is greatly compromised if only simple R, I, and S test results are available.

Data Analysis Module

The most important use of WHONET is the analysis and exploration of microbiological findings with a special focus on the management and interpretation of antimicrobial susceptibility test results. Applications include:

- Characterization of the local and regional epidemiology of evolving microbial populations
- Characterization of resistance mechanisms and multidrug resistance linkage
- Clinical decision support and the development of standard treatment guidelines
- Timely notification of new threats including hospital and community outbreaks and novel strains and resistance determinants of public health concern
- Recognition of deficiencies in laboratory test performance and reagent quality
- Evaluation of antimicrobial resistance containment interventions including education, infection prevention measures, and the implementation of antimicrobial stewardship programs and activities
- Benchmarking/comparisons of threats and challenges
- Quantifying risk factors for resistance selection, assessing the impact of *in vitro* documented resistance on clinical outcome, and estimating the burden of disease attributable to resistance
- Alerting laboratories to predesignated types of isolates to be saved for special testing

WHONET ANALYSIS FEATURES

WHONET offers a user-friendly interface to support flexible and interactive data exploration, as illustrated in Fig. 4. The user selects (i) the type of analysis to perform, (ii) organisms to study and (iii) data files to be included. For additional flexibility in selecting data subsets, the user may also click on "Isolates" to further filter data subsets on the basis of any available WHONET data field, e.g., blood isolates from the nursery resistant to imipenem. The feature "One per patient" allows a configurable definition of

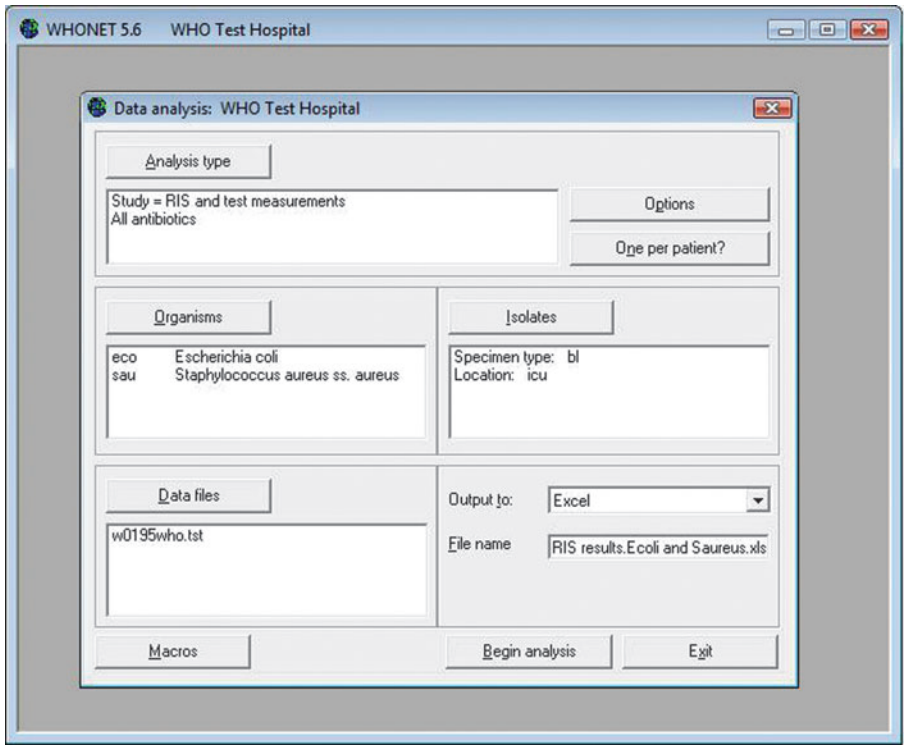

FIGURE 4 The WHONET data analysis control screen in which the user selects the type of analysis, organisms, and data files to include and isolate filters. In this example, the user has selected the %RIS and test measurements analysis for *E. coli* and *S. aureus* from blood isolates collected in the intensive care unit for a January 1995 data file. Results are to be exported to an Excel file with the name provided.

"repeat isolates" to address bias introduced when multiple clinical samples are taken from individual patients.

The principal types of analysis are described in the following sections. Analysis results can simply be copied and pasted or exported to other applications, such as Microsoft Excel, Microsoft Access, or Microsoft PowerPoint, and the WHONET "Macros" and "Report" features allow users to save query parameters for later reuse, batch processing, and scheduled automated (e.g., daily or weekly) analysis.

Isolate Listings and Frequency Distributions

The simplest WHONET analysis option, yet one of the most commonly requested by laboratory and infection control staff, is the creation of detailed listings of test results of patients or isolates that meet certain criteria, for example, a weekly list of patients with multidrug-resistant *S. aureus* or positive blood cultures from the neonatal intensive care unit. By activating the "Isolate alerts" option, the listing is enriched with text commentary and red highlights for important clinical or public health findings meriting notification, confirmation, epidemiological investigation, and where appropriate, patient isolation, as well as possible errors in laboratory test performance.

The "Isolate listing summary" feature permits the tabulation of results stratified by up to three row variables and one column variable, for example "Organism by month," "Organism by patient location by month," and "Facility by age group by specimen type." This analysis is similar to a classic statistical "cross-tabs" analysis except that the default unit of study is "Number of patients" rather than "Number of isolates/records." If the column variable represents a date field, such as specimen collection date, then WHONET permits the user to select the "Cluster alerts" option for the detection of statistical clusters of results suggestive of a possible outbreak.

Antimicrobial Susceptibility Test Statistics

The most common format for presenting susceptibility test results to clinicians, pharmacists, and health authorities is the percentage of tested first patient isolates found to be resistant (R), intermediate (I), or susceptible (S) to a given antimicrobial agent, as described by CLSI (47, 48). WHONET offers two output formats for this analysis:

- Detailed report: WHONET generates one output page per species including breakpoints, %R, %I, and %S, zone diameter and MIC distributions, and ancillary MIC statistics (MIC range and geometric mean, MIC_{50}, and MIC_{90}). This format is especially useful to laboratory personnel and researchers requiring this level of result detail.
- Summary report: WHONET provides a compact summary of priority findings (for example %S or %R) with multiple organisms in the output table, a format more suitable for distribution to clinicians, pharmacists, drug and therapeutics committees, and health authorities.

For both output formats, results may be stratified by date (e.g., year-by-year trends, location type, facility) to permit the monitoring of trends over time or benchmarking the experience of individual facilities within a national laboratory network.

Frequency Distributions of Susceptibility Test Measurements

The two traditional methods of susceptibility testing—disk diffusion testing and MIC determination—recognize that microorganism susceptibility to antimicrobials lies along a continuum. Most strains without resistance genes are observed to have typical wild-type susceptibility, while a few may exhibit hyper-susceptibility. Organisms possessing genetic determinants for resistance may exhibit decreased susceptibility to an antimicrobial agent (which may or may not impact expected clinical outcome), moderate-level resistance (the antimicrobial inhibits growth of the microbe to a degree but not sufficiently to ensure successful therapy), or high-level resistance (for the concentrations tested, no inhibition of microbial growth is observed). Figures 1 and 2, above, display the frequency distribution of disk diffusion zone diameters in the form of a histogram, highlighting different levels of resistance consistent with distinct genetic determinants.

As stressed earlier in the description of WHONET data entry, it is strongly recommended that laboratories record quantitative test results (zones of inhibition and MIC values) into WHONET data files.

- Data quality: Simply put, quantitative test measurements should be recorded because it is the correct way to do the test. If measurements are not systematically recorded, there is a tendency for laboratory staff to visually estimate ("eyeball") both test measurements (notably for disk diffusion zone diameters) and their interpretations. Given the marked differences in breakpoints between antimicrobial agents and organisms and the fact that breakpoint criteria may change over time, it is highly probable that laboratory staff will frequently provide incorrect test results to clinicians if measurements are not systematically recorded with computer-based interpretation.
- Assessment of data quality: Two common strategies for assessing laboratory test performance and reagent quality are (i) the frequent use of well-characterized standard test strains such as *Escherichia coli* ATCC 25922 and (ii) participation in external quality assurance programs. While these approaches permit an assessment of a laboratory's capabilities under special test circumstances, they do not necessarily reflect how well laboratories perform with routine clinical samples. With regard to assessing the reliability of antimicrobial susceptibility test results, disk diffusion and MIC measurement frequency distributions can be utilized effectively to explore problems in test performance and reagent quality including incorrect or inconsistent disk potency, consistency of test inoculum, pH and cation concentrations of test media, and biases in the recording of measurements. For laboratories reporting measurements, a systematic assessment of many aspects of test performance is possible. For laboratories reporting only R, I, and S results, it may be impossible to distinguish between laboratories with excellent test performance and those with unreliable results.
- Breakpoint changes over time: Official reference breakpoints do not change frequently over time, but when changes are announced, it is typically for scenarios of great clinical and public health importance, such as the recent revisions in CLSI breakpoints for carbapenems and *Enterobacteriaceae* or past modifications in vancomycin breakpoints for *Enterococcus* spp. and *Staphylococcus* spp. and beta-lactam breakpoints for *Streptococcus pneumoniae*. If only R, I, and S results are recorded, it is not possible to do a reliable comparison of temporal trends in resistance before and after the breakpoint changes.
- Mechanisms of resistance: The degree of resistance (decreased susceptibility, moderate-level resistance, and high-level resistance) assists in identifying resistance mechanism candidates, for example, some beta-lactamase-deactivating enzymes confer complete resistance to certain antimicrobial agents, whereas others confer only moderate decreases in susceptibility. Similarly, the degree of resistance to fluoroquinolones often reflects the number of gene mutations in the DNA gyrase or topoisomerase IV loci (49–52).
- Epidemiological markers for microbial strains: Even without knowing the specific mechanism of resistance, quantitative test measurements are of great value to infection control staff and public health epidemiologists in recognizing and tracking distinct microbial subpopulations. A practical application of reviewing the resistance profiles is the prompt identification of possible outbreaks of multiresistant organisms, which can then be followed by epidemiologic investigations.

Antimicrobial Test Scatterplots

While the analysis formats presented above focus on individual antimicrobial agents and their results, WHONET scatterplots permit characterization of cross-resistance and correlation between two antimicrobial agents, as in Fig. 5. Specific applications of this analysis include:

- Evaluation of treatment alternatives: In addition to their value in studies of molecular epidemiology, studies of cross-resistance are useful to pharmacists and clinicians for developing policies for the use of first-line and second-line treatment alternatives based on resistance profiles.
- Comparisons of results: Comparing results for antimicrobial agents within a class or subclass can provide insights into the potential mechanisms of resistance and linked resistance between two subclasses of antimicrobial agents.
- Epidemiological strain markers: Application as an epidemiological strain marker for infection control to identify clones causing hospital-acquired infections.
- Identification of quality assurance deficiencies: Discrepancies in test findings between similar antimicrobial agents may suggest errors in laboratory test performance or reagent quality. For example, an isolate of *E. coli* that was reported to be "resistant" to ciprofloxacin yet "susceptible" to nalidixic acid would be an unexpected finding and most likely due to a laboratory error.

Antimicrobial Multidrug Resistance Profiles

The emergence of multidrug-resistant organisms is increasingly recognized as a unique threat with important clinical and public health consequences. While resistance to a single or to a few antimicrobial agents poses an important diagnostic dilemma to the clinician (Which antimicrobials must the physician avoid to have a successful clinical outcome?) multidrug resistance poses a threat of even greater magnitude, which cannot be resolved through laboratory

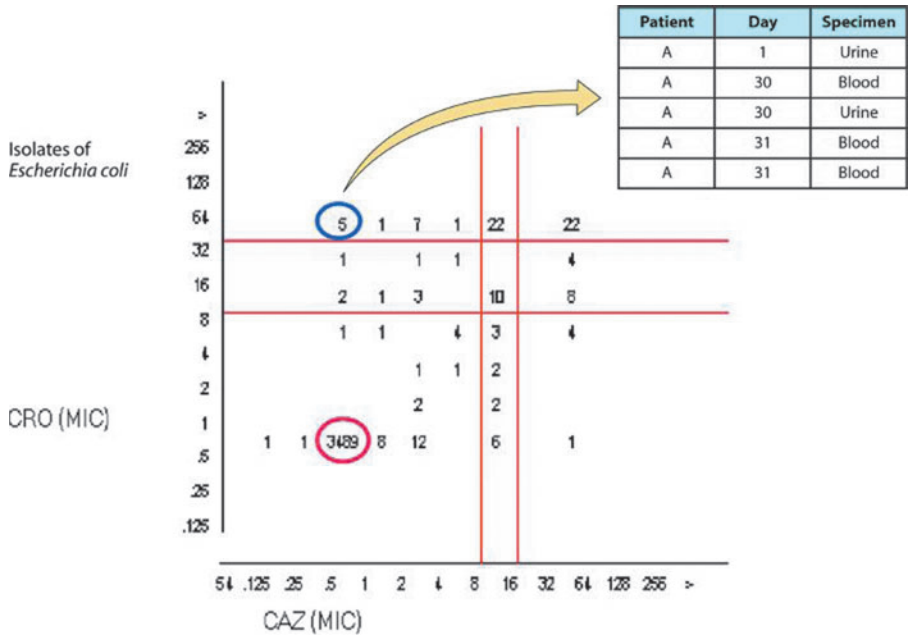

FIGURE 5 WHONET scatterplot of MICs of ceftriaxone (CRO) and of ceftazidime (CAZ) for all isolates of *Escherichia coli* at one hospital during 1 year. The circle in the lower left corner encloses the 3,489 isolates that had MICs of 0.5 μg/ml (susceptible) for both agents. The circle in the upper left corner encloses the five isolates that had an MIC of 0.5 μg/ml for ceftazidime (susceptible) and an MIC of 64 μg/ml for ceftriaxone (resistant). All five isolates were from patient A, who, as shown in the inserted table, had initially had a single urine and a month later urine and blood isolates with that unique-for-the-year combination of MICs. This patient had received a kidney transplant 3 months earlier on another continent, which illustrates how the systematic screening of phenotypes may detect the incursion of a foreign strain. (Reprinted from reference 66 with permission.)

testing: the risk that no commercially available (or locally feasible) therapeutic option may exist.

WHONET permits the systematic characterization of microbial strains on the basis of resistance phenotype using the "resistance profile" analysis, highlighted in Figs. 6 and 7. Users may classify strains on the basis of their susceptibility to all antimicrobial agents tested or to a select subset of greatest value in strain discrimination. The WHONET group has used this approach, i.e., correlating observed resistance phenotypes with confirmed genetic determinants of resistance, in tracking the geographic distribution and temporal trends of microbial "phenotypic clones" and in the detection and confirmation of community and hospital outbreaks. A new WHONET software feature for geo-referenced data permits the creation of KML files compatible with Google Earth, as shown in Fig. 8.

Isolate Alerts

WHONET offers two alert features to support the recognition of important, unlikely, or distinctive resistance characteristics. These alert features are available through WHONET data entry (users are notified immediately of important findings), the WHONET "isolate listing" analysis, and the WHONET "isolate alert" analysis.

- Microbiological rules: WHONET includes a list of over 180 fixed, predefined rules drawn from a number of sources, including microbiology textbooks, EUCAST materials, and the CLSI M100 table "Appendix A. Suggestions for Verification of Antimicrobial Susceptibility Test Results and Confirmation of Organism Identification" (53). WHONET rule categories include "important species" (e.g., *N. meningitidis*), "important resistance" (e.g., carbapenem-resistant *Enterobacteriaceae*), "quality control alert" (e.g., ciprofloxacin-resistant plus nalidixic acid–susceptible"), "inform infection control" (e.g., methicillin-resistant *S. aureus*), "send to a reference laboratory" (e.g., vancomycin-resistant *S. aureus*), and "therapy comment" (e.g., aminoglycosides should not be used in the treatment of *Salmonella* spp. infections). Facilities may also create their own user-defined rules for local alert notification needs.
- Frequency-based rules: In this approach, WHONET will provide alerts for isolates with findings of low frequency as defined by the facility's own historical data. For example, gentamicin-resistant *E. coli* is very common in many parts of the world (for example, in hospital A), but rare in others (for example, in hospital B). With a frequency-based approach, hospital B, but not hospital A, will receive an alert for any new isolate of *E. coli* resistant to gentamicin. This frequency-based approach is auto-configured based on historical findings and applies automatically to all antimicrobials and species tested. This approach is simple to implement and automatically provides locally relevant commentary based on prior findings.

Cluster Alerts

Since its earliest versions, WHONET has permitted the detection of possible hospital and community outbreaks

HOSP	Location	Specimen date	Specimen type	AMP	CEP	IPM	FOX	CTX	CAZ	SXT	GEN	AMK	CIP	TZP
A	uro	12/8/2007	Urine	6	17	22	17	24	19	6	14	14	6	17
B	out	12/21/2007	Urine	6	10	30	19	30	24	6	6	12	6	18
C	med	1/5/2008	Urine	6	10	27	17	30	24	6	6	14	6	17
D	uro	1/16/2008	Urine	6	16	28	22	30	25	6	6	14	6	15
D	rehab	1/16/2008	Urine	6	6	27	18	30	24	6	6	16	6	14
D	med	2/12/2008	Urine	6	6	29	20	32	24	6	6	16	6	19
E	uro	2/13/2008	Urine	6	6	25	14	23	22	6	6	13	6	12
F	sur	4/30/2008	Urine	6	14	30	18	34	27	6	8	10	6	19
G	out	5/13/2008	Urine	6	6	29	13	28	25	6	6	16	6	14
A	id	6/5/2008	Bood	6	18	22	20	23	22	9	6	13	7	19
H	med	6/12/2008	Urine	6	12	30	16	30	26	6	6	15	6	18
B	id	10/7/2008	Urine	6	20	22	20	26	24	10	6	15	6	18
C	gyn	12/20/2008	Urine	6	6	24	20	29	24	6	6	15	6	14
E	uro	12/30/2008	Urine		6	25	20	26	28	6	6	16	6	14
H	icu	1/4/2009	Blood	6	11	24	22	28	24	6	6	6	12	18
I	med	1/8/2009	Wound	6	6	30	20	26	25	8	6	16	6	15
I	icu	3/20/2009	Urine	6	6	28	16	31	19	6	6	6	6	14
J	obs	3/28/2009	Skin	6	10	27	21	28	20	11	6	16	6	15
G	med	3/31/2009	Urine	6	6	26	20	31	18	6	6	16	6	9
K	icu	6/5/2009	CSF	6	6	26	18	28	20	15	6	13	19	16
L	id	7/1/2009	Urine	6	6	25	21	25	20	6	6	8	6	18
M	sur	10/24/2009	Urine	6	12	29	15	24	23	13	6	16	6	17
G	med	1/21/2010	Urine	6	13	28	20	30	28	6	6	15	6	18
G	icu	2/6/2010	Urine	6	6	27	19	30	27	11	6	14	6	13
N	out	2/19/2010	Urine	6	6	30	21	31	28	6	6	16	6	18
O	out	6/16/2010	Urine	6	8	33	18	30	33	6	6	6	6	20
P	sur	8/7/2010	Abdominal	6	8	30	15	29	22	6	6	15	6	13
Q	obs	8/10/2010	Skin	6	9	30	20	33	25	6	6	15	6	20
R	id	8/24/2010	Urine	6	20	30	20	30	20	6	6	15	6	20
S	sur	11/3/2010	Urine	6	6	24	17	27	20	6	6	14	6	15
S	sur	11/23/2010	Urine	6	6	27	17	27	20	6	6	15	6	15
S	icu	11/30/2010	Urine	6	6	27	17	29	24	6	6	15	6	14
S	sur	12/31/2010	Urine	6	6	29	18	30	30	6	6	13	6	16
S	icu	2/2/2011	Blood	6	6	26	16	29	22	6	6	9	6	16
S	icu	2/19/2011	Blood	6	6	24	17	27	23	6	6	14	6	16
S	icu	3/5/2011	Urine	6	6	22	16	29	21	6	6	14	6	14
S	icu	3/5/2011	Urine	6	6	26	18	29	23	6	6	15	6	16
S	icu	3/7/2011	Urine	6	9	25	16	28	22	6	6	15	6	17
S	out	5/9/2011	Urine	6	8	26	18	28	22	6	6	15	6	16
S	icu	6/3/2011	Blood	6	6	29	18	39	24	6	6	15	6	18

FIGURE 6 Antimicrobial resistance phenotype subtyping and tracking in space and time. Disk diffusion zone diameters (mm) around susceptibility test disks are listed in columns for each of 10 antibiotics tested at 76 medical centers using WHONET in Argentina. Dates have been modified from the original data submitted. Test results represent all 40 isolates of *Klebsiella pneumoniae* from 2008 to 2011 with a particular antibiotype. These isolates are highly resistant, susceptible to only IPM, CTX, and CAZ. Of the 76 medical centers, this phenotype was seen in only 19 centers from 12 provinces. Until November 2011, most facilities had only a single patient with this phenotype, and at most three. Then from November 2010 through June 2011, this phenotype was only seen in a single facility: hospital S, with 11 patients in an 8-month period, primarily in ICU patients. This variation in time and geographic distribution is striking and highlights the heterogeneity and poorly appreciated epidemiology of strain subpopulations, as well as opportunities for prevention of spread with prompt notifications in real time to health care providers, infection control staff, and public health authorities.

through visual examination of temporal trends in organism, resistance phenotype, and location frequencies. But this type of manual, *ad hoc* review of a few priority organisms is not practical for real-time detection of and response to outbreaks.

In 2006, the WHONET center integrated into WHONET software the free software SaTScan for Monte Carlo simulation-based detection of "statistical clusters," as displayed in Fig. 7. A number of publications have validated this approach for the timely, sensitive, and specific detection of statistical case clusters meriting investigation as possible outbreaks. We have explored cluster detection at facility, network, and national levels using species identification, multidrug resistance phenotype, patient ward/clinic, patient medical service, serotype, and facility latitude and longitude. Among these variables, we have consistently found resistance phenotype to be of greatest value in defining microbial "phenotypic clones" suitable for SaTScan tracking (54–57).

In addition to the SaTScan Space-Time Permutation Model of cluster detection, we have also implemented the SaTScan Poisson Model, the CDC-defined cumulative

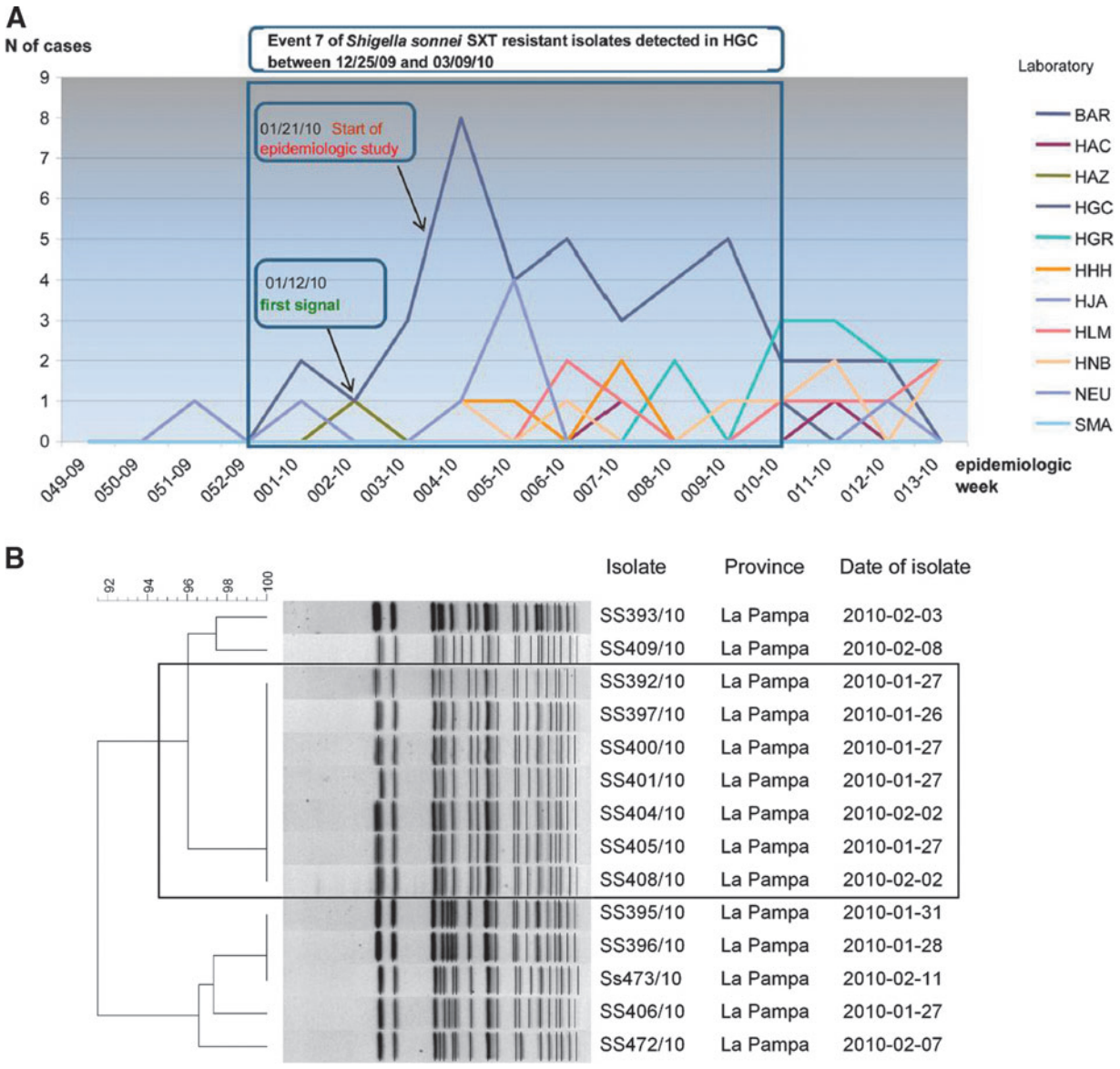

FIGURE 7 Collaborators in WHONET-Argentina utilized the SaTScan features in WHONET for real-time (weekly) detection of possible outbreaks of shigellosis (56). (A) One such confirmed outbreak of *Shigella sonnei* nonsusceptible to trimethoprim/sulfamethoxazole, detected using antimicrobial resistance phenotypes as a strain marker, is presented, highlighting detection and investigation of the event associated with hospital HGC. (B) Strains were collected for PFGE typing, verifying the clonality of the strains associated with the cluster period. (Reprinted from reference 56 with permission.)

sum "CUSUM" algorithms, and exponential weighted moving averages (58).

FUTURE DEVELOPMENTS

Since 1966, our goal has been the development of informatics tools to track and understand the appearance, movement, and evolution of emerging microbial threats and to guide interventions to contain them. We have developed tools for capturing data, statistical algorithms for managing them, and user interfaces to display them. Since those early years, both the laboratory technologies for recognizing microbial subpopulations and informatics tools for tracking them have advanced enormously.

As we look to the future, we are integrating advances both in laboratory sciences and in information technology into WHONET's data management and analysis capabilities including data mining approaches, outbreak algorithms, web-based phenotype libraries, and map interfaces to support the recognition, monitoring, and containment of emerging threats.

WHONET and Microbial Subtyping

For over a century microbiology laboratories have been identifying microbes they find in patients to genus/species categories. Those categories are usually broad, but they infer corresponding clinical syndromes well enough to inform management of the patients. Our group realized years

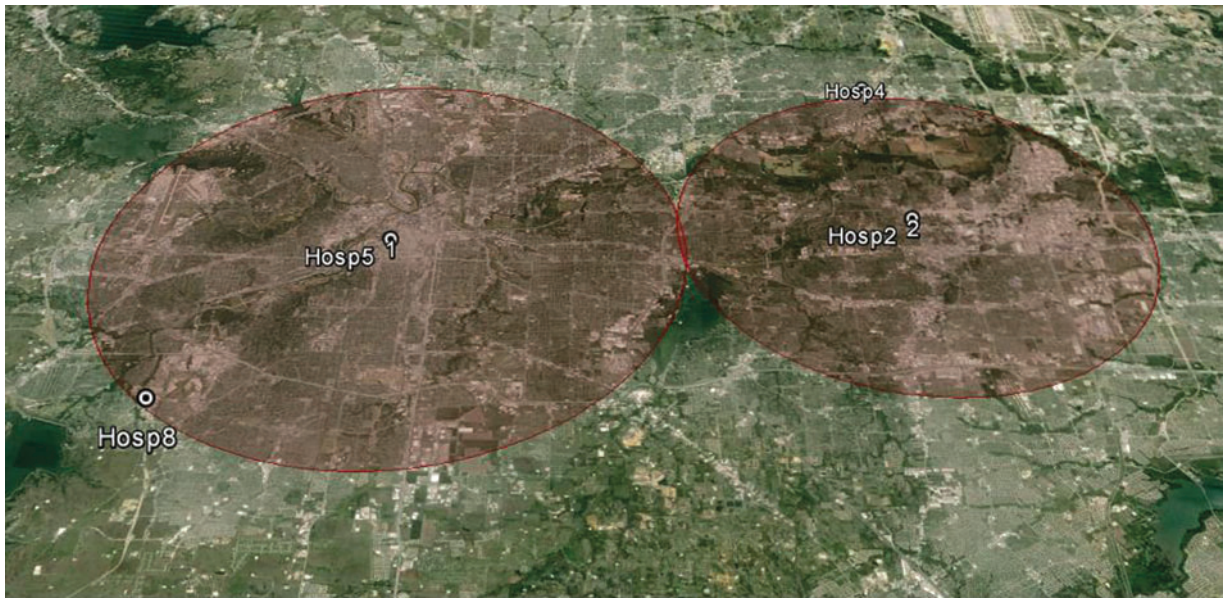

FIGURE 8 The most recent version of WHONET takes advantage of the new feature in SaTScan to generate Google Earth-compatible KML files. The map indicates the location of two statistical clusters identified by SaTScan, each of which involves two health care facilities.

ago that resistance phenotypes can be used to further discriminate subtypes within these genus/species categories to further enhance detection and tracking of microbial events, outbreaks, and epidemics, as described above. We have also explored the use of routine biochemical phenotypes from laboratory instruments such as Microscan and Vitek for this purpose, as illustrated in Fig. 9 (59).

As we look to the future, new instruments based on molecular approaches are already rapidly transforming traditional microbiology practices developed over the past century. Within our software development plan, we are currently looking at two of these technologies in detail:

- MALDI-TOF (matrix-assisted laser desorption ionization–time of flight): MALDI-TOF is a new mass spectrometry–based method for detecting ribosomal and DNA-binding proteins, and it has the remarkable ability to determine organism species identification from a pure culture within minutes, with low reagent costs (60–62). Given the significant initial expense (but

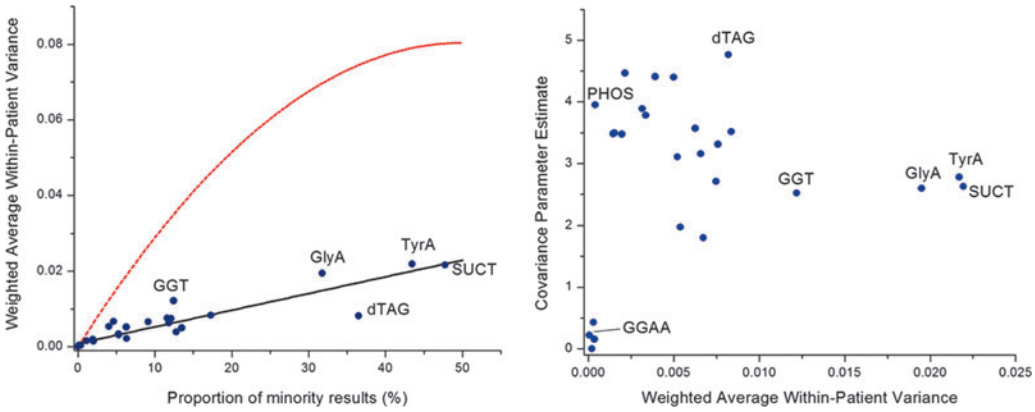

FIGURE 9 From a series of patients with multiple isolates of *Klebsiella pneumoniae* per patient tested against a standard set of 47 Vitek biochemicals, the left-hand graph compares the observed weighted average within-patient variance (lower points with black regression line) against the theoretical weighted average within-patient variance (upper red line) if there were no correlation between results of individual patient isolates. The right-hand graph plots the covariance parameter estimate obtained by generalized linear mixed-model variance component analysis using the weighted average within-patient variance (58). In both analyses, GlyA, TyrA, and SUCT stand out as "nuisance" variables, exhibiting relatively higher levels of variability (less reproducibility) than other biochemicals. By excluding such variables from WHONET analyses, we have demonstrated improved ability to recognize phenotypic clonal populations and improved detection of possible outbreaks. (Reprinted from reference 58 with permission.)

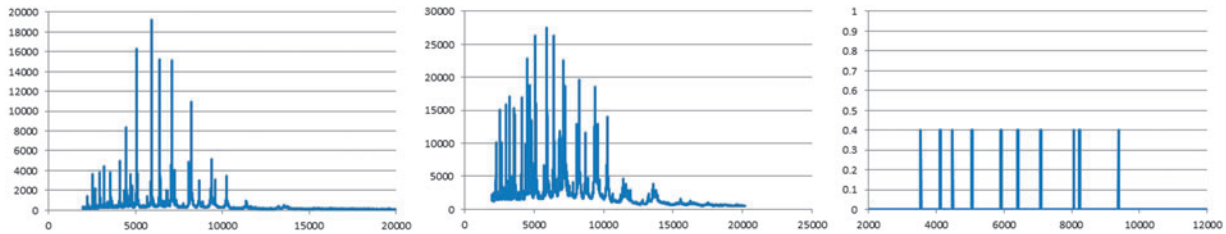

FIGURE 10 The first two graphs display unprocessed MALDI-TOF signal spectra (horizontal axis is molecular mass, vertical axis is number of ions) obtained from the Bruker Biotyper from two isolates of *Neisseria gonorrhoeae* from a WHONET-Argentina laboratory, highlighting significant differences between the two strains with regard to both peak numbers and peak heights. The third graph indicates a set of major "consensus" peaks common to both signal profiles, suggesting a set of signal peaks that may be uniquely indicative or probabilistically suggestive of *N. gonorrhoeae*. If one were to focus on the unique peaks of distinct strains, it may be possible to ascertain the clonality and phylogenetic relatedness of strains in real time.

practically negligible ongoing cost for materials), the rapid adoption of MALDI-TOF instruments, even in low-resource countries, is remarkable. The complex signal profiles MALDI-TOF instruments display appear to have considerable potential for real-time subtyping of routine clinical isolates (Fig. 10).

- Next-generation sequencing, including whole-genome sequencing: Efficient sequencing technologies are advancing rapidly, instrument and reagent costs are falling, and tools for genome management and annotation are maturing to a degree that it is now making it feasible for non–bioinformatics experts to start exploring the application of sequencing methods to public health and clinical applications (63): near real-time confirmation and monitoring of nosocomial outbreaks, prediction of antimicrobial resistance phenotypes, "virtual" multiplex PCR through detection of any set of predefined genes (resistance, virulence, species identification), new gene discovery, and phylogenetic classification and exploration of gene mutations and transfers.

Developing a Global Microbial Sensor Network

In recent years many examples have been developed of what have come to be called "electronic sensor networks." Each deploys multiple copies of some type of sensor device throughout a region, or even over large areas of the world, and transmits signals from them electronically to a central database. Examples of such sensors range from ocean buoys that measure ocean currents and water temperatures to land-based weather-recording instruments to bar-code readers recording inventory in a supply-chain network. Sensors in remote parts of the world can transmit signals by satellite.

Incoming signals from each type of sensor often need translations specific for that type into messages that convey whatever information is needed by those responding to that sensor's signals. Some sensor systems have also predesignated who should respond to what type of signal and will even automatically notify such predesignated responders. Large amounts of information collected over vast areas may thus be sifted through in real time, now often by evolving algorithms, to discern impending problems and bring them to the immediate attention of those best positioned to deal with them. Such systems may also have the capacity to discover problems not otherwise recognized.

The application of this sensor network model to the discernment and tracking of infecting microbes and their antimicrobial resistance elements now seems both possible and opportune (32, 64–66). The sensors for such a network are already in place. A microbiology laboratory is an exquisitely discerning sensor of pathogens and their antimicrobial resistance phenotypes, and many thousands of such laboratories are already in place around the world. Each of these laboratories adds new reported pathogens and their resistance phenotypes to its growing files each day.

The main obstacle to linking most of these laboratories into a global microbial sensor network is that the files of their reported pathogens are now inaccessible, either still on paper or in diverse electronic laboratory information systems that are not interoperable. The use of WHONET, however, has now circumvented this obstacle in a growing subset of those laboratories. Those laboratories have electronic WHONET files of their reported infecting microbes either directly entered or translated from their laboratory information systems by WHONET's data conversion utility, BacLink, and all the WHONET files are interoperable. More than 2,500 microbiology laboratories in more than 100 countries thus have files that can now be linked into the database that is needed to initiate a microbial sensor network.

Besides linking these files, a microbial sensor network also needs to develop algorithms to monitor the files and systems to send specific predesignated responders particular alerts generated by the algorithms. Integration of these components can be facilitated by experience from their prior use separately, e.g., SaTScan in WHONET detecting clusters, monitoring of phenotype prevalence, etc. but can be expected also to need successive review and revision in practice. The same is true for issuing alerts. An excess of a specific resistance phenotype at a medical center should alert its infection preventionists of a problem. If the same resistance phenotype is detected at a second medical center potentially in the same region, then not only its infection preventionists should be notified but so should a predesignated responder at a state or national facility.

A global microbial sensor network could be envisioned not only as an advance enabled by widespread use of WHONET but also as a potential fix for WHONET's two major limitations: underanalysis and late analysis, its analyses being largely operator-driven and rarely in near real time. WHONET users who find time to analyze their files (operators) with all the system's types of analyses often produce significant findings, but few of the existing WHONET files can be this fully analyzed, and fewer in time to inform patient reports or meet the now-growing need to

save specific isolates for further molecular testing. Automated processing of large-scale integrated data by progressively refined sets of algorithms can increasingly relate each laboratory's report to prior reports of all laboratories. Doing this in near real time could automate each laboratory's prompt response to critical data. More importantly, it could automate a laboratory's adding to any report to a caregiver contextual commentary on how the particular pathogen being reported relates to all the world's others.

For advancing the work described here, we acknowledge the significant contributions from 1966 through 1988, as advisors or technologists or as software filers or developers, of Antone A. Medeiros, Richard A. Norton, Ralph Kent, Margaret McLaughlin, Arthur Gershkoff, Richard Hogaboom, Susan Servais, John J. Farrell, Lee Chao, and Jiang Hua. For their contributions to WHONET development and support from 1989 on, we value the contributions of Christopher Tullen, Manuel Eskildsen, Christopher Fallon, Herbert Lison, Jeremy Bristol, Robert Peters, and Adam Clark. For his role in introducing to one of our group, and eventually to the world, standardized practical methods for clinical laboratories to measure and track antimicrobial susceptibility and resistance, we recognize the value of the contributions of John C. Sherris.

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Cloud-Based Surveillance, Connectivity, and Distribution of the GeneXpert Analyzers for Diagnosis of Tuberculosis (TB) and Multiple-Drug-Resistant TB in South Africa

WENDY S. STEVENS, BRAD CUNNINGHAM, NASEEM CASSIM, NATASHA GOUS, AND LESLEY E. SCOTT

49

Cloud computing is emerging as a new paradigm in health care information technology strategies and the growth of the industry. Despite reports emerging as early as the 1950s hinting at the potential of this approach, the strength of this is only now being realized in the health care sector. This is a simple principle of service-orientated architecture delivering a service around data rather than a product. The main enabling technology for cloud computing is virtualization. Virtualization is the ability to allow the system to operate independently of the hardware from the cloud via the Internet, so one can provide information to other users of hardware or software. In addition, resources can be shared within and between organizations to improve economies of scale. Data can be transferred in a computer network that is able to compartmentalize your needs.

In clinical medicine, there is often frequent opposition to using cloud-based technology, which is attributed to concerns around patient confidentiality, ownership of the data, and the security thereof. Despite this, large numbers of health-care-related organizations are shifting from traditional data exchanges for health information to the cloud arena. This approach has the ability to exchange and promote integration of electronic medical records (EMR), provide storage for the vast amounts of data that are stored for purposes of patient care, medico-legal issues, and the development of new technologies such as microarray and next-generation sequencing analysis engines and to simplify reimbursement. In spite of the strengths of allowing third parties to manage your data, security needs to be carefully designed up front with policies detailing government data access permissions, encryption, and the segregation of patient-identifiable information from the information itself.

To fully utilize cloud servicing, a broadband Internet and wireless expansion is required, which is not accessible in many countries, especially developing countries. Advantages cited include increased speed, flexibility, and a reduction in costs and labor. New work discusses the use of the "mobile cloud," which combines the use of mobile devices and the cloud (1). In this approach the devices can be personal digital assistants, smart phones, and tablet computers that can be used at clinic sites for data collection and can be tracked using a global positioning system facility where enabled on these technologies. However, there is the option for this to become, in various contexts and formats, part of a comprehensive surveillance strategy. In Australia, for example, data collected from very remote sites can be used for identifying outbreaks of emergent arbovirus without involving complex surveillance strategies using mosquitoes. The data can be uploaded using a cloud-computing platform across the country (2).

In South Africa and other developing nations, most of the patient care for human immunodeficiency virus (HIV) and tuberculosis (TB) occurs at the lowest level of the health care system, namely, primary health clinics. These are challenged in the collection of clinical data, which is frequently manual or paper-based. This is largely due to shortages of resources, both human and financial. They are challenged frequently by the lack of unique patient identifiers and inconsistent (or disconnected) laboratory and clinic record keeping. Solutions to these challenges are being explored by numerous groups using cloud computing technologies and services. In these situations, computing resources are shared via a cloud-based virtual desktop infrastructure to serve multiple primary health clinics and/or hospitals that have no or very different hospital information systems (HISs). An example from China demonstrates a cloud-based hospital information service center. Costs were significantly less than establishing enormous individual hospital-based information systems (3). Barriers are still interoperability with electronic health care records. Cloud computing could benefit a number of stakeholders such as patients, health service providers, and those responsible for reimbursement (4). Edwards and colleagues, following a large literature review, established that barriers to implementation are quite substantial and include standards, security issues, and potential economic loss to competitors (5).

However, a study conducted by Salka and Kiechle (6) showed that the implementation of an instrument management platform for a single point-of-care (POC) glucose

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instrument interfaced to an HIS resulted in an annual cost saving of $119,095 after implementation in 61 testing facilities. The largest benefit of the introduction of the instrument management platform was the alleviation of manual tasks which were estimated to accumulate to $140,185 per annum. The other major findings from the Salka study were that connectivity increased operator compliance with regulatory guidelines, achieved cost savings for both nursing and POC testing (POCT) coordinator staff, and would yield a return on investment within 14 months of operation. Although the initial setup costs for establishing the necessary infrastructure may be high, these systems provide high cost-saving benefits and increased functionality.

An HIS promotes and provides standardized interfacing (HL7, ASTM, POCT1-A). Interestingly, the use of point-to-point interfaces has rapidly been changed to management by third-party applications facilitating exchange-enabled interfaces. The move to interface engines is being driven by reducing costs, reducing dependency on laboratory information system (LIS) vendors, developing rapid interfaces, and strengthening monitoring, alerting capabilities, and maintenance (7). To achieve proper EMR and LIS links, the EMR needs to be able to perform order entry and the laboratory results need to be exported via an exchange interface and parsed by an LIS/HIS or EMR system. Laboratory analyzers usually work to the same standard (ASTM/HL7) facilitating integration and interfacing of analyzers on a large scale. The development of the instrument interfaces and the interface management engine must be clearly defined. Interface development for information exchange (for systems like the GeneXpert; Cepheid, Sunnyvale, CA) requires a once-off driver configuration for a management engine to be able to correctly parse and interpret the exported information. The problem, however, lies in the fact that the systems which parse and interpret the information (LIS/HIS/EMR) often charge a fee-per-interface or instrument license fee. This means that the initial costs include not only the development of the interface, but a charge per instrument utilizing the interface. The scope of work and all hidden costs such as data transfer fees, port fees, and interface development need to be clearly defined up front. The National Health Laboratory Service (NHLS) in South Africa, for example, only paid for one development task, but license fees were required per instrument (e.g., one interface was built for all 306 analyzers in the South African National GeneXpert program).

BIG DATA EMERGING IN HEALTH CARE

The collection of enormous amounts of data provides the ability to data-mine and answer public health questions quickly, using the correct data set. Problems encountered in the past centered around cost concerns, time to change, poor Internet coverage in certain areas, and poor data storage capabilities. Moving from traditional company capital, high-investment, structured infrastructure models to a model with no more investment in infrastructure over time must be an advance. The maintenance of the LIS described above, for the South Africa public sector laboratory services, is extremely expensive for both hardware and software.

Using the cloud facilitates the analysis of big data and what is being referred to as deep data; the former enables a question to be put by the analyst, allowing the emergence of frequently surprising data, and the latter addresses a single well-defined question on a well-structured data set with an expected answer.

As new technologies such as next-generation sequencing (with varying chemistries) are used more frequently, the combined outputs from thousands or millions of sequences will need to be unraveled. Another example used in-country that requires sophisticated analysis of large amounts of data is the microarray platforms that analyze RNA expression profiles or single nucleotide polymorphisms. Both big data and deep data have applications in mining for information on these data sets. The trend with data on these kinds of data sets is that their worth is not always known up front, and the data which can be extracted from them is generally unknown. In these instances the general trend is first go big, then go deep. Big data is useful for identifying trends and correlations and identifying new links for information. Once these links have been validated, deep analysis allows the researchers to look for answers to specific questions about the hypothesis.

TB AND HIV LANDSCAPE IN SOUTH AFRICA

South Africa is a democracy of close to 52 million individuals (8) and is classified as a middle-income country with government health functions organized at three levels, namely, national, provincial, and local (the latter with coterminus boundaries between district and municipal authorities). Health policy and legislative development are national functions in health care, while the nine provinces are responsible for operational service delivery, mainly through district and subdistrict government entities, including clinics and hospitals. Although it is a middle-income country, due to historical, social, and economic inequalities and development, first- and third-world conditions coexist in the country, which now has a poor Human Development Index of 121 (9).

An extraordinary number of South Africans die of infectious diseases. There is a realization that infections such as multidrug-resistant TB (MDR-TB) or general antimicrobial resistance, SARS (severe acute respiratory syndrome), and other emerging communicable diseases are a massive global security risk. South Africa has one of the highest burdens of HIV, TB, and coinfection, with an estimated 6.4 million HIV-infected individuals. Estimates suggest that at least 2.4 million infected individuals are on antiretroviral treatment. South Africa has one of the worst global TB epidemics, notifying over 300,000 cases/year (http://www.who.int/topics/ehealth/en/). Annually, the testing requirements for these two communicable diseases amount to one third of all laboratory expenditure at the NHLS (W. Stevens, personal communication, 2013). In 2013, 2.8 million HIV viral loads, over 4 million CD4s, and over 300,000 early infant diagnosis HIV PCR assays were performed (NHLS, Central Data Warehouse [CDW]). South Africa has 20% of the world's HIV-associated TB cases and is the fourth largest reporter of MDR-TB (http://www.who.int/topics/ehealth/en/). Over 65% of all suspected TB cases are infected with HIV. In 2012, TB incidence rates reached 1,000/100,000 of the population, with vulnerable groups in correctional facilities, mines, and the pediatric population having far higher rates (10). It is estimated that 1% of the population develops TB disease annually (http://www.who.int/topics/ehealth/en/). In addition, MDR-TB rates are increasing,

and over 15,000 new cases were reported in 2012 (CDW, 2013). Currently, of the high-burden countries, South Africa ranks second in TB prevalence and first in TB incidence (11). Thus, an enormous number of diagnostic assays have to be performed in laboratories, with routes to clinics for collection being improved with relatively recent geographic information system mapping of clinics and laboratory facilities with drive times and distances allowing for the assessment of needs or gaps in service.

With increasingly high rates of smear-negative TB and liquid culture not being clinically relevant for screening due to long delays, once the World Health Organization (WHO) endorsed the GeneXpert (a simple molecular test for tuberculosis) (12), it was implemented immediately in South Africa in a phased manner as a front-line screen for both TB and rifampin resistance. To speed up access, microscopy centers at all levels of the health care system were involved. To date, over 306 analyzers of varying sizes, including 7 GX 80 Infinities, have been placed in 216 centers in South Africa (NHLS, CDW, personal communication) as seen in Fig. 1. Between March 2011 and February 2015 over 5 million tests had been completed. As the project expanded from high-burden to lower-burden districts, the percentage of TB positivity dropped from a country average of 16% of all sputa tested in 2011 in higher-burden districts to 10% in 2014. Rifampin resistance is detected simultaneously in the assay, by molecular beacon probes that cover the relevant point mutations in the 81-base-pair region amplified in the *rpoB* gene. While geographical differences occur, approximately 7% of the population are rifampin resistant, and more than 50% of these are new TB cases (N. Ishmael, personal communication, TB reference laboratory). When isoniazid resistance is tested, over 85% of samples collected demonstrate probable multidrug-resistant cases.

Currently, collecting basic TB, MDR, and HIV testing statistics is relatively easy at the laboratory level using the LIS and the CDW (large centralized server able to store all LIS data). Far more difficult is the management of the various TB and HIV registers that have to be filled in manually at the tier 1 and some tier 2 clinics. All TB data are transferred into the electronic TB register (ETR.net) and the drug resistance register (the edr.net) at the National Department of Health. The clinical and laboratory data are not easily linked due to the absence of an in-country unique identifier, and thus, probabilistic matching is used by the laboratory for reporting data trends. In 2013, it was mandated that all patients use their national identification number for health care, although this is not yet being widely practiced.

From a data collection perspective, the following is the status quo: (i) a large number of instruments needed to be interfaced in South Africa to an LIS, including the 306 GeneXpert analyzers; (ii) large data sets which are getting larger as we move toward novel technologies for routine HIV drug resistance testing or whole-genome sequencing for TB (big data); (iii) delivery of results to a health care system that has fragmented clinic electronic health care records with poor availability of hardware required at clinics; (iv) poor linkage of laboratory to clinic records (few have HISs that are fully integrated); (v) POC assays being increasingly used in the field (for strip-based assays, mobile

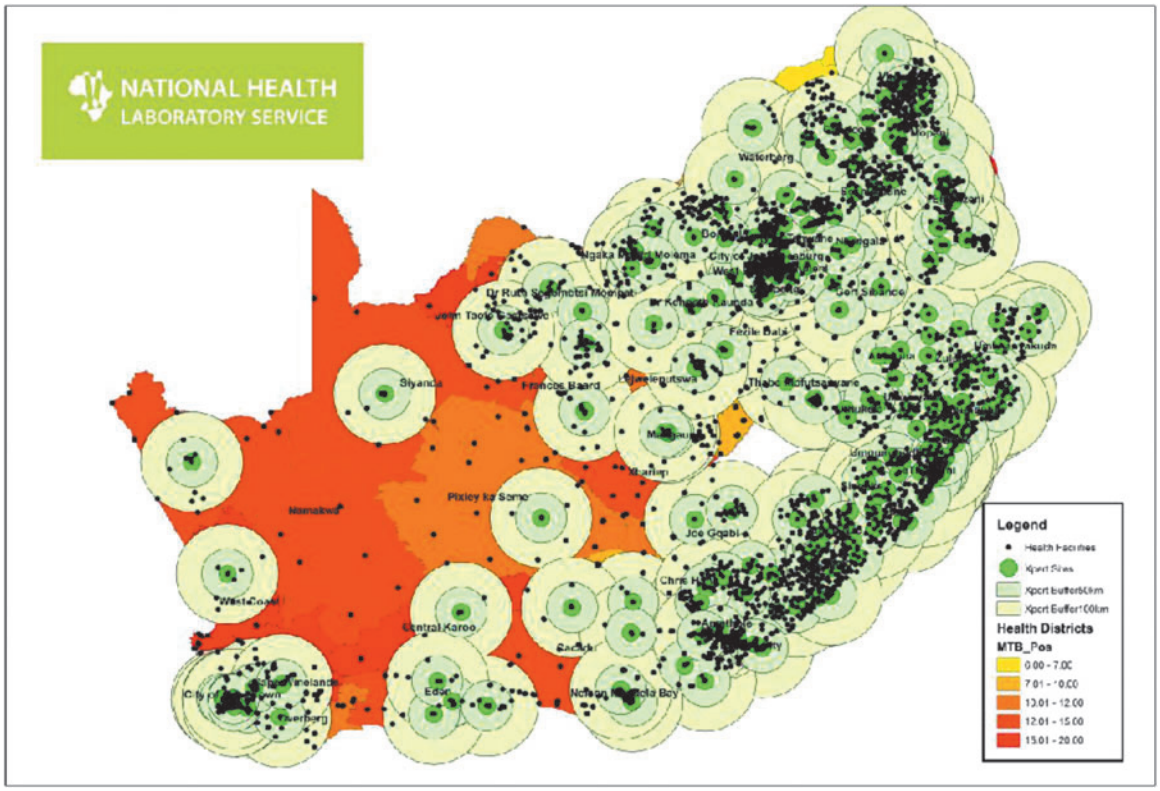

FIGURE 1 Current GeneXpert placements and clinic sites.

devices/readers may be possible for monitoring quality and entering patient information into the CDW; for instrument-based POC testing, connectivity remains a difficulty and is discussed in more detail below); (vi) Internet and bandwidth not optimal in all areas; and (vii) mobile technology available in most areas: an estimated 69.2 million active SIM cards in use and 14.4 million people estimated to have smart phones in South Africa (Vodacom, personal communication). The discussion that follows describes the status quo followed by insights into how this could be expanded upon or changed in the future.

INFORMATION TECHNOLOGY LANDSCAPE IN LABORATORIES IN SOUTH AFRICA

There are many different clinical and laboratory systems in use in-country for health care. Historically, health care systems in South Africa were characterized by fragmentation, lack of coordination, high prevalence of manual systems, and lack of interoperability between systems. Recently, the National Department of Health and the Council for Scientific and Industrial Research wrote a framework document providing recommendations for the implementation of e-Health strategies in South Africa (13). The Protection of Personal Information Act has been signed into law in South Africa and governs the definition of personal and special personal information and the legal requirements for collecting, storing, and transmitting data (14). The WHO defines e-Health as the use of information and communication technologies for health to, for example, treat patients, pursue research, educate students, track diseases, and monitor public health (http://www.who.int/topics/ehealth/en/). In the United States, while still in its infancy, different bodies are emerging to address the regulatory issues, the Food and Drug Administration (FDA) (15) being the most developed in its software policies for medical devices. The large number of legacy systems in middle-income countries such as South Africa may actually delay or compromise innovative new approaches.

Current Public Health Information Technology Laboratory Infrastructure

South Africa has only one national LIS that connects data from analyzers directly to the public sector, which lies at the core of all laboratory operations. The NHLS is currently the custodian of the National Department of Health for laboratory data, which is stored in a CDW with massive server capability. This is a major achievement since this level of data collection and storage was characterized by a number of different LIS systems or paper systems, resulting in remote sites in the not-so-distant past and required a huge investment in hardware and software. The country is in a unique situation to have the bulk of national laboratory results stored in one system. The NHLS serves over 87% of all testing needs in the public sector and thus provides a valuable national resource and is used both for surveillance purposes and individual patient management.

The Library Information Management System is used for the management of data related to samples received, instruments used to test these samples, and other functions such as result distribution, results storage, and quality control monitoring. Currently, the NHLS has 265 laboratories serving the public sector, with all using the centralized LIMS. Data are captured into the LIMS using requisition forms. Each set of specimens from a single request form is allocated a unique laboratory number. A process is under way to use a unique patient identifier, the identity document number. Most results move from the analyzer directly into the LIMS to avoid transcription errors. For certain analyzers and assays, rules are established to direct release of accurate results and to allow automatic reporting if quality control is deemed accurate and the complicated results or cases are held back for review by senior pathologists, technologists, or scientists, thus making sure scarce high-level technical skills focus on the complex cases (a process called autoverification, which has predefined rules and algorithms).

NEW TESTING METHODS MEET OLD INFORMATION TECHNOLOGY IN THE TB AND HIV ARENA

Trends in laboratory technologies are in constant flux, changing rapidly with the development pipeline of high-end, fully automated analyzers and an increase in the pipeline for assays suitable for testing conducted at POC. The information technology requires exceptional flexibility to deal with these massive changes in scale and context.

POC Testing

In South Africa, centralized LISs manage laboratory testing and result distribution well. The proposal to expand to POC testing in remote sites and primary health care clinics will require stricter control of instrument data and quality assurance. The concern around interfacing POC testing is the diversity of physical interface connections and protocols used by available analyzers. Few manufacturers comply with the standards and recommendations of the Connectivity Industry Consortium and the IVD Industry Connectivity Consortium to provide and implement open, standard protocols, and proprietary interfaces are still present in the market (16), although the adoption of these recommendations is improving.

A recent randomized controlled trial conducted in South Africa is reaching completion and is called Investigating the Feasibility of a Multidisciplinary POCT Laboratory in an Active HIV Clinic and Determining the Impact on Patient Outcome (Grand Challenges, Canada Grant). Instrumentation was randomly selected from that which was readily available, including the PIMA for CD4 testing (Alere), the GeneXpert for TB (Xpert MTB RIF assay; Cepheid), Reflotron (for alanine transaminase and creatinine; Roche Diagnostics, Germany), and the HemoCue HB201DM (HemoCue AB, Angelholm, Sweden) for hemoglobin. This does not mean endorsement of these products by the NHLS; these systems merely provided examples to test the feasibility of connectivity in this context. The information technology component was to establish whether these instruments could all be connected to the NHLS central LIS. The instruments used in the study quickly highlighted the connectivity disparity between both physical connections and the standard communication and exchange protocols. To interface these instruments, evaluations of both available middleware and instrument management systems (IMS) were undertaken. The middleware layer generally acts as a translator, extracting the data into a standard format and passing this on to the next layer of the IMS, which is responsible for

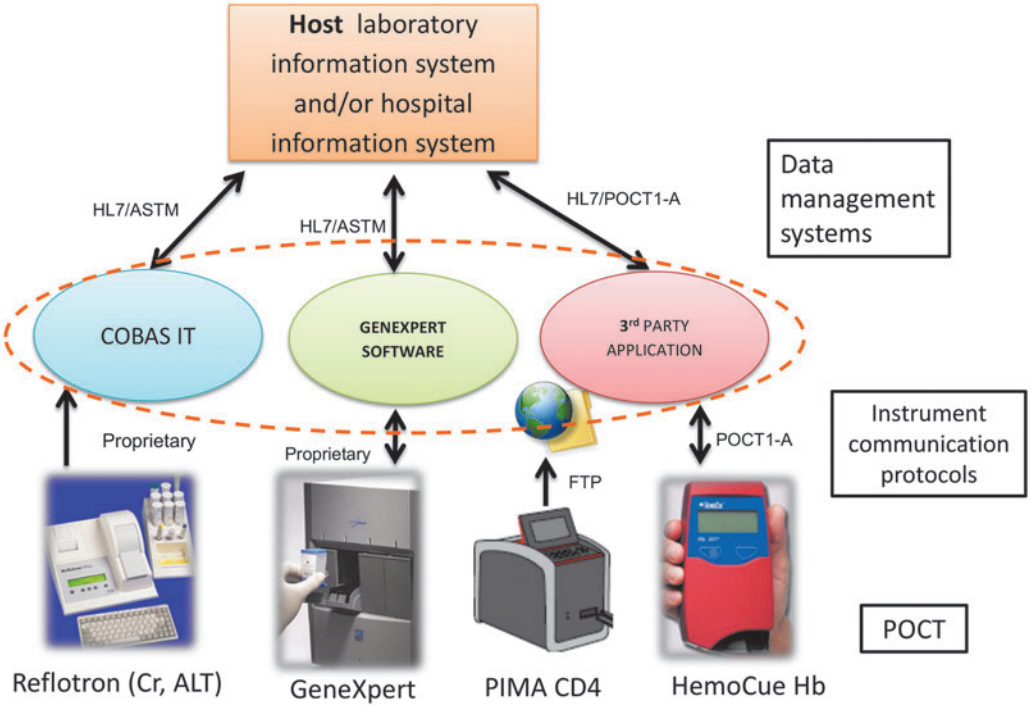

FIGURE 2 Standard connections and protocol disparity. (Courtesy of Brad Cunningham.)

data processing and analysis and implements all of the user functionality (see Fig. 2).

The GeneXpert and HemoCue systems have standardized communication protocols that could be interfaced with the NHLS LIS. In most instances the interfacing is a once-off development cost, and then a license fee is levied for the LIS for each instrument interfaced. Using a middleware or IMS solution to interface instruments to the LIS makes it possible to limit the number of required licenses to a single interface. Many LIS developers are obviously reluctant to support the latter model and base the charges on a per-instrument rather than per-device charge. Table 1 lists a few off-the-shelf IMS and clinical management solutions which were investigated as part of this study and highlights that no single solution achieves the ideal functionality required to produce a total solution covering all aspects from clinical to data management.

Upon completion of the initial validations, connectivity of instruments was identified as one of the most important components in the development of a sustainable POC model with potential links to clinic and laboratory monitoring and monitoring and evaluation purposes. Challenges were the following: (i) temporary network downtime remains a problem, and even though instruments cache these results locally, this slows the process down dramatically; (ii) operational dashboards are manufacturer specific; and (iii) frequently captured test results are delinked from patient clinical data, so stored data cannot be forwarded into HISs or LISs.

A number of additional connectivity solutions were explored: (i) Short Message Service (SMS), (ii) satellite technology, and (iii) General Packet Radio Service (GPRS)-SMS.

- SMS is an option for data transmission and coverage and generally exceeds the performance of GPRS options due to lower bandwidth requirements and lower susceptibility to interference. However, SMS is generally more costly than data, is limited in the number of character fields allowed for transmission, and introduces security concerns in terms of information susceptibility and encryption.
- The largest barrier to implementing satellite technology is the cost of the hardware and monthly connection fees, which are still significantly higher than the more freely available communication media. Advantages include good coverage and high-bandwidth transmissions.

TABLE 1 Instrument management systems, middleware, and patient management systems

System	Instrument interfacing	Training and certification	Quality control and instrument management	Patient history	Result management	Clinical information	Visit management
AegisPOC	Extensive	Yes	Yes	Yes	Yes	No	No
POCcelerator	Extensive	Yes	Yes	Yes	Yes	No	No
Cobas IT	Limited	Yes	Yes	No	Yes	No	No
Identicare	Development	No	No	Yes	Yes	No	Yes
Therapy Edge	None	No	No	Yes	Yes	Yes	Yes

- Currently, the most effective coverage would be achieved with a GPRS-SMS hybrid model which could dynamically switch between the two transmission methods sharing the data and transmission requirements, depending on the data destination and type of information being transmitted. This facilitates cost-effectiveness of data and standard and robust encryption schemes.

An SMS approach is being evaluated in a linkage-to-care project attempting to link all patients with MDR-TB to care nationally, using alerts from the laboratory data system where the diagnosis is first made (see example in Fig. 3). SMSs containing results are generated directly off the LIS, and alerts are sent to the clinic coordinator, the linkage coordinator, the facility manager, and the patients themselves. A number of linkage-to-care pilot programs are being undertaken in-country for HIV and TB to address the high loss to follow-up characteristic of these patients along the chronic continuum of care. Figure 3 illustrates one possible approach to MDR-TB linkage.

CLOUD FOR REAL-TIME SURVEILLANCE USING THE REMOTE CONNECTIVITY SYSTEM: DEVELOPED FOR THE GENEXPERT TECHNOLOGY FOR TB

Real-Time Cloud-Based Solutions for Monitoring Testing Quality

Remote connectivity in the laboratory medicine context refers to the ability to replicate data from the laboratory analyzer, which performed the test, to a central server hosted by the analyzer supplier or an additional third party at a secure site. Essentially, the analyzer instrument is managed by an information system that is associated with the analyzer. In this setting, the computer hosts an application that will send IP-based data from the various laboratories to the remote connectivity server.

The GeneXpert technology and Xpert MTB/RIF assay are a good example of how effective remote monitoring can work together with an LIS (see Table 2, which highlights system differences). The GeneXpert technology applies the ASTM communication protocol and could be interfaced to the NHLS LIS. All 289 GeneXpert analyzers in the field could then forward all results to be made available to the LIS for immediate resulting and then to the central data warehouse for storing and future data manipulation. Several variables are generally collected from the LIS as listed in Table 2. These data are then extracted within the CDW and mapped for additional functionality and overall monitoring and evaluation, providing reports at different levels of the health care sector. The data supplied include (i) the number of tests performed per province, district, and subdistrict facility and summarized national reports; (ii) the number of tests reported as negative and positive (with rifampin susceptibility or resistance); and (iii) the number of tests reported as invalid, error, or no result (incomplete testing). The LIS/GeneXpert interface and data extraction through the CDW was found to be limited, in that no information about the individual instrument's performance or associated functions was easily retrieved to assist with internal quality management. This sparked the new development of the Software as a Service from

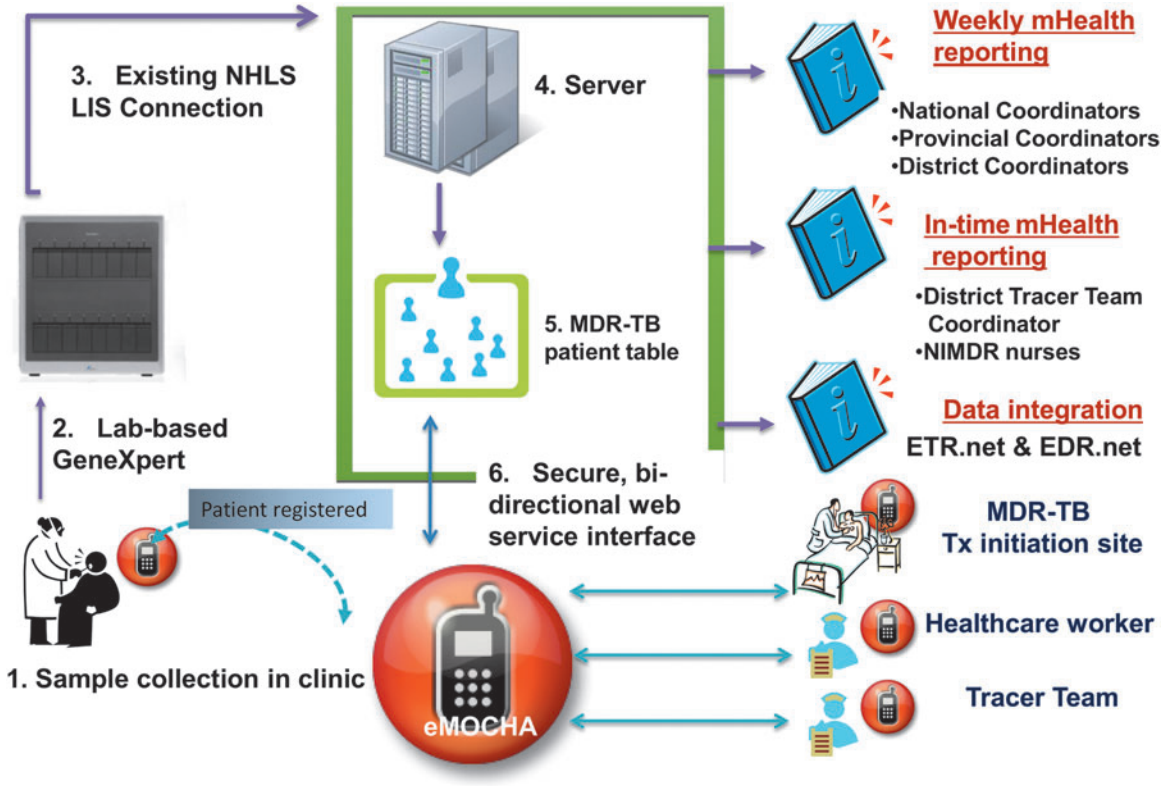

FIGURE 3 Linkage-to-care using analyzer interface, SMS result distribution, and m-Health solution combined with training of nurses to trace contacts and administer MDR care.

TABLE 2 Variable description of the National Priority Program LIS CDW and the Cepheid RM monitoring tools

Variables	CDW/LIS/data extraction	Cepheid RM
Patient name and hospital ID[a]	Yes	No (customer server)[b]
Date and time of assay run	Yes	Yes
Laboratory number	Yes	Yes
Cartridge number	Yes	Yes
Duration of run	No	Yes
Test type (test code)	Yes	Yes
Lot number	No	Yes
Result	Yes	Yes
Error	Yes	Yes
Error code	Yes	Yes
Test user name	Yes	Yes
Module ID (serial number)	No	yes
Pressure and temperature curves	No	Yes[b]
Error log per module	No	Yes[c]
Instrument status	No	Yes[c]
Module status	No	Yes[c]
Monitoring and trending	?	Yes[c]
Module usage	Manual analysis per laboratory	Yes
Central data control	Requires data extraction (analyst)	Directly
Historical data	Yes (requires extraction)	Yes
Real time	Yes	Yes
Technical status	No	Yes
Share with external applications	No	Yes (login user-specific)
Cycle threshold value and sample processing control	Yes (data extraction, manual analysis)	Yes[c]
Probe frequency	Yes (data extraction, manual analysis)	Requires extraction[c]
Turnaround time	Yes for result upload	Yes for time cartridge placed in instrument
Usage	Manual for monthly	Yes (day, month, etc.) and time of day schedule testing
Calibration	No	Yes[b]
External quality assessment/quality control	Only if identified	Only if identified
Duplication and serial testing	Yes	No[b]

[a]May be date of birth, hospital number, or national ID.
[b]Expected in future versions.
[c]New upgrade version 2013/2014.

Cepheid that would be preconfigured on all newly installed GeneXpert instruments.

For Xpert, remote connectivity allows for real-time monitoring of instrument and site performance (17). Cepheid has developed a remote connectivity operational dashboard for real-time monitoring of results, errors, reagent data (batch and lot numbers), calibration data, instrument serial numbers, resistance, and positivity rates (17). This has been an exciting pilot program in collaboration with the University of the Witwatersrand, Johannesburg, South Africa, and the NHLS that is highly involved in the initial system requirements, set-up, troubleshooting, and testing of the prototype system, which is now in the process of complete deployment (18). In the next version, as part of the intention to scale up the existing architecture to accommodate international providers, the separation and definition of user rights have needed to be incorporated. Potentially, in phase 2, designated organizations such as the WHO and CDC could have, with country permission, delinked global surveillance data. This provides enormous strength for surveillance across the continent. The recent and rapid spread of the ebola virus reinforces the ability to use cloud computing across countries.

For remote monitoring, the only requirement for the laboratory service is to provide a network point at the laboratory. Additionally, the laboratory firewall must permit network traffic to the remote connectivity server (IP address). Due to bandwidth connectivity challenges in South Africa, data transmission to the remote connectivity server takes place during periods of low demand. While the discussion to follow will focus largely on GeneXpert, it should be noted that other examples already exist, such as AbbotLink, which states that 60% of the laboratories worldwide that use their Architect are connected to a remote service. The same connectivity is available for Roche viral load testing (19). This not only facilitates data collection, but enables remote monitoring and troubleshooting.

In addition to the functions described above, data are collected for the metadata around the various reporting probes' cycle threshold values, which may be useful, for example, in monitoring Xpert probe data to detect strain

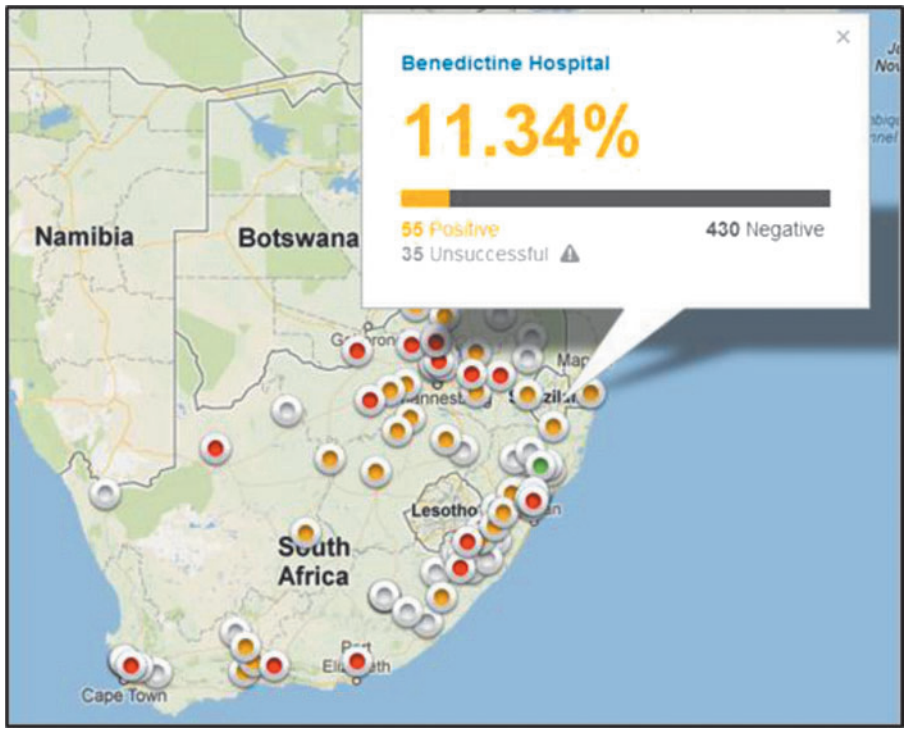

FIGURE 4 Geo-located devices. (Courtesy of Cepheid.)

diversity by region and over time (17). One of the benefits for the client or supplier is the ability to use remote monitoring to assess which sites require proactive technical support, instead of waiting for a failure to occur, at which time extensive troubleshooting is generally required (17).

The remote monitoring dashboard allows for real-time audit indicators (configured graphically on-the-fly) that are beneficial to any size TB program (at the national, provincial, and district levels and all the way down to individual modules). The dynamic content generated by the geo-located device's landing page uses Google maps (Fig. 4).

The system also features a powerful error-tracking tool which allows the user to view the error rates as a percentage of the total error types per week (Fig. 5). This assists greatly in responding to batch- and lot-related errors in real time as the tests are being run.

One of the more powerful features of the site is the ability to download all of the test results which have been transmitted and do any number of functions in Excel to be able to view data such as (i) number of errors per module, (ii) types of errors per module, and (iii) number of user errors per user (identify training needs).

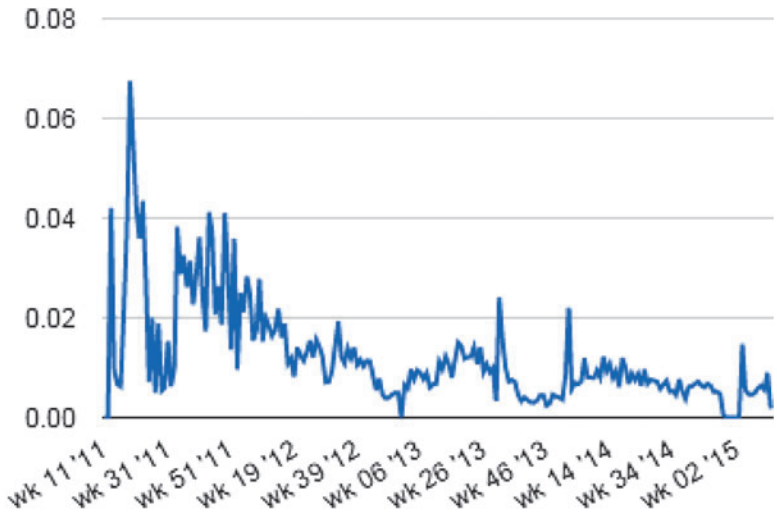

FIGURE 5 2% errors per week.

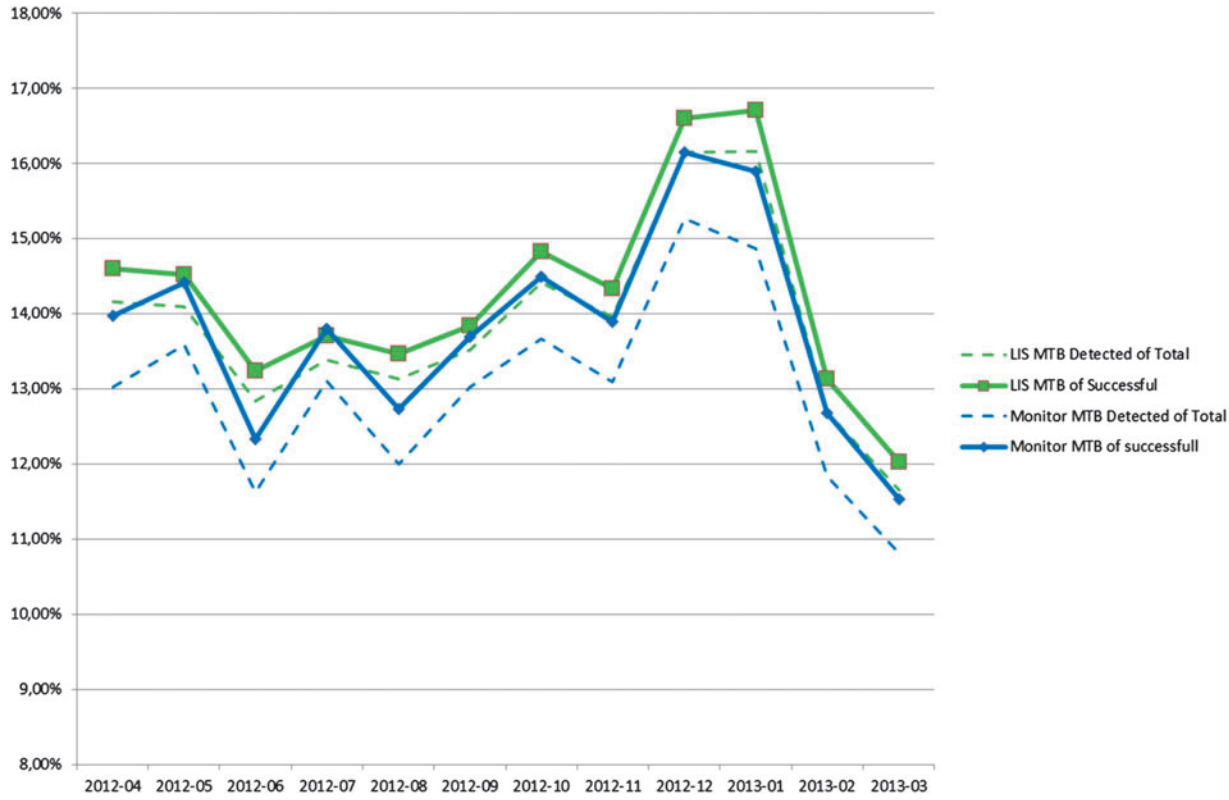

FIGURE 6 LIS versus remote connectivity data.

The experience with the Cepheid pilot operational dashboard has shown the potential use of cloud-based systems for monitoring operational and epidemiological components of a national/international diagnostic intervention program. The features of the program are not limited to the graphics generated, and the data collected in the cloud can be downloaded for any number of functions in MS Excel. The Cepheid RM solution can also be used as a validation tool of existing LIS data. The graph in Fig. 6 shows a 1% bias between the NHLS LIS and the Cepheid monitoring system, which is due to duplicate removal and repeat testing on specimens generating an error, which is not extracted from the LIS. Fig. 7 reveals further examples of audit indicators.

Monitoring the dashboard and reviewing the data becomes an exceptionally difficult task which would require at least one full-time employee to effectively derive the necessary information. The main issue introduced in the volume of data is the loss of detail.

Once this type of system surpasses the point where anomalous results no longer affect significant figures of the averages, they become very difficult to detect. The overall effect of a series of anomalous results (say *x*) only affects the total average by *x*/total results, and even at the point where the total exceeds 10,000 results, 100 anomalous results (which is a significant enough number for concern) would affect the identifiable reported averages by 0.1%.

At this point in a system implementation, it proves that real-time data do not inherently translate into real-time intelligence, and mechanisms for analyzing and reporting the data become as important as the data delivery. This field of study introduces a number of topics such as big data, deep data, and intelligent (intelligence) systems; these are all aimed at promoting business intelligence and are emerging in the health care industry.

The advantages of systems like the Cepheid dashboard, which are able to deliver information in real time, are only truly realized if the data can be acted on in real time. A simple distinction needs to be made between real-time data and real-time intelligence. Intelligence is derived from information. Information is derived from data. Simply delivering data in real time does not translate into real-time intelligence (see Fig. 8).

These kinds of real-time systems stimulate further innovation in an already innovative health care field and present opportunities for interdisciplinary engagement to further the state of technology.

Intelligent systems are able to analyze data and extract information to answer defined questions, but they also provide higher levels of intelligent output than simple reporting percentages and stats. One example would be utilizing the GeneXpert operational data set to evaluate the instrument error rates and derive information beyond the normal reporting criteria (Table 3).

The primary purpose of intelligent systems (or the development of "smart dashboards") is not simply to create a number of data tests which a user would have to run at defined periods, sort through the information, and select which actions require attention (although this is a component of their purpose), but to automatically derive intelligence from the data and alert the user to the events.

The inclusion of intelligent systems on data sets being provided by Cepheid on platforms such as the operation dashboard would realize the advantages of the real-time delivery of data and allow, as nearly as possible, near-real-time

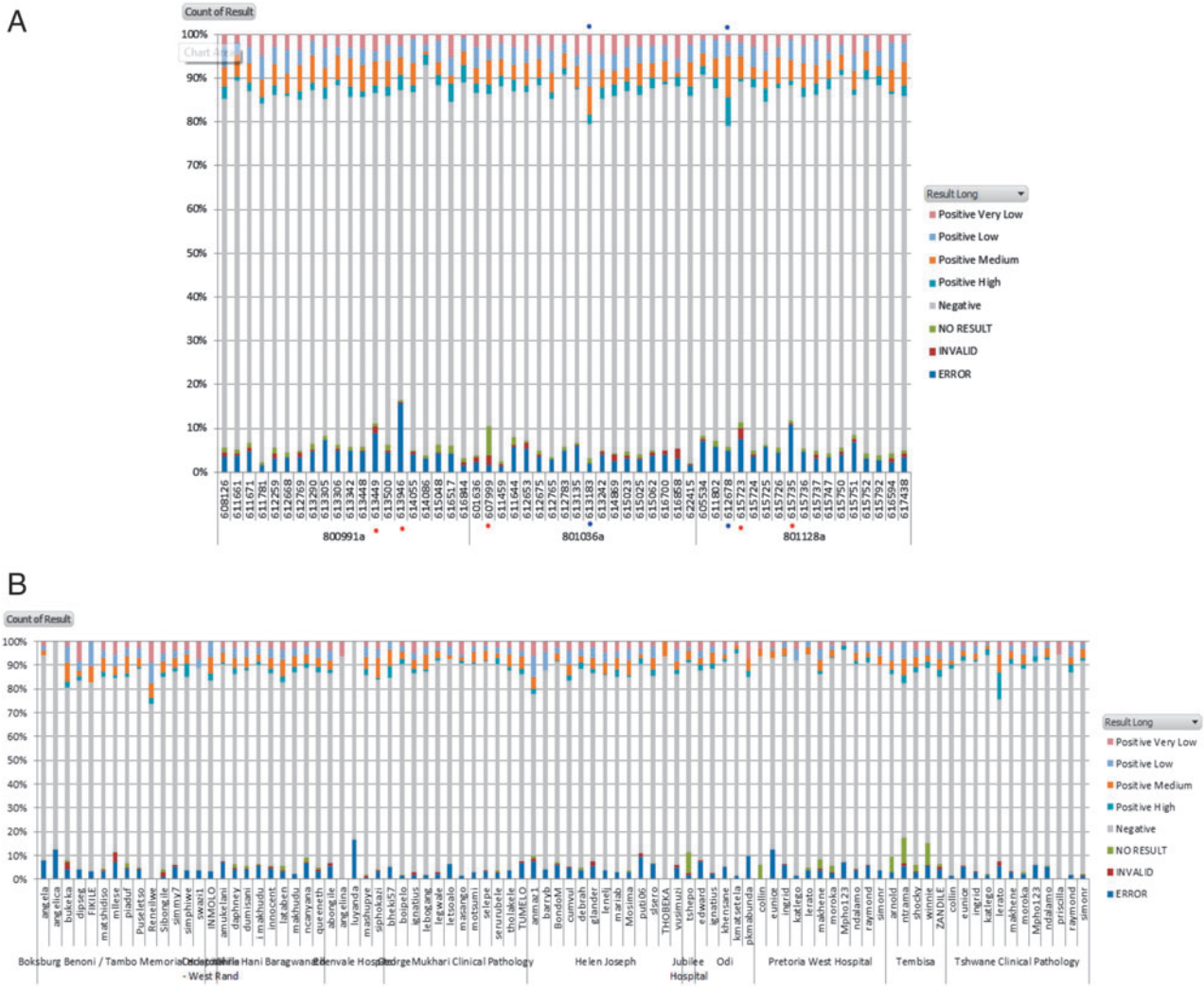

FIGURE 7 Examples of audit indicators.

intelligence and action on the data. Some potential scenarios currently being investigated by the NHLS with intelligent systems are:

- Identification of potential sample contamination within laboratories on a daily basis
- Identification of users with consistent and/or above-normal training-related errors
- Identification of potentially malfunctioning/misreporting modules per instrument
- Identification of potential regional outbreaks
- Identification of potential lot-based cartridge issues

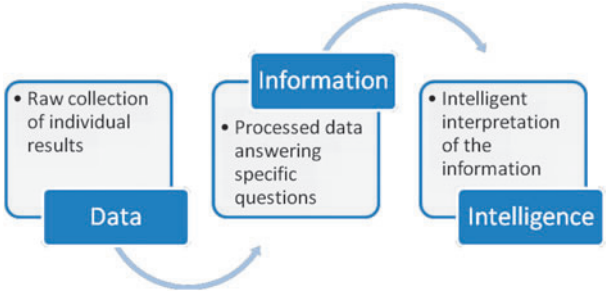

FIGURE 8 Journey from data to intelligence.

DATA SECURITY

Information Security and Risk of Operational Dashboards

One of the common concerns with the adoption and growth of cloud-based diagnostic instrument reporting

TABLE 3 Example of data processing

Step	Description
Data	Raw collection of individual results
Information	Report defining overall and the error rates per province, laboratory, and instrument
Intelligence	Identifying that the cause of a number of errors beyond the norm is related to an individual operator within a laboratory and that the operator requires (re)training

systems (i.e., dashboards) is the sensitivity of the information they carry. The term "operational dashboard" implies that the systems carry and process operational data from the laboratory information system, which requires that the data be delinked from patient-identifiable information. Operational data, for the purposes of this article, is defined as the data set produced from a diagnostic platform which contains the reportable data being produced by the instrument, with the exception of any fields containing sample or patient-identifiable information.

The fact that the data are, by explicit design, delinked from any fields which could lead to the identification of a sample significantly reduces the medico-legal implications and risks of storing the data. The fact that no patient-identifiable information is stored should not, however, be used as a justification for not designing the necessary security measures and access control into such systems.

Transmission of Data into the Cloud

If secure storage can be implemented, how can one be sure it reaches the cloud securely? Web-based systems are a daily component of most businesses worldwide, and security measures for Web transmissions of data (https, hashing, RSA encryption, to name a few) are widely used, well established, and thoroughly tested and used by other systems which contain sensitive and more attractive information to intercept such as bank payments and financial information.

Surveillance and the Detection of Outbreaks

In addition to real-time monitoring of quality indicators, for optimal laboratory service and patient care, a real-time monitoring system can also be used for monitoring regional and global population disease burden and therefore interventions aimed at reducing the diagnostic delay or disease outbreaks (hot spots) and increasing intensified case findings as well as individual patient responses to treatment (20–22) (much like HIV viral load testing) (23). This is becoming a rapidly growing area for use in global disease surveillance by organizations such as the CDC as a tool for surveillance. An example is the use of whole-genome sequencing and cloud computing for influenza surveillance (24).

If it is geographic information system–mapped, a population can be monitored in near real time with the ability to provide the data needed for appropriate public health interventions. A public health task force set up by the CDC to standardize, develop, and train on the use of global health information exchange with epidemiological reporting capabilities developed a roadmap referred to as ISO TR 14639-1:2012.

Connectivity for Quality Assurance

Material Preparation

Xpert remote monitoring and the NHLS centralized LIS data collection and analysis are just two of the options available to GeneXpert users in South Africa. Since the global implementation of the technology, the need for quality monitoring for verification and external quality assessment was required, but no suitable testing material was available. This was addressed by the development of the dried culture spot (DCS) quality program that was used in the largest implementation program in South Africa (25). DCSs are manufactured using *Mycobacterium tuberculosis* cultures grown in single cell format, followed by chemical and heat inactivation, quantification, and spotting onto Munktell TFN filter cards (LabMate, Cape Town, South Africa). The DCS cards are barcoded and shipped by post at room temperature. Processing of the DCS on-site follows the Xpert MTB/RIF manufacturer's testing protocol. The program involves two components: (i) verification (results unblinded) and (ii) external quality assessment (results blinded). Other quality control programs with different material formats are also available to users (N. Gous, L. Scott, R. Prieur, B. Kana, and W. Stevens, presented at 3rd SA TB Conference, June 2012, South Africa). Verification is conducted to ensure the instrument is "fit for purpose" and is suggested at the time of installation, after module swap-out or replacement, if the instrument is moved, and more recently, in agreement with the Global Laboratory Initiative, after calibration. Verification is now also used in mobile vehicle testing, where instruments may have been moved in transit before testing is initiated. The external quality assessment is required for accreditation and frequently includes strains other than *M. tuberculosis*.

Online Platform for Management and Reporting

One factor that became evident with the advent of the DCS quality program to support the South African national program was the need for the automation of result analysis and reporting. Initially, the DCS program data management and report generation was performed manually, but as the rollout gained momentum, the feasibility of this approach was questioned. An automated, Web-based platform (TBGxMonitor, www.tbgxmonitor.com) was then developed to manage the data collection and report generation for the national rollout within the NHLS. TBGxMonitor is a simple interface for end users of the system, the testing laboratories, and clinics. After DCSs have been tested, the GeneXpert result file (.csv) can be uploaded onto www.tbgxmonitor.com, and reports are automatically generated in real time. Identification of these quality-specific specimens (and not patient results) tested on GeneXpert is through a unique barcode provided with each DCS.

TBGxMonitor highlights the advantages of having specialist cloud servers which promote rapid implementation and use due to the low overhead requirements for client systems to be compatible for use. Cloud-based systems such as TBGxMonitor provide a number of advantages over local systems (installed on individual PCs):

- Have a central server which can be accessed globally
- Require a low-bandwidth connection (typical file size for external quality assessment /verification is ~8 kb)
- No installation required
- Support all web browsers
- Remove the burden of processing from local PCs to the server
- Centralized troubleshooting and reporting

Two other connectivity solutions have become available to GeneXpert users. These are GxAlert and XpertSMS. The GxAlert (http://www.gxalert.com/) was developed in 2012 by Abt Associates and to date is active in India and Nigeria. This software is designed for managing result reporting with patient information directly to the cloud, which can then also be provided to existing LIS users or become the data repository where existing LIS infrastructure is lacking. This solution also manages drug demand and supply, with added messaging pushed directly to ministries of health, clinics, and laboratory testing sites. The

latter retrieve information on instrument error rates, assay cartridge expiration, and stock control, as well as test utilization and deployment planning.

XpertSMS (http://www.ihsinformatics.com/xpert-sms) was developed in 2011 by Interactive Health Solutions in Pakistan to automatically receive test results from GeneXpert instruments. These results are then sent to several individuals and groups in the clinic, laboratory, and directly via SMS to patients for disease management such as MDR treatment. The data are stored in servers.

This is an exciting time for global disease surveillance options using technology such as cloud computing. We can foresee a future where organizations such as the WHO and the CDC may be able to quantitate and manage epidemics on a global scale in real time. Bioinformatic tools can thus potentially be made available as services to anyone anywhere and through any device.

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section XI

Quality Assurance

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Molecular Method Verification

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50

This chapter is intended to introduce the basic principles of method verification to laboratorians who have not yet been exposed to statistical concepts for method verification and provide a refresher for those who already use these analytic methods and concepts.

In clinical microbiology laboratories, molecular method development takes place in a variety of settings and is regulated by both guidance documents and federal law. In general, the required characterization of method performance and error assessment is divided into two categories: verification and validation. Verification is a one-time process performed to characterize and compare the diagnostic accuracy of a new method to that of a reference method which is accepted by the laboratory community as a standard of care (also known as "gold standard") for a particular analyte or disease (1). The method verification process documents that laboratory methods are assessed for accuracy as well as clinical utility prior to initiating patient testing. It also defines the errors and limitations of the assay that is being verified. Method verification for FDA-approved methods and laboratory-developed tests (LDTs) is performed according to regulations detailed in the Clinical Laboratory Improvement Act (CLIA '88), Code of Federal Regulations, Title 42, Part 493, Subpart K, 493.1253, Standard: Establishment and Verification of Performance Specifications (1). All methods, whether FDA approved, FDA cleared, or created as LDTs with analyte-specific reagents (ASRs) (2), are subject to the CLIA regulations for method verification. Method verification allows a laboratory to assess the risks of error, bias, and imprecision to assess the probability of causing a change in the interpretation of a test result, or treatment decision, which may affect patient care.

In contrast, validation is an ongoing process which is performed for quality assurance of a verified assay. The process of validation documents the assay performance over time and under various conditions and ensures that the method continues to perform as originally described during the verification process. Validation allows a laboratory to document continued accuracy of the method as well as operator competency over time. Of note, there are various definitions of verification and validation (3); however, in this chapter, we use the definitions originally established in the CLIA regulation (1) and in the *Cumitech* publications from the American Society for Microbiology (4).

When performing method verification for a molecular assay, several aspects of assay development must be considered. First, several definitions, each with its own caveats, exist. For instance, the reference method is commonly called a reference standard or gold standard method in the context of the verification process, but it may not always be the most sensitive of the methods. Second, the "new" method may not be new with regard to its history, but rather, it is new to the particular laboratory that is performing the method verification. Finally, there are many definitions, regulations, and guidance documents which must be considered and whose history is important for understanding the challenges that laboratory leadership faces and the choices they make as they plan to establish a new test method. It is the latter aspect, specifically the strategies and processes of method verification, on which the remainder of the chapter will focus. Under CLIA '88, method verification requirements include the establishment of assay performance specifications (1). These specifications include accuracy, precision, analytical sensitivity, analytical specificity (including interfering substances), reportable ranges, reference ranges, and any other characteristics required for test performance and interpretation of results. This chapter will elaborate on these aspects of method verification as well as the common statistical analyses that are used to assess data acquired during method verification.

HISTORY OF REGULATIONS AND GUIDANCE

For diagnostic tests classified by the FDA, such as *in vitro* devices, there are two paths to market. One path is through the FDA, aiming at FDA clearance or approval with commercial distribution. The other path is the LDT, formerly called a "home-brew," "in-house test," "off-label test," or "research use only" test (RUO). When performing method verification for a molecular assay, there are several aspects of assay development that must be considered. FDA-approved and -cleared assays do not require the same intensity of verification as LDTs, since the former have already undergone a battery of rigorous assay development parameters, design control, and clinical trials. Nevertheless, FDA-approved methods do require verification data and proof of laboratory competency prior to their use in a clinical laboratory setting. Typically, with such assays, verification is minimal and may consist of running 20 to

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50 positive specimens and 50 negatives (4), while for verification of LDTs, the number of required samples is more substantial and must be performed in accordance with CLIA '88. In addition, for LDTs it is prudent to consider adherence to the principles of the Standards for Reporting of Diagnostic Accuracy (STARD), which describe minimal criteria for characterizing and reporting diagnostic accuracy (3, 5–8). Lack of adherence to these principles is the cause of inaccurate performance of laboratory methods, which is well documented in the literature (9–19). In addition, as standards for evidence-based laboratory medicine best practices evolve via the Centers for Disease Control and Prevention's collaboration with the American Society for Microbiology (ASM-LMBP), use of the STARD criteria may improve the likelihood that clinical verification data could meet the stringent data requirements for inclusion in ASM-LMBP meta-analysis and guideline creation, which could stabilize and further the cause of clinical laboratories as they strive to document improvements in laboratory services.

The use of LDTs is a well-established practice but is not well defined with regard to method verification. The FDA states that "clinical laboratories developing [in-house] tests are acting as manufacturers of medical devices and are subject to FDA jurisdiction under CLIA"; however, the FDA has generally exercised enforcement discretion over LDTs and had not actively regulated them until 31 July 2014. Historically, the FDA decided to try to ensure the quality of the reagents used in LDTs by creating the ASR Rule in 1997 (CFR 21, CFR 864.4020, 809.10, and 809.30) (2).

ASRs are defined as "antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reaction with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens" (2). The ASR guidance was instituted from a desire to ensure that reagents used in LDTs for clinical use were manufactured using good manufacturing processes. It was a deliberate effort to create a safe harbor for LDTs and ensure transparency in labeling such that the responsible party was the laboratory, not the manufacturer of reagents. The intent of the 1997 guidance was that laboratories would take responsibility for the design, verification, and validation of the test, a condition that was not possible with "kits" and "pseudo-kits" that were commonly sold subsequent to the first ASR rule. Therefore, in 2007, the guidance was clarified to eliminate inappropriate use of such reagents (2). Following the July 2014 notice, the FDA posted draft guidance on 30 September 2014 and formally published notices in the *Federal Register* on 13 October 2014, with a 120-day comment period that ended 2 February 2015. The *Federal Register* notice for the draft guidance documents is identified as Docket No. FDA-2011-D-0360 for Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories: Framework for Regulatory Oversight of Laboratory Developed Tests (LDTs), and Docket No. FDA-2011-D-0357 for Draft Guidance for Industry, Food and Drug Administration Staff, and Clinical Laboratories: FDA Notification and Medical Device Reporting for LDTs. At the time of this writing, molecular microbiology laboratories across the United States anxiously await the final revisions to these important documents and issuance of the final federal regulations.

TRANSLATIONAL INFECTIOUS DISEASE DIAGNOSTICS

Many of the molecular assays that are used clinically today were originally created in a research laboratory setting and therefore were formerly called "research use only" tests (RUOs). While the research laboratory is an avenue for creativity and ingenuity, methods developed in a research setting require commitment to a defined translational research process to reach the bioindustry development stage and eventually the clinical laboratory bench with a role in patient care. Before these assays can be used in a clinical laboratory, they must undergo rigorous testing and evaluation.

Two common deficiencies can occur when research laboratories develop tests aimed at the clinical market: (i) impractical method design and (ii) inadequate error assessment. In the research laboratory, state-of-the-art technology is generally tested for "proof of concept," and test methods are assessed based on their innovation and use of the latest scientific principles. However, in the clinical laboratory, assays must also be supported by standardized testing practices and quality systems with indicators that have proven ability to monitor test outcomes. Thus, it is the translational research process that is responsible for the actual technology transfer to health care, modifying the assay and the experimental design to support expectations for standardized testing practices.

LABORATORY DIRECTORS AND RUOs

Of note, under regulations in CLIA '88, all laboratory directors of record are legally responsible for their own laboratory as well as for the investigation of the performance and suitability of any reference laboratory that they choose to supplement their own clinical service (1). When sending specimens to any research or reference laboratory that offers RUO testing, it is critically important to inquire about the method verification strategy and data summaries and to be wary of under-verified methods. Some would argue that, as an RUO, it is not appropriate to issue results from these tests in the medical record. In either case, when there is an inconsistency in any test results, one must ask pertinent questions and get answers that will help in the interpretation of the test results. Issues and practices related to LDT assay verification via CLIA '88 (1) are detailed in the following sections by discussion and by example.

THE PATH TO ASSAY VERIFICATION

When a laboratory is designing an assay, the initial focus is to define the assay requirements in daily use. By defining these requirements, the laboratory is able to facilitate characterization of assay performance and error assessment to define the assay's limitations. The verification process and assessment should mimic clinical reality as much as possible and be performed in the specimen matrix planned for actual patient testing. It is critical to statistically determine if the assay performance characteristics are acceptable when compared to a defined standard reference method and that limitations are minimized or mitigated. The extent of the test verification should be predetermined by the laboratory director and must reflect the criticality of clinical decisions which are to be made based on results of a given assay. For example, the assay requirements for a herpes simplex virus (HSV) test to be performed on a cerebrospinal fluid specimen may be quite different from those of a genital

specimen. In the cerebrospinal fluid specimen, the requirements for assay sensitivity will be much more critical than that of a genital specimen, in which the viral density may be much higher.

Additionally, when an assay demands high specificity, the assay conditions required to achieve those requirements may impact its performance for sensitivity. Stevenson et al. showed how polymorphisms in the probe target can affect the performance of an HSV real-time PCR assay (20). Such polymorphisms can result in decreased sensitivity of an assay, which is critical for a cerebrospinal fluid specimen. In this study using the crossing threshold (C_T) as a cutoff for assay sensitivity, it was estimated that as many as 15% of the HSV type 1 (HSV-1)-positive and 7% of the HSV-2-positive specimens would be missed when comparing two real-time HSV PCRs based on commercially available ASRs. It is important to note that assessment and interpretation of "melt curves" along with the amplification curves for a real-time PCR assay can help to flag such specimens. In such cases, a probe may bind enough to produce a melt curve but not enough to produce an amplification curve. Since native viruses commonly mutate, polymorphisms are a common problem with real-time PCR (20, 21); therefore, a strategy to mitigate those risks must be created and deployed.

One way to formalize risk management decisions within the laboratory is the Individualized Quality Control Plan (IQCP), currently under development. By 42CFR493.1250, IQCP is the CLIA QC policy allows an alternate quality control (QC) option. The guidance and concepts for IQCP are a formal representation of many laboratory practices already in place to ensure quality test results, but IQCP allows customization of a QC plan according to test method and use, environment, and personnel competency while providing for equivalent quality testing.

Verification Strategy

Each new method or genetic target should have a verification document, which is a packet of information that serves as documentation and overview of the analytical method performance. In the packet, the laboratory provides a summary of what the assay is; when, why, where, and how it was verified; and by whom. It should include statistical characterization of the assay and a summary of test performance, including graphs and charts to describe results. Test limitations and recommendations for use should also be documented. The verification packet holds policies and procedures related to the test method. Inclusion of a description of the document control process, including raw data and the process to create data summaries, is also useful, as are any relevant cost analyses, documentation of clinical utility, and manufacturer's information. Essentially, the verification packet contains all information from the initial method design and project planning to the data summary, review documents, and ultimate approval and signature of the laboratory director or consultant.

When planning the initial design of an assay, there are several key factors that can limit the usefulness of the study, and therefore, those attributes must be considered in planning. It is important to clearly define all the specimen requirements, including sample type, quality, and volume; collection method and device; and transport and storage requirements. Suppliers of appropriate control materials are also critical. Obtaining controls may be as simple as growing a strain of bacteria and preparing a McFarland standard or may be very costly when the controls must be purchased from a commercial source. One must consider the variability inherent to the purchase or preparation of a commercial control for quantitative assays. Unless the control is standardized or harmonized, such as those from the World Health Organization (WHO), there is no way of knowing how your control material will perform when compared to the literature. Widespread variability is observed for assays that rely on quantitative controls, since users may be using a different strategy to measure their material. This variability is evidenced in results for assays with no purchasable international standard, such as viral loads for cytomegalovirus (22) prior to initiation of a WHO standard. Tables 1 and 2 provide listings of supporting information that may be useful when planning verification of a new laboratory assay.

Other examples of common methodological errors to consider when planning the verification include false-negative results, false-positive results, and outright errors. False-negative results can occur due to limited sensitivity of the assay in the sample matrices, reaction to inhibitors in the sample, or antigenic and genetic variation of the organisms. False-positive tests can result from contaminating

TABLE 1 Test selection summary: important CLSI protocols for test verification

EP05-A3, *Evaluation of Precision of Quantitative Measurement Procedures; Approved Guideline*, 3rd ed.
EP07-A2, *Interference Testing in Clinical Chemistry; Approved Guideline*, 2nd ed.
EP09-A3, *Measurement Procedure Comparison and Bias Estimation Using Patient Samples; Approved Guideline*, 3rd ed.
EP10-A3-AMD, *Preliminary Evaluation of Quantitative Clinical Laboratory Measurement Procedures; Approved Guideline*, 3rd ed.
EP12-A2, *User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline*, 2nd ed.
EP15-A3, *User Verification of Precision and Estimation of Bias; Approved Guideline*, 3rd ed.
EP24-A2, *Assessment of the Diagnostic Accuracy of Laboratory Tests Using Receiver Operating Characteristic Curves; Approved Guideline*, 2nd ed.
C28-A3C, *Defining, Establishing, and Verifying Reference Intervals in the Clinical Laboratory; Approved Guideline*, 3rd ed.

TABLE 2 Requirements for verification of molecular microbiology assays[a]

Category of the verification requirement	Type of assay		Required by:	
	Qualitative	Quantitative	CLIA '88	CAP
Accuracy	X	X	R	R
Method comparison	X	X	NR	R
Sensitivity	X	X	C	R
Specificity	X	X	C	O
% Carryover	X	X	NR	R
Precision	NA	X	R	R
Linearity	NA	X	NR	R
Reportable range	NA	X	R	R
Reference interval	NA	X	R	R

[a]Abbreviations: X, the verification requirement characteristic is applicable to the type of method; NA, the verification requirement is not applicable to the type of method; R, required; C, required under certain conditions; O, optional; NR, not required.

nucleic acid template or amplicon, limited specificity testing during assay verification, or unknown organisms in the environment that may cross-react with assay reagents. Outright errors can occur due to new and faulty lots of reagents, primers, or probes as well as a poor method design, for instance, lack of appropriate controls built into the assay. Sample processing errors can occur when a specimen is inappropriately processed; these errors can occur at any step from extraction to amplification to detection and may have nothing to do with the reagents themselves. Because of these issues, it is important to include sample processing controls and verify individual instruments as well as assay reagents.

Another important verification method consideration is the "improved gold standard" phenomenon. Since many molecular assays create a situation in which the new method may be more sensitive than the reference standard laboratory method, assessment of such improved gold standards can create complexity. Organisms that may have been missed by traditional reference methods now may be detected by newer, more sensitive assays. Therefore, it is often necessary to test specimens by a third assay, by broth enrichment to boost culture sensitivity (if possible), by genetic sequence-based detection by another target or sequencing method, or by clinical assessment of the disease state to differentiate true-positive from false-positive results. Under these circumstances, "discrepant analysis" is often performed by using a second molecular method that targets a different area of the genome to confirm that a specimen is a true positive. Interpretation of discrepant analysis should be performed with extreme caution, since statistically speaking, retesting of only the discrepant specimens is likely to confirm some of the results, even if that occurs by chance alone (8, 11, 12, 17). It is preferred that all specimens be retested by a second reference method, when possible. Additionally, when utilizing archived specimens for verification of discrepant results, it is important to note how old they are and how many freeze-thaws the specimens have undergone at that point. Degraded specimens can create confusion during performance of discrepant analysis.

Finally, it is important that data management not be overlooked. It is prudent to keep the assay verification data organized by creating directory folders, both hard copy and electronic, as well as by appropriately naming data files (i.e., Date_Descriptor_initials). Data organization allows laboratories to track operators, instruments, and reagent lots as well as experiments, making record keeping and documentation of the data much easier. In this era of computers, an electronic backup of the data can make organization of the data and records simpler for both the laboratorian and for laboratory inspectors who will review the method verification data. There are many useful statistical software packages and tools that can help to organize verification data, summarize the performance, and define the limitations of the assay (these tools are discussed in subsequent sections).

COMPONENTS OF QUALITATIVE ASSAY VERIFICATION

Determining Analytical Sensitivity

Qualitative assay characterization is required for the development of microbial detection methods. The first step is to determine the analytical sensitivity or limit of detection (LOD). Optimizing the LOD may require some adjustment to the specimen input volumes and extraction or PCR requirements, which a laboratory will want to determine prior to running additional specimens. The LOD is defined as the 95% LOD of the organism in sample matrix. This means that an analyte is detected 95% of the time at that particular microbial density. It is a mistake to define the lower detection limit, the lowest concentration of analyte that can ever be detected by the assay, as the reportable LOD. The lower detection limit may only be detected a small percentage of the time. Of note, the LOD is also different than the limit of quantitation (LOQ), the lowest concentration of analyte that can be accurately quantified by the assay. The LOQ will be further discussed later in this section.

One of the ways to determine the assay LOD is by testing several microbial densities (commonly and incorrectly referred to as concentrations) and applying a probit analysis to the data to calculate the assay's 95% detection limit. Probit analysis is discussed in more detail in the statistical section of this chapter. Typically, for an LOD assessment, one could perform at minimum 12 repetitions of each density, bracketing the expected LOD and diluting the sample 2- to 10-fold, until one reaches at least 1 log below the expected LOD. The selection of densities to be tested can be an iterative process, which includes some trial and error, particularly when the target LOD is unknown. Often, clinical laboratories do not have resources for the optimal design and may substitute as few as 6 replicates or simply try to test approximately 20 samples at the published LOD in the hope that 19/20 or 20/21 will yield positive results. Often, one can find verification data sets and publications with reports of 19/21 positive samples, indicating that the laboratory added an extra specimen to reach the 95% LOD once their original 20 samples were tested. This practice, while not optimal, is practical and can save resources when resources are limited.

The probit analysis process can also help with the assignment of the assay's positive cutoff value, which is typically based on the 95% confidence interval (CI), of the C_T value associated with the LOD derived from the probit analysis. When applying this process to real-time PCR, since the target quantity is known, the C_T value of the calculated LOD can be determined and used to assign the assay's cutoff C_T value. The cutoff C_T value represents the highest C_T value that can be interpreted as true positive without concern of fostering false-positive results, based on nonspecific amplification. The cutoff C_T value represents a low microbial density, sometimes found at the assay's 95% LOD or by other means such as careful assessment of assay specificity at relatively high C_T values. In many cases, any C_T value higher than the cutoff C_T value represents a result in an assay's equivocal zone with regard to assay interpretation. Some of the samples in the equivocal zone may be truly positive; however, there is a possibility that false-positive reactions can occur in the equivocal zone due to nonspecific amplification. Some laboratories scrutinize internal (inhibition) controls for these C_T values to determine if repeat testing or dilution of inhibitors may be warranted.

For LDTs, the assay cutoff value should be revalidated periodically according to clinical regulations and guidelines, for example, every 6 months (refer to chapter 56). Assessment of the data from 12 repetitions also yields the intra-assay variability, because the calculated mean, standard deviation (SD), and other variance measures can be obtained at several analyte densities.

Determining Analytical Specificity

Once the analytical sensitivity is determined, analytical specificity can be determined by testing the assay with a

battery of closely related or genetically similar organisms to assess any cross-reactivity with other organisms. Analytical specificity can also include common organisms found in the biome for which the assay is intended. This testing can be performed with spiked specimens by using organisms that are related or may be commonly found in the sample matrix. A variety of "known negative" patient samples can also be used, because they provide an unknown but widely varied diversity of the local biome. Another strategy, "virtual specificity," (also known as *in silico* assessment) can be assessed by examining sequence alignments of the primer and probes used in the assay and comparing them to all known genetic sequences for potential cross-reactivity. It is important to note that the *in silico* assessment should include a wide range of organisms commonly found in the normal specimen type(s), not just those that are thought to be closely related; it should also include human genome sequences.

Specificity assessment is an activity that continues through the life of the assay as new organisms are identified. Using a broad approach to specificity assessment can identify potential cross-reactions in your assay but cannot predict the evolution of microbes or the emergence of newly identified microbes that may cross-react with primers and probes in the future; therefore, *in silico* assessment may also become part of a laboratory's validation process and be repeated at regular intervals.

Defining Qualitative Accuracy

Qualitative accuracy (percent agreement) of the assay is determined by comparing assay performance to a reference standard method. Ideally, this is accomplished by using known positive specimens over a known range of target densities. These target densities should include a large sampling of weakly positive specimens to provide a suitable challenge to the system being tested. At minimum, 50 positives and 100 negatives should be tested; however, depending on assay performance, additional specimens may need to be tested to obtain enough statistical power to compare assay performances and provide a legitimate reason to replace an existing assay. For rare pathogens or difficult to obtain sample types, laboratory directors may determine sample size and method according to CLIA guidelines for method verification.

Since positive specimens may be very challenging to obtain for assay verification purposes, proficiency panels and spiked specimens may be substituted if no other options exist. It is important to ensure that specimens are tested in a random fashion (i.e., do not test all the positives in one run and all the negatives in another) so that testing mimics reality as much as possible.

Documenting Other Method Characteristics

Qualitative analysis in natural sample matrices allows a laboratory to perform *post hoc* calculations for percent inhibition and percent contamination as part of the assay verification process. In addition, use of a checkerboard layout (alternating positive and negative specimens) for extraction and testing will challenge the process or instrument system to document that there is no carryover of sample throughout the entire process.

Describing Precisions/Variability

There are two types of variability that are important to characterize for LDTs: intra-assay variability and interassay (between run) variability. For FDA-approved methods, no requirement for characterization of precision exists. For those interested in assessing variability, simple numerical means (averages), SDs, and 95% CI can be calculated when at least three triplicate samples are tested; however, sample sizes of at least six specimens tested on the same run will provide more accurate assessment of within-run variability and can provide coefficient of variation (%CV) data. The College of American Pathologists (CAP) requirement for 20-day QC provides an opportunity to calculate interassay variability, even if it is not required. Assessment of variability provides an assessment of operator competency for the various technologists involved in the process. Refer to the quantitative assay section for more information about precision measurements.

Calculating Assay Efficiency for PCR

Assay efficiency depends on the purity of the nucleic acid extract and PCR efficiency. Each real-time PCR instrument has its own specified equation to calculate efficiency. The equation is based on the slope of a standard curve line, plotting C_T versus microbe density or quantity, displayed as $\log_{10}$ dilutions of CFU/ml in Fig. 1. Assay efficiency is not commonly calculated for non-PCR methods.

Characterizing Clinical Test Performance

Risk assessment of the assay is important for clinical outcome analysis. The clinical sensitivity of a diagnostic test is defined as the proportion of truly diseased people, as measured by the reference standard, who are identified as diseased by the test under investigation. Specificity of a diagnostic test is the proportion of truly nondiseased people, as measured by the reference standard, who are identified as nondiseased by the diagnostic test under investigation.

Receiver operating characteristic (ROC) analysis evaluates test accuracy and produces a distinct graphical representation of test performance, balancing both sensitivity and specificity. A ROC analysis, sometimes called a ROC curve, gives a graphical description of test performance by plotting the true positives (sensitivity) versus (1 – specificity) the measure of false positives for a binary classifier, such as disease or no disease, as its discrimination threshold is varied. The plot graphs the clinical accuracy in terms of sensitivity and specificity, which is displayed over the entire spectrum of decision levels and can be used to help determine assay decision thresholds or cutoffs (CLSI EP24-A2). The ROC analysis can be represented equivalently by plotting the fraction of true positives (true-positive rate) versus the fraction of false positives (false-positive rate) (Fig. 2).

Prevalence data are used to calculate positive predictive values (PPVs) and negative predictive values (NPVs) of medical assays. The PPV and NPV vary depending on the prevalence of disease in a given population. Prevalence data describe the value of a test when used at the actual disease prevalence of a given population. For example, the PPV is always lower in a population with a lower prevalence of disease than in a population in which prevalence is high. The terminology used to describe these testing categories is depicted in Fig. 3.

COMPONENTS OF QUANTITATIVE ASSAY VERIFICATION

Quantitative assays require more testing and analysis than qualitative assays. Similar to qualitative assays, the initial strategy is to determine the analytical sensitivity of the assay. Determining the LOQs allows a laboratory to

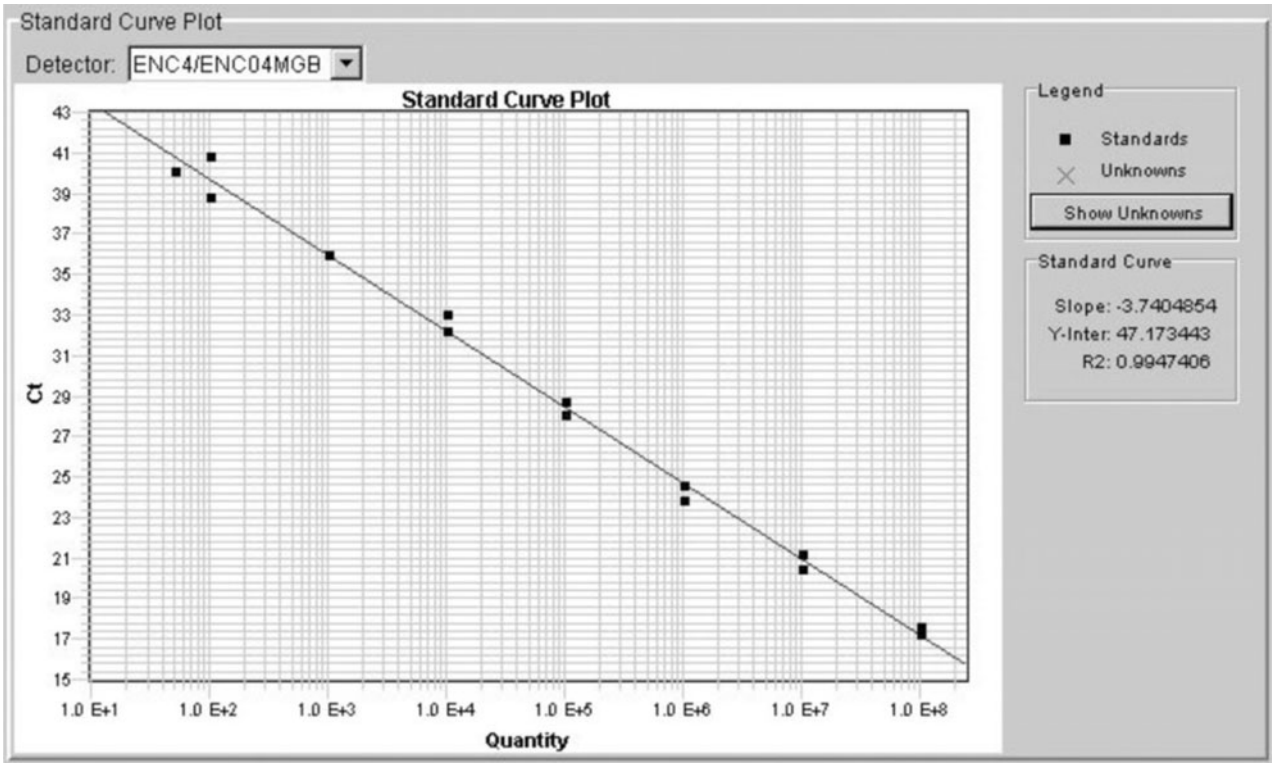


FIGURE 1 Depiction of a PCR efficiency calculation and graphical representation of PCR efficiency. Dose-response relationship for C_T values and microbe density depicts a linear and inversely proportional relationship at high microbe concentrations. Linear regression shows 99% correlation with linearity to the highest dilutions tested; however, a visual check of C_T values at 100 and 50 organisms per reaction depicts a breakdown in the precision of the data points at that concentration and may predict the microbial density at which assay linearity may begin to decline.

characterize the entire linear range of quantitation of the assay. One of the primary ways to determine the quantitative capability of an assay is by evaluating the precision or reproducibility of the assay at various analyte densities. Typical quantitative methods in microbiology include viral loads, but other quantitative assays exist and are characterized in largely the same way, with the exception of quantitative gene expression analysis.

Measures of Precision (Variability)

Simple measures of intra-assay and interassay variability are required for quantitative method verification to characterize assay robustness and operator competency. Quantitative assay verification must include a broader assessment of variability and determination of precision at extremes of microbial density, both on the high and on the low end, to help characterize the assay's linear range. For quantitative assays, more stringent requirements for sample size must occur.

Interassay variability (i.e., precision or reproducibility) is monitored over the course of assay verification via ≥20-day QC so that a statistically acceptable range and CI can be determined for various parameters of the assay. By means of trend analysis, the stability of the controls should be assessed at analyte densities that are 1 log above the determined LOD of the assay.

Typically, results from an external positive control can be charted over a period of time and graphed against the mean to describe interassay variability. For these assessments typically, the upper and lower confidence limits are determined. Attempts to perform and characterize 20 to 28 separate test runs using samples of external positive control or equivalent will establish a sound interassay variability against which new lot numbers can be assessed as time goes on (4). When an assay can detect as well as type an analyte based on the melt curve temperatures, both the C_T and melt curve temperature of the assay should be monitored and statistically characterized. Once the director has characterized and reviewed the assay for clinical use, assay validation (ongoing QC of the assay) will continue to be monitored.

For practical purposes, or under conditions of emerging pathogens or emergency use of assays in a pandemic situation, 20 to 28 samples for precision testing may not be practical; each doctoral director must then assess the assay's %CV and standard error of the mean to determine if the assay is precise enough to use clinically and then monitor each run, reviewing QC closely until statistical conditions are met. A good place to start is six replicates; then update your final data when 28 runs are reached. The strategy of three samples is typically not optimal to produce an acceptable %CV or SEM for an accurate precision assessment.

Measures of Quantitative Accuracy Across the Assay's Linear Range

Precision testing determines the agreement between replicate analyses. It is a key performance component for quantitative assays. Precision limits will ultimately define the assay LOQ and are intimately related to the assay accuracy, particularly at the low end of microbial density tested. Typically, quantitative assays are used to measure viral loads

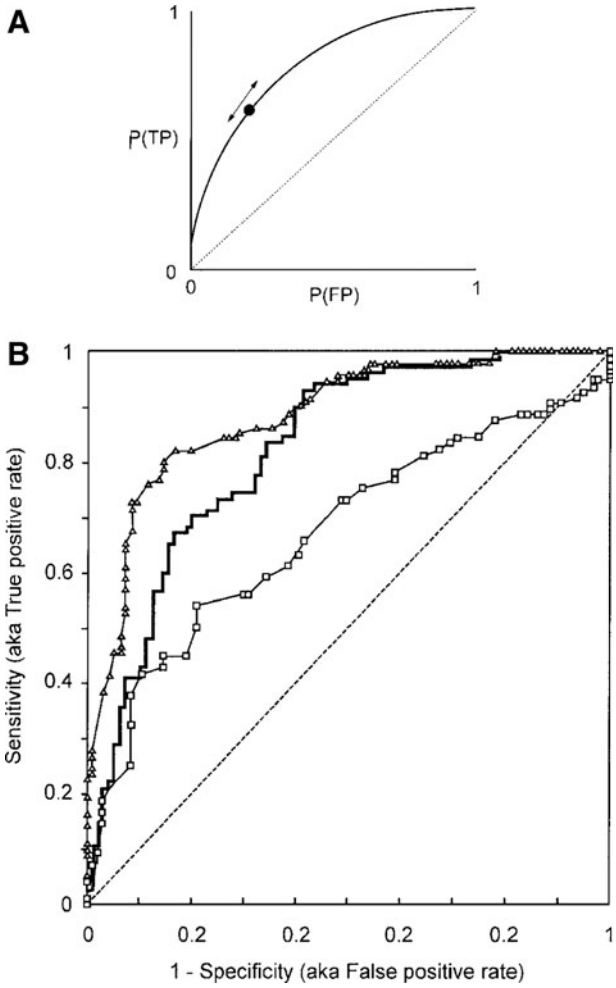


FIGURE 2 (A) Example of an ROC plot for an imaginary diagnostic test. The true-positive rate (sensitivity) is plotted against the false-positive rate (1 – specificity) for every known threshold value. The dotted diagonal line shows the case when the test has no discriminatory power. A test that gives no false-positive or false-negative results has a curve that passes through the top left-hand corner (dotted line), i.e., the distributions of the test results in the two groups are completely separated, and the area under the concentration-time curve equals 1.0. (The test is perfect.) The double-headed arrow shows the effect of varying the threshold value. (B) Comparisons of ROC plots for different test methods; the top line has the highest discriminatory power.

and monitor treatment response, so it is important that an assay can meet the predetermined assay tolerance limit, defined as the difference between two sequential results that can be considered significantly different in terms of clinical care. This tolerance limit is also known as the medical decision interval.

The medical decision interval defines a clinically significant change in viral replication, which is equal to the sum of the biological variation and the intra-assay imprecision (23). For example, the medical decision interval for an HIV type 1 (HIV-1) quantitative assay is 0.5 log (3-fold), with the biological variation making up 0.3 log and the assay imprecision accounting for 0.2 log. Thus, a quantitative assay would need a precision of ≤0.2 log SD across the quantitative dynamic range of the assay to meet the tolerance limits of the assay (Fig. 4A illustrates precision).

The preferred way to assess variability in a quantitative assay is with precision calculations based on the use of selected multiples of SD, because exclusive use of the %CV to assess precision for quantitative assays can be misleading. Figure 4B shows an example of a way to depict precision that represents the absolute error of the assay, while depicting variability in %CV, to represent the relative error of the test method. For example, an assay with a 0.2 log SD over the entire quantitative range of an assay will yield a 12% CV at 50 copies and a 4% CV at 50,000 copies. If %CV is used to characterize assay variability, it should be reported with the corresponding %CV at various analyte densities and should be specifically assessed at low concentrations of analyte, where relative variability will be the greatest.

The LOQ is determined based on the precision of the assay at the lowest analyte density. As stated previously, this is different than the 95% LOD, because an assay may still be able to accurately detect an analyte at the low end of microbial density but will not be able to quantify it accurately. At the low end of the detection limit, an assay loses precision and can muddy the distinction as to whether a change in viral load is due to a biological change in the virus or the imprecision of the assay (Fig. 5). One of the advantages of applying real-time PCR to viral load assays is that those assays allow the user to combine a qualitative and a quantitative result in one test.

When assessing precision, a common concern is the question of how many replicates, over how many days, are required to establish a calibration curve and determine LOD, LOQ, and precision. Many of the reference documents suggest different numbers of replicates. For example, CLSI EP5-A suggests two repetitions/level for 20 days (20-day QC), while CLSI EP15-A suggests four repetitions/level for 5 days. Optimal performance of probit analysis requires a minimum of 12 repetitions (to determine LOD and LOQ). Establishing the LOD, LOQ, and precision should not be done with a single run of two to three repetitions per density, because this is not statistically sound. Tools such as Levy Jennings plots can be applied to help examine interassay variability and perform trend analysis.

Relationship to Reference Standard

As with qualitative assays, analytical specificity and accuracy should also be tested, and special care should be taken to test various subtypes or genotypes, because genetic variability may alter the assay's ability to quantitate the target analyte. Verification should ensure that different known genotypes are not under- or over-quantified due to genetic variability. For example, for a quantitative hepatitis C virus load assay in a patient population that has a broad range of genotypes, the assay validation should include analysis of these various genotypes, not only the predominant genotype in the region. Genotype analysis can be performed by assessing the correlation data via a graph of the new assay results versus the old assay results and examining the coefficient of determination (r^2) and the line of equality as described below and depicted in Fig. 5 (see also Fig. 13A and B, below).

The R^2 value is the mathematical square of the correlation coefficient, r, and is typically listed along with the equation for the line of equality in Excel (Microsoft, Redmond, WA) or other statistical software programs; it gives you a measure of the *strength* and *direction* of the relationship between two variables, x and y; it does not describe the agreement between variables. To further describe these statistics, r is derived from a statistical regression analysis, and

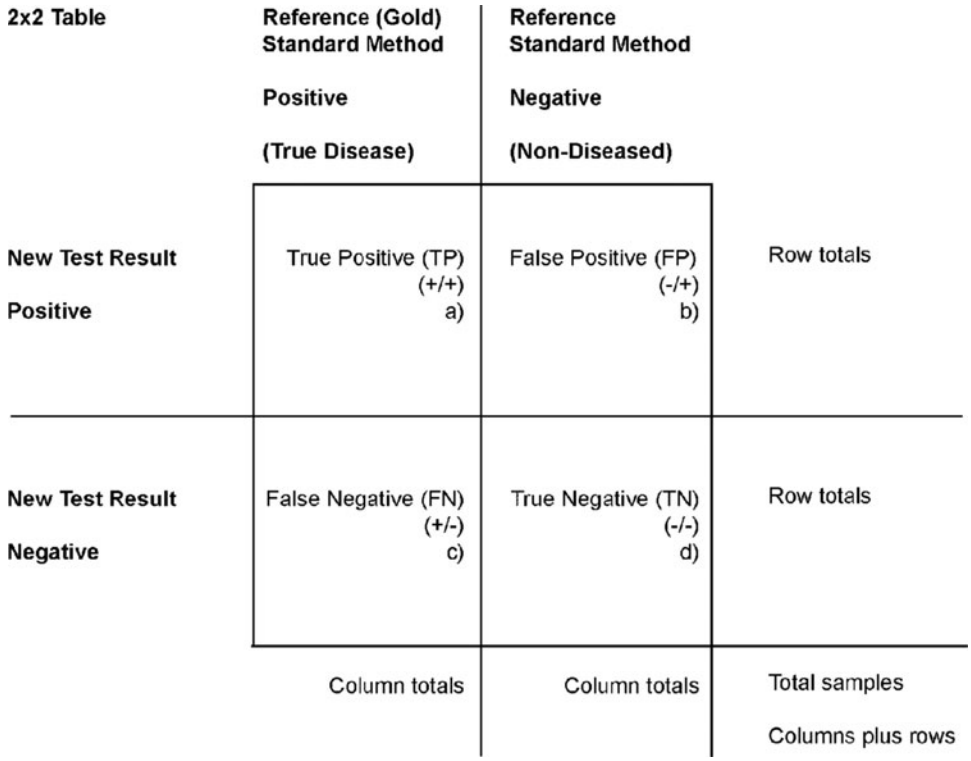


FIGURE 3 Illustration of a 2 × 2 table to diagram terms commonly used to describe assay performance during diagnostic method assessment. Fill in the boxes according to agreement status and calculate the column totals (down, up) or row totals (right, left), as indicated in Fig. 2B, to be used to calculate sensitivity, specificity, PPV, and NPV. Sensitivity = TP/(TP + FN) or a/(a + c) × 100. Specificity = TN(FP + TN) or d/(d + b) × 100. PPV = TP/(TP + FP) or a/(a + b) × 100. NPV = TN/(TN + FP) or d/(d + c) × 100.

the *r* value depicts the proportion of the change in *y* values (the new test values), which can be predicted by a change in *x* values (the reference test values). A positive *r* value depicts a positive relationship (correlation that graphs as a line with a positive slope, also known as a direct relationship), and a negative *r* value is depicted by a line with a negative slope (also known as an indirect relationship).

The line of equality is calculated from the *x* and *y* data points and represents the theoretical line which would result in a perfect or best-fit comparison. The line of equality provides a measure of agreement between the *x* and *y* values, because the graphic display allows for comparison between the actual data point to the calculated line of equality, depicted in Fig. 5, and shows increased variability at low microbial densities and less viability at higher densities. These differences are important, as is the *y* intercept of the equation of the line, which depicts shifts between the values of the old and new methods; one can have a high R^2 yet still have a shift in relative values between the old and new methods, typically representing an assay bias. When little or no bias is observed, the *y* intercept will be close to or at 0.

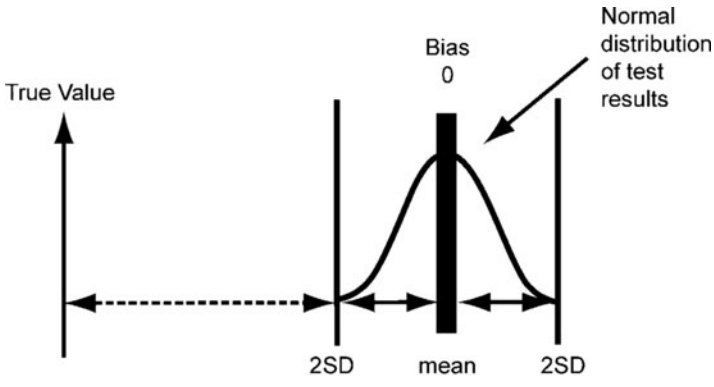
When comparing the quantitative values of two assays, especially when using methods for which multiple genotypes are being amplified, one should assess the difference between the new and the old methods, using regression analysis as described above, and determine how much the differences between the two conditions will impact results in the context of clinical decision making; in certain circumstances, what seems like a large difference can occur between two quantitative methods without causing difficulties in clinical interpretation (24).

Another useful tool, Bland-Altman plot analysis, supports assessment of the log differences between two assays or conditions and produces a graphical representation of the variability between the methods (Fig. 6). A Bland-Altman plot is a method of data-plotting used in analyzing the agreement between two different laboratory assays (10, 24).

Quantitative analysis, described above, should be performed for each genotype or subtype of the virus or microbe in question. Performing statistical analysis, as described in subsequent sections on groups of defined data, will help to assess whether or not the differences between two assays are statistically significant. Specificity testing among various genotypes/subtypes is important because it can affect assay sensitivity and quantitation. Quantitation can be affected by extraction efficiencies and contaminants in the eluate (i.e., salts) or by primer/probe binding due to sequence variation or secondary structures in the nucleic acid (20, 21, 26–27). Despite the fact that most viral load values are relative and not absolute values, the potential for under- or overquantitation/detection of a viral load or qualitative result may ultimately affect treatment or cause misdiagnosis of a patient depending on the treatment algorithm (28).

CALIBRATION MATERIAL

Calibration material and calibration curves play a role in assay performance. Performing calibration curves can be expensive when one considers the cost of commercially



	Integer IU/mL	Log_{10} IU/mL
Average	3,151	3.50
Standard Deviation (SD)	258	0.004
%CV	8.17	1.00
± 1SD	2,894 - 3,409	3.46 - 3.53
± 2SD	2,636 - 3,666	3.42 - 3.56
± 3SD	2,379 - 3,923	3,38 - 3.59
± 0.1 log or 0.2 SD	2,495 - 3,954	3.40 - 3.60

FIGURE 4 (A) Example of a medical decision interval defined by biologic and assay variability. Per CLSI MM06-A, the medical decision interval for HIV-1 quantitative assay is 0.5 log or 3-fold. Therefore, if the biological variability is already known to be 0.3 log, as in the case of HIV-1, then to meet the medical decision requirements for HIV treatment protocols, the assay precision must possess an SD of ≤0.2 log across the quantitative dynamic range. If this is true, the %CV will vary across the range, as in this example, which shows a 12% CV at 50 copies and a 4% CV at 50,000 copies. (B) Example of establishment of quantitative control limits by determining ranges based on assay tolerance limits, which are 0.2 log_{10} SD for a hepatitis B virus midrange control (n = 28 replicates). Note: for numerical values <10, two decimals are displayed; for values >10, no significant figures are displayed.

available controls and reagents. Historically, a calibration curve should be tested with at least two lots of reagents to ensure that there is no lot-to-lot variability. Initial testing of the calibration curve can be considered preliminary until at least 5 days of calibrators are performed. Since a calibration curve must be retested every 6 months, with each new lot, or if the controls reflect a problem, the ongoing evaluation of the calibration curve can be incorporated into assay validation as an ongoing QC/QA assessment. This will allow the continued monitoring of precision over the quantitative dynamic range.

ASSAY CONTROLS

Controls are also a component of LDTs. Whereas controls provided in a kit have acceptable ranges or cutoffs stated in a package insert, for LDTs it is the responsibility of the laboratory to determine what controls to use and what the ranges of these controls will need to be for a run to be considered acceptable. Controls for qualitative assays are different from those for quantitative assays because of the tolerance limits that are set for them.

Some laboratories have chosen to apply Westgard rules and 2 SD as a tolerance limit for their controls (29, 30). This strategy can be used but may result in an unnecessary number of failed runs. Other strategies include the use of 3 SD, 95% CI, 99% CI, or a simple C_T cutoff value based on the laboratory's experience with the breakpoints defined by true positives and true negatives as defined by the sample test set. These choices are under the discretion of the laboratory director; no single answer works for all circumstances. For example, when developing a qualitative assay, the control is typically tested at 1 log above the LOD. If the change in C_T values equals 3 C_T per log of analyte and the SD is 0.2 C_T, then using 2 SD as the range of variability will result in a control range of 0.4 C_T on either side of the mean (average) C_T value for the control. This calculates to <0.5-log difference as the entire acceptable range of variability, which may be too narrow for some assays. Ultimately, the acceptable tolerance level will need to be determined for each assay. Depending on how reproducible your assay is and on the precision of your technologists' pipetting during the evaluation period, the SD may create a tighter tolerance limit than required for the controls. The question that needs to be addressed when setting control cutoffs is "What is the tolerance limit for the assay?" (Do you select 0.5-log, a 3-fold change, a 2-fold change, etc.?) The same circumstances hold true for quantitative assays.

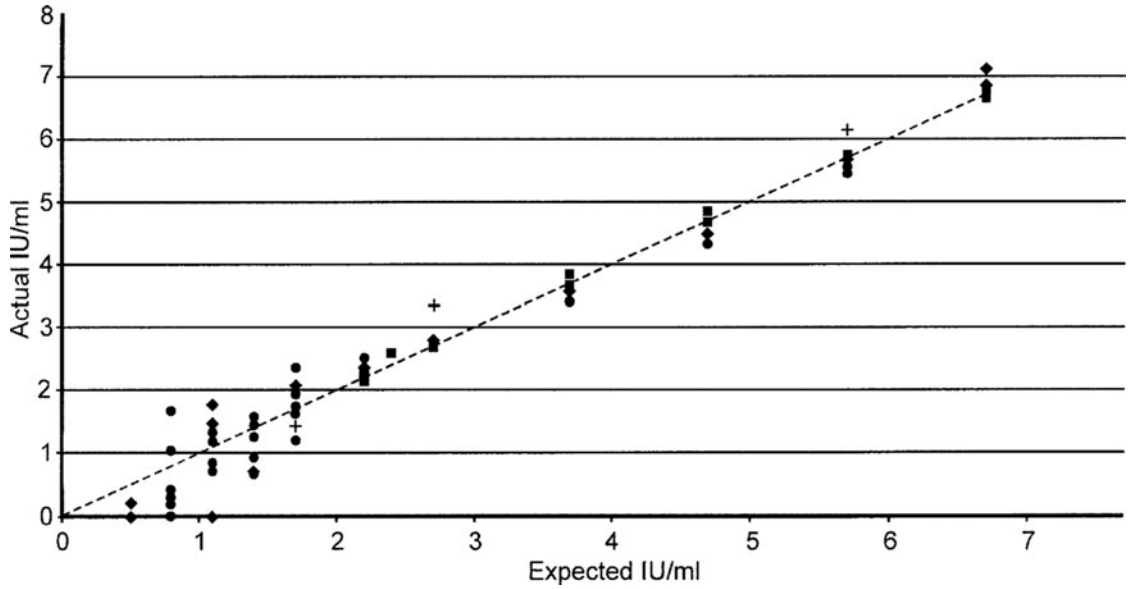


FIGURE 5 Example of calibration curves showing a standard curve with increased variability at the low end of microbial density (expected versus actual IU/milliliter, purchased standards [0–6.7 log IU/ml], real-time PCR, hepatitis C virus). The Poisson distribution characterizes the behavior of the analyte and precision at the LLOD.

In the previous section we discussed how precision can affect a quantitative assay. The medical decision interval for a viral load assay will be affected by both the biological variation of the virus in the body and the imprecision of the assay (CLSI Document MM06-A2) (23). For many laboratories, using 2 SD to establish control ranges is commonplace. However, given how good the precision is already in many real-time PCR assays, applying a 2-SD rule as the tolerance limit may result in a very tightly defined tolerance limit. For example, if your control has an average value of 3,151 copies/ml and an SD of 258, this would be equal to a log value of 3.5 copies/ml with a 0.035-log SD. The 2-SD tolerance limit for this assay would be 2,636 to 3,666 copies/ml or log 3.42 to 3.56 copies/ml. The 3-SD tolerance limit for this assay would be 2,379 to 3,923 copies/ml or log 3.38 to 3.59 copies/ml. However, if the tolerance limit of 0.2-log SD is applied, the range of the assay is now 2,495 to 3,954 copies/ml or log 3.40 to 3.60 copies/ml, which is closer to the 3-SD range (Fig. 4A and B). Thus, using 2 SD to set the tolerance limits may set the control range tighter than it needs to be for a valid run, resulting in unnecessary failed runs. All controls will need to be monitored on at least a monthly basis (more often if the assay is run more frequently) to make sure they maintain their performance and defined tolerance limits. The emerging use of Six Sigma metrics will help laboratories better define assay precision, predict defects, and help determine the number of QC runs that need to be included to fully control the assay process,

For both qualitative and quantitative methods for verification of molecular assays, a working knowledge of biostatistics is required. Many references are available. We

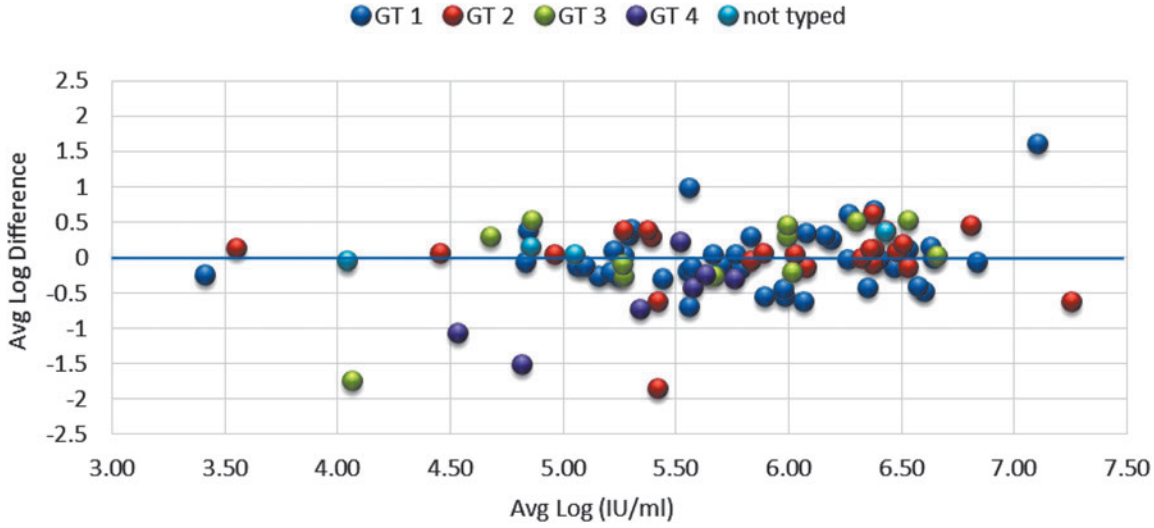


FIGURE 6 Example of Bland-Altman plot analysis to characterize the log difference between two assays with relationships to different genotypes.

cite two books that are helpful to analyze laboratory data (31, 32), as well as several statistical software packages with user-friendly interfaces, as described in the following sections.

BASIC BIOSTATISTICS IN CLINICAL MICROBIOLOGY

Statistics Are an Important Tool

Understanding statistics is vital for the interpretation of current literature, assessing the performance of a reference laboratory, and performing and reporting molecular method verification and validation for the College of American Pathologists, etc. In addition, mindful participation in clinical trials, translational research, basic research, and publication of your data require an even higher level of understanding. Even if one obtains the referral services of a biostatistician, to have a productive conversation with that expert, one must acquire at least a working knowledge of the language that is biostatistics.

Verification of LDT methods comprises a large proportion of efforts in many molecular microbiology laboratories. Clinical laboratory research is, in essence, translational research, in which our purpose is to describe, explain, predict outcomes, and control our systems. This review focuses on those aspects of biostatistics that are relevant to the verification and validation of new laboratory assays. In truth, we all know that variability is inherent to any assay; we accept that as an inherent aspect of any laboratory test. The question we must ask is "How much variability is too much?" The following sections define and describe key aspects of verification experiments.

Planning the Verification Process

To begin a process of method verification, one must adhere to the standard principles of experimental plans. Before data collection begins, it is best to establish parameters for your experiments. For scientific methods, there are nine steps that are useful when planning method development in a clinical microbiology laboratory.

1. Identify and document a problem.
2. Develop a definition of the problem.
3. Identify possible solutions.
4. Formulate a hypothesis based on critical medical decision points.
5. Select a statistical analysis plan.
6. Decide the best way to measure, collect, and analyze the data; develop data collection forms and collect data.
7. Analyze data/interpret results; determine how many measurements to take (determine sample size based on power analysis).
8. Formulate conclusions and action plan.
9. Document your entire process.

When you begin your verification experiment(s), it is important to plan and prepare appropriately. It is important that statistical principles be considered in this phase of planning and/or that a biostatistician be consulted. In general, the required planning steps and tips for creating a smooth and seamless process are as follows:

1. Plan experiments: use software and/or enlist the assistance of a biostatistician to help you plan.
   i. Select medically relevant decision criteria.
   ii. Choose your data entry software and data collection method.
   iii. Prepare data collection logs and set up log sheets or spreadsheets to avoid data entry error and incompatible file formats.
   iv. Establish a file naming system that reflects the types of testing date, operator, and purpose.
2. Develop your analysis plan and select statistical tools.
   a. Determine the data types of the variables (categorical, ordinal, or continuous) and assign scales of measurement.
   b. Document assumptions and potential confounders which will impact results.
   c. Determine sample size based on power analysis.

Formulating a Hypothesis

Hypothesis testing is meant to determine if the variation between two sets of data (sample distributions) can be explained through random chance or not. Therefore, a good hypothesis will:

1. Start with a research question that is concise, testable, and measurable.
2. Assume equivalence; you must reformulate (change) the research question into a format that is compatible with subsequent statistical analysis. This form is called a null hypothesis (i.e., it is a statement that says there is no change). This type of hypothesis is called a nondirectional hypothesis (it assumes the methods are equivalent).
3. Contrast the null hypothesis with an alternative hypothesis (i.e., a statement that says there *is* a change). Note that options for the clinical laboratory include those that assume a new test is better than an existing test. When this type of improved gold standard assessment is expected, it is acceptable to consider a directional hypothesis (i.e., ask if the method is better or worse), versus a nondirectional hypothesis (i.e., ask if the method is equivalent).

Developing and Selecting the Proper Statistical Measures and Test Parameters

Development of your plan for statistical measures requires the following: (i) an experimental design, (ii) a sampling strategy, and (iii) a study design, which can be classified as either nonexperimental or experimental.

Once a hypothesis is developed, you must select your variables. A variable is an "any" characteristic that can be measured or categorized. We often use descriptive statistics to characterize the variables (occurrence, distribution, frequency, etc.), offering no explanation or hypothesis. These types of descriptive statistics may generate a hypothesis for a later experimental study or give insight into the magnitude of the problem.

Some definitions are required to fully understand the experimental design. Dependent variables and independent variables have a relationship to each other that responds to change. The dependent variables are those that are observed to change in response to the independent variables. The independent variables are those that are deliberately manipulated to invoke a change in the dependent variables. In short, "given x, then y occurs," where x represents the independent variables and y represents the dependent variables. Depending on the context,

independent variables are also known as predictor variables, regressors, or explanatory variables. The dependent variable is also known as the response variable, the regressor, the responding variable, the outcome variable, or the experimental variable.

Determining the Type of Experiment to Use

An experiment occurs when one or more independent variables are manipulated to determine their effect on a dependent variable—a purposeful (controlled) manipulation of the independent variables to determine cause and effect. To perform a true experiment, one needs to define experimental and control groups. There are several experimental designs that may prove useful to method verification in molecular microbiology; they include the following:

- **Descriptive, exploratory, or observational studies.** Examples of this type of study might include assessment of one group of individuals with a pretest and a posttest, a static group comparison, or a prevalence survey.
- **Experimental, interventional, or analytical study designs.** These studies are the centerpiece of scientific study and apply to many aspects of clinical laboratories. Often clinical laboratorians ask questions but do not pose them in the form of a scientific study and therefore lose the opportunity to assess the true merit of their work.
- **Pretest and posttest.** Common laboratory experimental design may focus on a pretest and a posttest with a control group or perhaps simply a posttest with only a control group. For comparisons between subjects or within the same subject at different time points, inferential statistics are used.

Selecting an Experimental Design

There are several key approaches to designing experiments for the clinical laboratory. Often, a laboratory simply begins the process without thought to the design or experimental plan; however, thoughtful selection of the design may improve and even speed the process of verification. Two choices for experimental design are common: a simple method comparison and a clinical utility design.

Simple Method Comparison Design

With the simple method comparison approach, a selected population is used to represent a variety of conditions, for example, positive, negative, or indeterminate results. For this design, a general rule of thumb is to use a minimum *n* of 20 to 40 samples. The minimalist approach would be considered acceptable for FDA-cleared methods, which are well published in the literature and do not have particularly high risk or potential for liability. It may also be acceptable when large differences in performance are expected between two methods. With this minimal data set, there would generally be insufficient power to detect small differences between two methods. Likewise, it may not be sufficient to prove equivalence of methods. It would generally not be considered sufficient for verification of ASR or LDT methods.

Clinical Utility Design

The clinical utility approach is required for ASRs and LDTs. With this approach, "unselected patients" are used from within a population. This can be thought of as a "taking all comers" approach. This design is often selected when ASR or RUO methods are verified, since the sample population should closely mimic the population of samples that occur under normal operating circumstances. The methods can be assessed under conditions of typical disease prevalence; therefore, PPV and NPV are expected to be indicative of what they will be when the method is offered on a daily basis. Clinical utility is assessed in a typical patient population. The rule of thumb, used when a more thorough method of assessment is required, would be to have a sample population of >40 to 200 samples. This approach is useful for ASR assay verification, when clinical laboratories are required to document their own clinical utility. For this approach, it would be prudent to consider performing an ROC to maximize the clinical utility of a quantitative method.

Choosing a Specimen-Sampling Strategy

Akin to method design, sampling strategy is essential to the outcome of method verification, yet it is often overlooked. To begin, simple definitions and examples are provided. For instance, a sample is a subset of a larger population. Sampling is driven by experimental design, population demographics/profile, the required statistical power of the analysis, the resources available, and the original purpose of the sampling. As an example, laboratory staff may select a sample composed of positive and negative specimens with a preselected positive prevalence to determine an assay's general performance characteristics for that sample. Under these circumstances, it is prudent to test the assay's performance in a sample with a prevalence similar to that of the general or local population served by the laboratory.

Whenever one defines a population, one must critically assess the answer to the following question: "How representative is the sample of the large population?" For example, if one considers an infinite number of subjects in the universe, the definition is a true population; this is obviously impractical but true to the definition. Equally impractical would be a sampling of all subjects in the state of Pennsylvania or even all subjects in Danville, Pennsylvania, but in reality even a much smaller sample, such as all people in one intensive care unit from one hospital in Danville or all positive methicillin-resistant *Staphylococcus aureus* samples from an intensive care unit in a 5-year period, may be nearly impossible to obtain. As laboratory staff, our samples might be typically represented by a subset of positive methicillin-resistant *S. aureus* samples obtained during 1 week from an intensive care unit in one hospital in Danville, Pennsylvania. While this approach might be practical, it is not likely to be representative of the kinds of diverse samples the assay may eventually encounter. Because convenience sampling may be our reality, one quickly understands the limitations of performing statistical analysis or performance characteristics that are representative of a true population. We do our best to represent reality as we know it, but there are limitations to all laboratory assessments, and it is prudent for laboratory staff to minimize those limitations by understanding the pros and cons of each possible sampling strategy. The different types of sampling strategies from within a population are the following:

- **Random sampling.** Each individual in the population has an equal and independent chance to be selected *once*. This is the preferred sampling strategy of many clinical trials, but one limitation exists. Despite the advantages of random sampling, one must ensure that the

study is sufficiently powered; otherwise, if the sample size is too small, the sample may not represent all groups of interest.

- **Stratified sample.** The sample is selected from all groups of importance. For instance, one may choose to "match" the samples by age, gender, race, disease state, morbidity, etc. When a sample is matched, the sample is designed in such a way as to be proportional to the population. Many circumstances force us to assess a sample that is not proportional to the population, a situation which is less than optimal. For example, one may select more positive samples to test than exist in one's normal population of subjects. This situation can occur when laboratory training sets require bench technologists to test sufficient numbers of positive samples to achieve competency. While practical and expedient, whenever one tests a sample with higher prevalence than the usual hospital population, the assay's predictive values will be skewed. In this situation, the PPV will appear higher than it will actually be when the test is performed under normal operating conditions.
- **Convenience samples and volunteer samples.** These types of samples are found in many laboratory verification and validation sets; however, there are serious limitations to this approach. This type of sample is called a "nonprobability sampling," and under these circumstances, the probability that an individual sample is selected is unknown. This type of sample set produces results which are prone to bias. Do not assume that results derived from this type of sample are representative of a larger subject population.

Identifying Data Types Associated with Key Variables

To ensure that the data you collect can be efficiently and accurately entered into statistical programs for subsequent analysis, you must classify and identify the type of data that will be created in your experiments. Improper categorization of data type will lead to improper use of statistical analysis tools, and incorrect conclusions may be drawn from your data. There are four major types of data.

- **Categorical data** (nominal data) are data that are divided into several categories (e.g., high-risk, moderate-risk, low-risk or positive, negative, or indeterminate). Note: If only two categories are possible (e.g., yes/no), then the term "dichotomous data" is appropriate.
- **Ordinal data** are categorized and placed in order (rank) when categories are unevenly distributed (e.g., age categories such as 0 to 1, 2 to 6, 6 to 13, and >13 years).
- **Interval data** are categorized into evenly spaced categories (e.g., 1+ to 4+).
- **Continuous data** can be categorized, ranked, and placed on a number line. Continuous data require decimals (e.g., volume, temperature, viral load, C_T value, and time) and descriptive statistics such as the mean and variance. Of note, "discrete data" are the same as continuous data, except the numbers are *whole* numbers only.

Determining Acceptable Error, Power, and Sample Size

Perhaps among the most misunderstood aspects of laboratory method verification is the concept of statistical power, particularly as it relates to determination of sample size. It is critical to understand and select the appropriate statistical power and to use appropriate software to calculate the required sample size for your experiments, based on your assumptions and the medical criticality of your method or clinical practice. Confounding the issues of appropriate sample size, laboratory consultants are faced with high costs of method verification and, in some cases, the problem of finding enough samples to adequately characterize rare disease testing or rare sample types. Therefore, it is prudent to consult with a biostatistician until you are comfortable performing your own power analysis.

The first aspect of performing a power analysis is to select or determine the criteria for "allowable" or "acceptable" errors, i.e., the critical values outside of which the percentage of errors would be unacceptable to a reasonable clinician. Acceptable error is typically context dependent and integral to the study question(s). We often speak of results in terms of their "statistical significance," which simply means that the results are outside the range of predetermined acceptable errors in the hypothesis-driven framework of your experiments. Statistically speaking, a result is called significant if it is unlikely to have occurred by chance alone. A statistically significant difference means that there is statistical evidence of a difference; it does not mean the difference is necessarily large, clinically important, or significant in the usual medical sense of the word. Rather, the significance level indicates the percentage of results outside the chosen cutoff limits (critical values and acceptable errors).

There are two important error types: α (alpha, also known as type I) error and β (beta, also known as type II) error. When choosing a level of statistical significance, there is an inherent trade-off between the two types of errors.

α Error (Type I Error)

An α error is the probability of concluding that there is a difference when there is no real difference; the difference is by chance alone. It can also be thought of as the probability of a false-negative result, the *P* value, or the significance level. The α value is typically set at 0.05, which means that in 5 of 100 times, the observed difference is due to chance alone, and 95% of the time it is due to a real difference ($P = 0.05$ means that the concluded difference is real 95% of the time under the assay conditions).

β Error (Type II Error)

A β error is the probability of concluding there is no difference when there is one. It can also be thought of as the probability of a false-positive result. After selecting acceptable errors, one must determine or approximate the expected differences in the assay results. Differences are defined as differences in proportions of positive results or differences in actual assay values, for example, the percentage of true-positive results improved from 80% to 85%; or mean C_T value changed from 25.5 to 21.5, or % accuracy improved from 90% to 93%.

The probability of not making a β error $(1 - \beta)$ is called the "power" of the statistical test.

Power is the probability of concluding that there is a difference when there is a difference, in other words, the probability that your study will detect a difference, given that one truly exists. One example can be described in the following manner. A power of 0.90 indicates you are 90% sure that your study can identify a true difference at a preselected α error. For instance, if $\alpha = 0.05$ and power = 0.90,

then only 5% of the time could the difference be due to chance; you are 95% sure the difference is real, and you are 90% sure that the study is powerful enough that it could detect that "real" difference among the number and types of samples that were tested.

In statistical terms, "absolute power" = 1, and power will move closer to 1 when the sample size increases (as *n* becomes larger), the differences between numeric means increase, precision increases, i.e., sampling error decreases, and α increases (e.g., α of 0.01 increases to 0.05).

While absolute power is not an assay verification requirement, there are circumstances and conditions when we should choose conservative power limits (i.e., the highest power). High power should be selected for studies of very significant consequence, for studies in which the sample set is very diversified, for studies in which small or minute differences in results are anticipated, if variables are complex or difficult to control, or if test measures are relatively unreliable or imprecise. You should also select high power when you are expecting to encounter the need for subgroup analyses to eliminate bias from confounding variables.

The estimated sample size is based on the experimental power requirements. Sample size is defined as the count or the number of measurements and is also known as *n*. To determine the required sample size for your experiments, you must preselect α and β. Typically, α is set at 0.05, and a typical β value for laboratory studies is ≤0.2%, which makes the typical power ≥80%. The power of the analysis should be predetermined; ideally, it should be ≥90%; however, the resulting sample size can sometimes be cost-prohibitive. Nevertheless, developers of LDTs must remain cognizant of their legal responsibility for accuracy in LDT methods. If 80% power is selected, continued assessment and correlation with clinical data may be necessary. In all cases, as a laboratory consultant, one must be mindful of the assay limitations, including those related to sample size

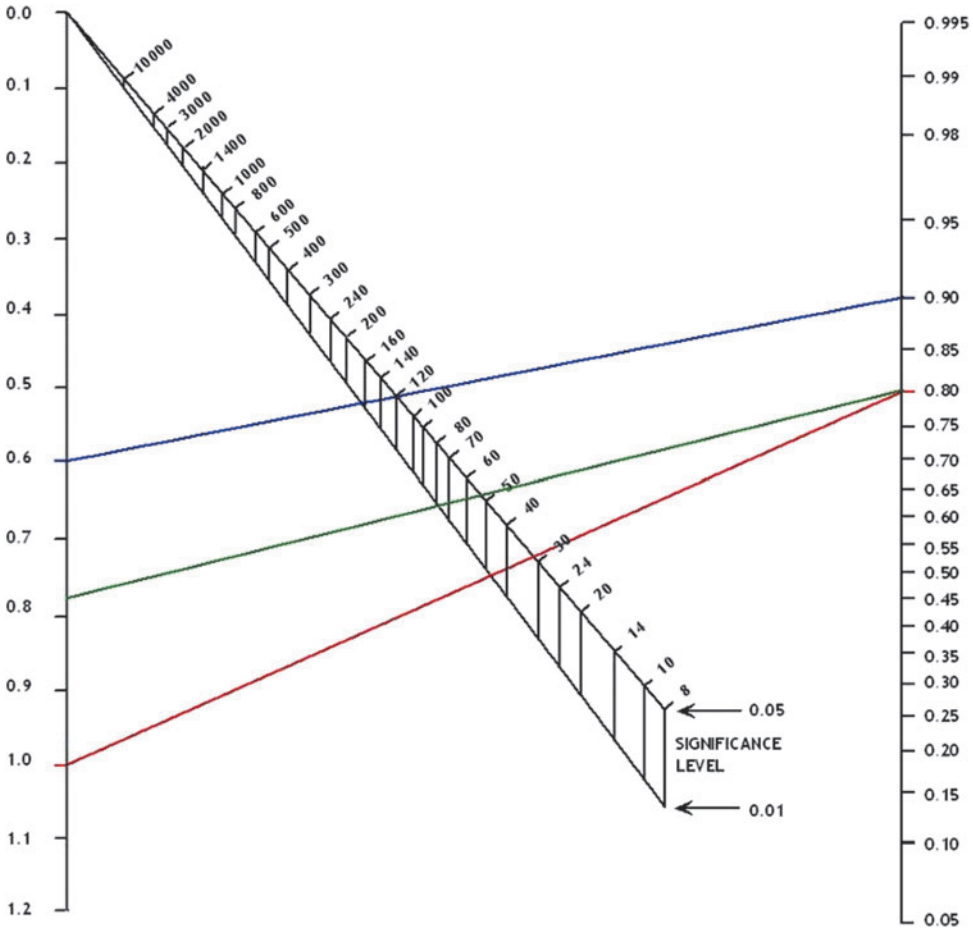


FIGURE 7 Altman's nomogram for estimating sample size is based on predetermined selections for alpha, and the expected differences in proportions of attributes between new and old methods. It is a graphical method that can be found and described at the following website: http://www.statsref.com/HTML/index.html?sampling.html. To use the nomogram, mark the standardized difference on the left vertical scale and the statistical power you require on the right vertical scale, then draw a line between these points and read the sample size from the ladder section, depending on whether you chose an alpha of 0.05 or 0.10. In the figure, the point at which the blue line (0.6 proportional difference and 0.9 power) intersects the ladder-like sloping lines will provide the estimated sample size for significance levels 0.05 and 0.01, respectively. Reading from the scaled ladder, we can read $N = 120$ and $N = 160$ for P values of 0.05 and 0.01, respectively. Thus, the total sample size required in each group you compare (e.g., new method and old method) could be 120/2 = 60 each for alpha 0.05.

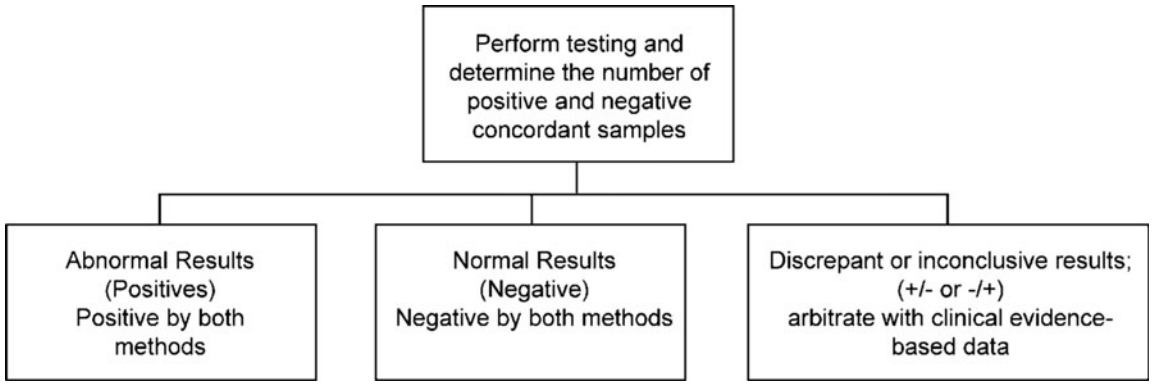


FIGURE 8 Flow diagram depicting categories of results and options from a qualitative method verification. Describe data with descriptive statistics and 2 × 2 tables, and then analyze data with inferential statistics.

and power. Power nomograms, as depicted in Fig. 7, can be used to assist in crude estimates of sample size.

Selecting a Statistician or an Appropriate Statistical Software Package

Until a laboratorian becomes fully competent with statistical analysis, there is no substitute for collaboration with a biostatistician who is familiar with the needs and operating environment of the clinical laboratory. Good communication and an understanding of simple statistical terms will be required to make that collaboration useful and successful.

Notwithstanding, there are many ways the clinical lab staff can self-educate to improve this collaboration and perform the appropriate statistical analysis themselves. Most laboratories have access to a copy of Microsoft Excel, which contains a number of statistical commands. Excel is not optimized for clinical laboratory use, nor is it equipped with detailed help files for the neophyte in statistical analysis. Several software programs will provide laboratory staff with a higher level of support for statistical analysis. EP Evaluator (David G. Rhoads Associates, Kennett Square, PA) is available for purchase or as fee-for-service via EP Evaluator online (http://dgrhoads2.com/cgi-bin/framework.exe?PG=Home). EP Evaluator is a software program designed for the clinical laboratory user. It meets all CLIA '88 and College of American Pathologists requirements for validating and evaluating methods. Another, more diverse program is JMP Statistical Discovery software (SAS Institute); JMP (http://www.jmp.com/software/) is a complete statistical package and also includes Sigma Six quality analysis and help files that are very useful for the new user. A variety of packages, ranging from analysis to power and sample size calculators, are available from GraphPad Software (http://www.graphpad.com), whose GraphPad and StatMate programs are friendly for the new user. Full statistical analysis programs, such as JMP, STATA (StataCorp), and SPSS (SPSS, Chicago, IL), are programs that are often used by biostatisticians and represent full capacities for data analysis, data mining, discovery, and statistical analysis.

Collecting and Validating Data

Data collection and validation are tasks that are easily understood by the clinical laboratory employee. Data must be accurately collected and checked (validated) for accuracy. If manual data collection occurs, it is more prone to human error and should be scrutinized closely. Computerized data transfer is less prone to error but must also receive audits on a regular basis to check data for validity. As with clinical laboratory results, data input should be checked by a third party to maximize accuracy. Determine the concordance of positive and negative specimens as well as the discrepant or discordant results, as depicted in Fig. 8.

Using Software/Statistician to Describe and Analyze Data and Assay Performance

Descriptive Statistics

Descriptive statistics are commonly used for assessment of qualitative molecular methods. Examples of descriptive statistics, which can be calculated and used as a basis for method comparisons, include proportions or percentages, such as percent agreement between a new method and the reference method, percentages of samples whose matrix is inhibitory to PCR, and percentage of contaminated tests, if any. Descriptive statistics can also be used to characterize the typical performance criteria created by the 2 × 2 tables, such as analytical and clinical sensitivity, analytical and clinical specificity, and PPV and NPV. Instructions for calculating a 2 × 2 truth table are depicted in Fig. 3, above.

Another perspective is that descriptive statistics can describe two aspects of method performance: probability and inference. Probability is defined as the number of likely outcomes divided by the number of possible outcomes. Probability ranges between 0 and 1, with 1 being 100% probability. Inference moves one step further, defined as the prediction of an outcome based on the calculated probability.

Descriptive Measures of Central Tendency

Perhaps one of the most important aspects of data distribution analysis is its use for the selection of the proper downstream statistical analysis as the choice for analysis where parametric or nonparametric statistics is required. There are two common data distributions that affect those choices. One is the normal distribution, or bell curve (for data that achieve a normal or near-normal data distribution), and for data with normal distribution, parametric tests (such as the *t* test) are the appropriate data analysis tools. However, they are inappropriate unless the data set achieves a normal distribution. It is a common error to overuse parametric statistics. The other data distribution is skewed distribution, for which data analysis requires the use of nonparametric

statistical tests instead. To determine the appropriate analysis statistic, it is best to graph the data and describe data characteristics with graphical or numerical representation of the data distribution. Histograms or curves are especially useful to display data distribution and characterize the populations or samples that were tested. Displays of midpoint and central tendency help the user to assess whether the data are normally distributed or skewed. The various displays illustrated in Fig. 9 and 10 are clear and useful for this purpose.

- Histograms depict distributions and percent simple probability (Fig. 9).
- Frequency distributions portray the frequency of a given result, *x* (Fig. 9).
- Relative frequency depicts the probability. Probability = *x*/*n*.
- In a box-and-whiskers plot, whiskers are ranges, with outliers depicted outside the whisker (Fig. 10).
- Quantiles are a type of box-and-whiskers plot that provides information about values at which a specific percentage of the data is less than or equal to that value (Fig. 10): the 75% quantile, also known as the upper quartile, is where $P = 0.75$; the 50% quantile is called the median, or midpoint, and is the data point beneath which 50% of actual data lie and $P = 0.050$; the 25% quantile, also known as the lower quartile, is where $P = 0.025$.

Three Important Numerical Measures of Central Tendency

In addition to graphical representation, three calculated parameters are used to describe central tendency; they are the mode, median, and mean. The mode is used as a measure of central tendency for categorical data, and one can observe more than one mode for categorical data, depending on the number of categories. The median is used for ordinal data or for continuous data that are skewed; for example, at the 50% percentile, one-half of the data are above the median and one-half of the data are below the median. The mean is the average and is calculated by the sum divided by the sample size. When used for continuous data with normal distribution, the mean typically provides the most information. However, the mean can be skewed by outliers and is not the best measure for data containing legitimate outliers.

Descriptive Measures of Variation

Descriptive statistics can also be used to characterize variation, also known as dispersal. The definitions below explain terms that are used to discuss variability.

- **Range:** The spread between the highest value and the lowest value.
- **Outlier:** A value so far away it will disproportionately influence the estimate of the mean.
- **Standard deviation (SD):** Also known as *s*. The square root of variance, a measure of variation around the mean, the average deviation from the mean. Calculators or software can compute the SD, and it is common to represent data as the value ± 2 SD. For typical data sets, an *s* of >2 or 3 is rare (Fig. 11).
- **Quantile:** Subrange with an even number of data points instead of even intervals.
- **95% confidence interval** (95% CI): A region around the mean.
- **Coefficient of variation** (%CV): The ratio of the SD to the mean (commonly used but provides little information of value)
- **Variance:** Measure of spread about the mean. Variance is SD squared (s^2), with $n - 1$ degrees of freedom (*df*).
- **Standard error of the mean:** The average deviation from the mean as it relates to sample size.

Characterize and Understand Bias

To assess dispersal error, consider that error is unavoidable; the question we must ask is "How much is too much?" The answer to that question depends on the criticality of the assay and the need for accuracy (precision). Typically, error is acceptable when error is random, is not biased, has the same magnitude across groups, and has a relatively small or inconsequential effect on the clinical outcome. Bias presents the laboratorian with more challenges. To understand bias, one must understand the difference between systematic error and random error as depicted in Fig. 12. Systematic error typically leads to bias in the results of the new method. One must determine what, if any, bias is acceptable in the outcome of the verification before the test is performed to derive patient results. Two major threats to diagnostic result validity, spectrum bias and test review bias (33), can be circumvented by adoption of the STARD criteria (5–7), i.e., guidelines for the quality and reporting of test accuracy. A recent publication by Bachman et al. elucidates a mechanism to improve upon the STARD criteria (33), adding a requirement for multivariable statistical adjustment and not only reporting variation of diagnostic accuracy across subgroups but also performing multivariable adjustments on test performance, if they are warranted.

INFERENTIAL STATISTICS

Inferential statistics are used when one is performing hypothesis testing of tests of association. These statistics are used to make inferences about a population based on the results from a sample of that population and thereby predict characteristics of a larger group. Inference involves probability calculations, which are a type of prediction. Based on probability theory, calculations yield numbers that indicate how likely it is that your claims are correct. Therefore, the calculated probability means "This is how sure I am, knowing I could be wrong." There are two categories of inferential statistics: nonparametric and parametric statistics.

Nonparametric Inferential Statistical Tests

Nonparametric statistics are used in the following situations.

- Data are categorical (qualitative or ordinal): both independent and dependent variables are categorical or nominal.
- The data distribution is not normal.
- The variance is not homogeneous.
- The assumptions required of parametric statistics are not met.

Chi-Square Test (Cross-Tabs)

An example of a nonparametric test is the chi-square test, a qualitative test that is based on a 2 × 2 table and is also des-

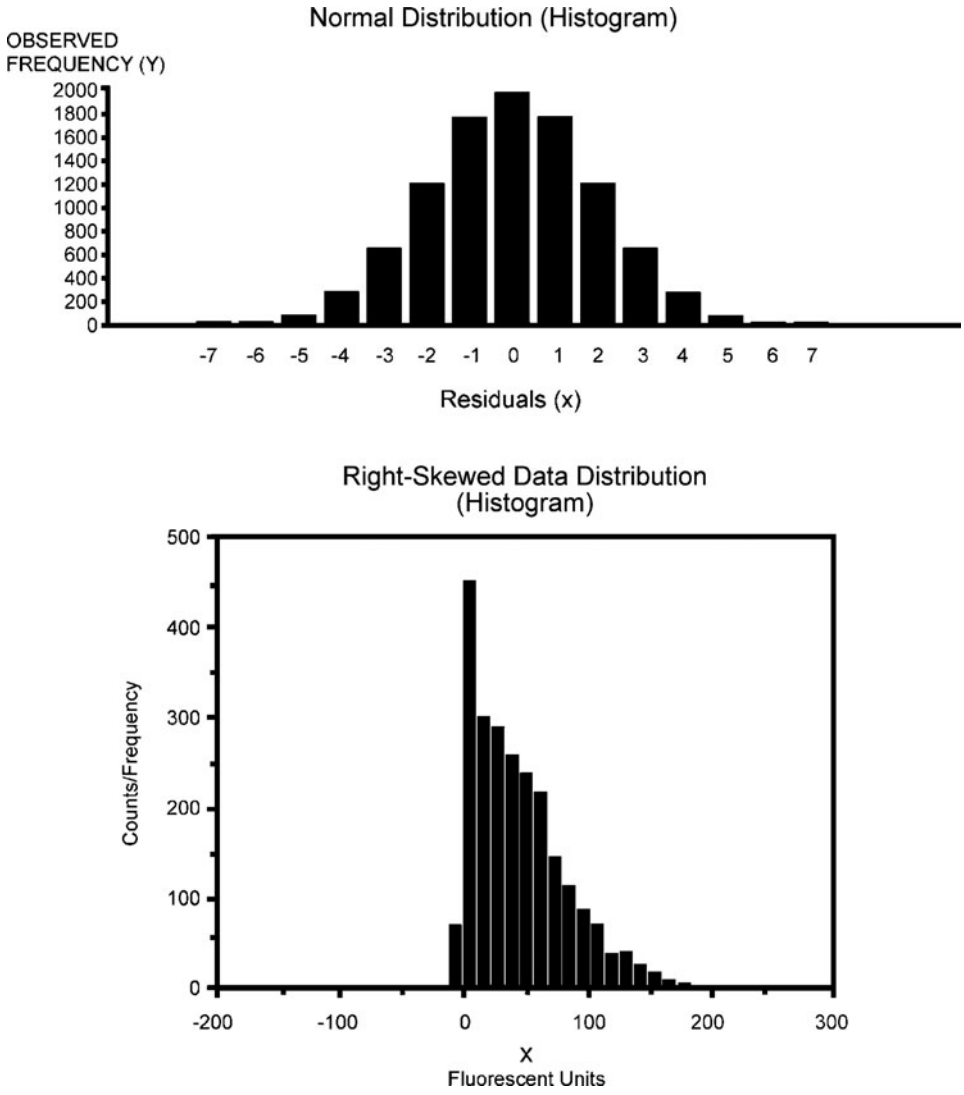


FIGURE 9 Graphing the distribution of data. One can use histograms or curves to display data distribution in the form of frequency distributions, which are important to assess whether or not the data have a normal distribution, as depicted in the top diagram. If the data do not have a normal distribution (e.g., bottom diagram), one cannot use parametric statistics, such as the *t* test, to compare the means; one must use nonparametric statistics. One can use box-and-whisker plots and quantiles (described in Fig. 10) to help assess the midpoint of the data.

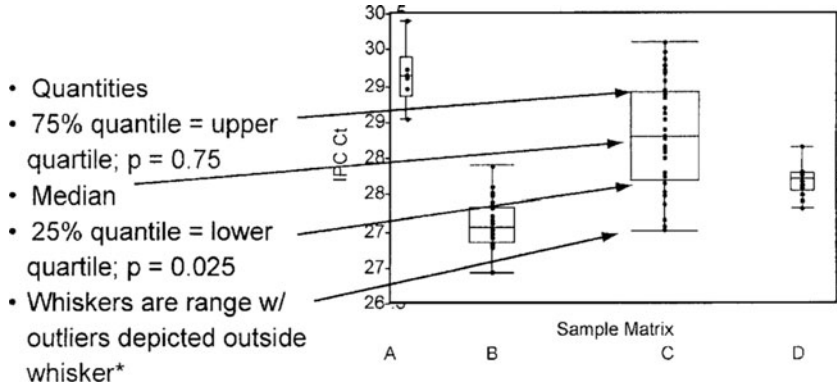


FIGURE 10 Descriptive midpoint measurements, depicted in a box-and-whiskers plot, displaying the major quantiles. Outliers have undue influence on the mean; therefore, the mean may be misleading, depending on the magnitude of the variation from the median.

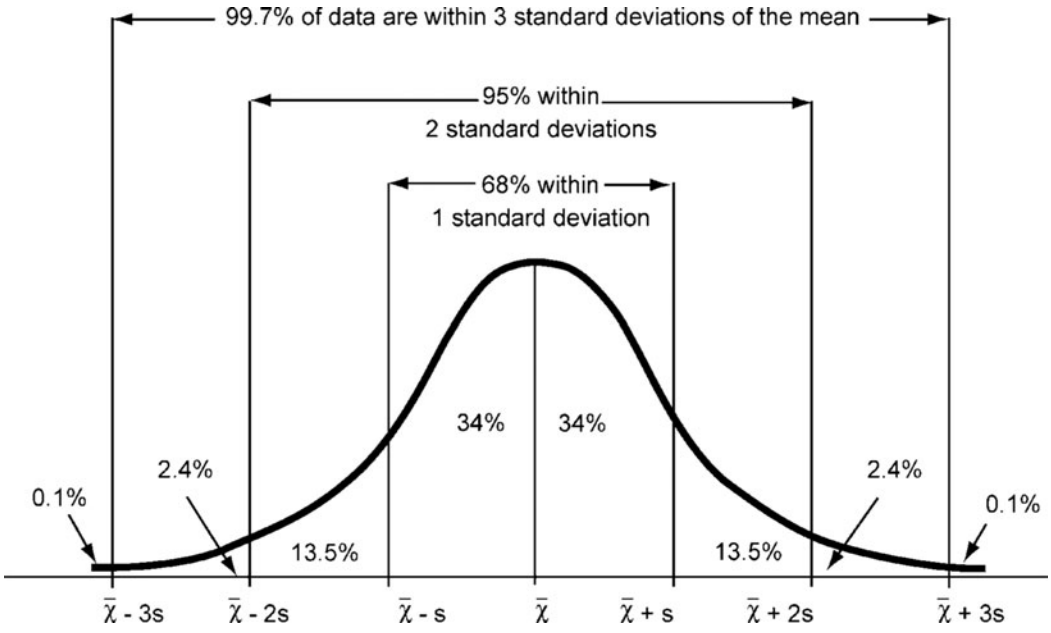


FIGURE 11 The empirical rule. For data sets with a normal bell-shaped distribution, the following properties apply: about 68% of all values fall within 1 SD of the mean; about 95% of all values fall within 2 SD of the mean; and about 99.7% of all values fall within 3 SD of the mean.

ignated chi^2 or χ^2. The chi-square test is used to compare frequencies of data (the distributions) occurring in different categories (groups). It tests whether data distributions are different by group or vary within different groups. It compares the observed frequencies to the expected frequencies of the categories. It also determines the association between independent variables and dependent variables by counting the frequencies within the distribution. The validity of the chi-square test is best if each category has more than five data points per chi-square cell, but it is acceptable if at least 80% of the categories have at least five data points per category and all have at least one data point per category. An example of a chi-square hypothesis might be as follows.

The Null Hypothesis (H0)

- Qualitative H0: the frequencies of the control (reference population or gold standard) = the frequencies of the experiment (new test) (i.e., there is no difference between the two tests in a 2×2 table format).
- Qualitative H0: the proportion of controls – the proportion of the experiment data = 0.
- Quantitative H0: the mean of the control – the mean of the experiment = 0.

Alternate Hypothesis (HA)

- Qualitative HA: the proportions are not equal or the proportion is higher (e.g., % positives).
- Quantitative HA: the means are not equal or the mean is higher (e.g., C_T values) (hypothesize by how much).

For any cell, the expected cell frequency is the product of the sample size and the probability: cell frequency = (n) × (probability of condition). In a population, we compare observed frequencies or proportions with expected frequencies or proportions. The chi-square, goodness-of-fit, compares observed frequencies with expected frequencies (expected = gold standard or theoretical) and determines if the differences are real or if they are due to random sampling variation; i.e., it is used to determine if experimental frequencies (i.e., those observed with the new test) are significantly different from the expected frequencies (those obtained with the gold standard test).

To perform a chi-square test, arrange data in contingency tables (2×2, 2×3, $2 \times n$); normally, the criterion for rejecting the null hypothesis is set at $\alpha < 0.05$. Then, compute the chi statistic: determine the status of the null by comparing the calculated chi-square to the critical chi-

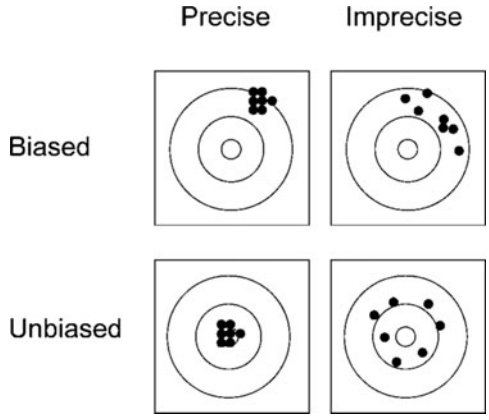


FIGURE 12 Systematic error (bias) versus random error. A calculation can correct for a bias, but imprecision cannot be corrected by calculation. (Top left) Good precision with poor accuracy gives a biased result (the average is off center). (Top right) Poor precision and poor accuracy give a biased result (the average is off center). (Bottom left) Good precision and good accuracy give an unbiased result (the average is on center). (Bottom right) Poor precision with good accuracy gives an unbiased result (the average is on center).

square values or use software to obtain cross-tabulations and chi-square statistics. (Note: The CHITEST function in Excel will show only a *P* value; other statistical software will provide more information.)

For the chi-square test, if *P* is <0.05, your decision is to reject the null hypothesis (that there is no difference) and conclude that there is a difference. Caution must be exercised in the performance of the chi-square statistic, for it is prudent in that an inability to reject the null hypothesis does *not* prove test equivalency but merely proves that there is either no difference or that there were not enough samples tested to prove a difference; one cannot tell one explanation from another.

What if we don't want to prove a difference? One must appreciate the fact that calculating a statistical comparison that is not statistically different does *not* mean that it is statistically equivalent. To prove performance equivalence, one must use noninferiority statistics, also called tests of equivalence, to demonstrate the "absence of a difference." For a hypothesis of equivalence, improvements are not the focus; rather, equivalent results are the focus. These analyses require more complex calculations, typically not found in most statistical packages; however, you can form a hypothesis and consult with a biostatistician if noninferiority statistics are required. To create a hypothesis for these circumstances, one simply replaces the typical null hypothesis with the typical alternate hypothesis.

Parametric Statistics

Parametric statistics are used to compare numerical data that are normally distributed. With these tests, means and proportions derived from data from different laboratory methods can be compared. Examples of parametric statistics include the following:

- The Student *t* tests, which compare the means of two groups (Fig. 13).
  - An independent *t* test is used to compare two groups that are mutually exclusive (e.g., experimental versus control groups or males versus females).
  - A dependent (paired, correlated) *t* test is used to compare pretest and posttest groups, i.e., to compare two groups that have been matched in pairs (e.g., split blood samples).
- Analysis of variance (ANOVA) is used instead of the *t* test when more than one variable is being measured, and it assesses the effect of more than one variable. It compares the means of more than two populations or the means and effects of two or more factors. Analysis of variance takes into account two sources of variability: the within-group variability and the between-group variability.
- Variance is compared by using the *F* ratio: *F* = (variance between groups)/(variance within groups).

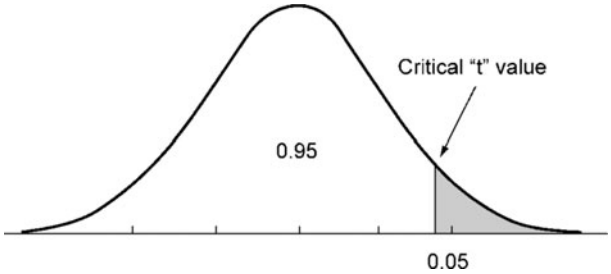


FIGURE 13 A one-tailed *t* test in the positive direction. A one-tailed test determines whether a particular population parameter is larger than some predefined value. The *t* test uses one critical *t* value, defined from a table. If the sample mean falls outside the accepted area (95%), then the null is rejected (the area shaded in gray is the area for rejection of the null hypothesis).

COMPARATIVE STATISTICS: COMPARE RELATIONSHIPS WITH CORRELATION AND REGRESSION

Correlation

One uses the correlation coefficient (*r*) to indicate the degree of relationship, otherwise known as the association between variables. One can evaluate the strength of relationship, because the correlation coefficient ranges from 0 (no correlation) to +1 or −1 (Fig. 14A). The sign indicates the direction of association: a positive association or a negative association, respectively. One must keep in mind that correlation is not causation.

Association

Association is typically represented by a scattergram, a graph showing the line of best fit between variables *x* and *y* (Fig. 14A). The Pearson correlation coefficient is based on the actual data values and is a measure of linear association. It is used when the data have normal distribution and is related to linear regression (R^2). For the simple linear model we can test hypotheses regarding the estimated β: for H0, $\beta = 0$, and for HA, $\beta \neq 0$. In contrast, the coefficient of determination (r^2) indicates the proportion of variance in one variable that can be explained by the variance in the other variable.

Analysis of Residuals

When the data are plotted against the mean and the difference from the data point to the mean is depicted, the plot is called a residual plot (Fig. 14B).

Linear Regression (Prediction)

Linear regression is used when the measures of association are strong enough that one can predict the values of continuous dependent variables by the values of continuous independent variables; in other words, *x* predicts *y*. We assume that pairs of observations are collected and that one variable (*x*) is used to predict another (*y*) in situations where variable designations (dependent or independent) are based on the research question itself; *x* is the independent, or predictor, variable; *y* is the dependent, or response, variable.

Modeling the relationship between *x* and *y* requires the specification of two components: the systematic component and the random component. The systematic component is visualized when the *y* intercept does not cross the *y* axis at zero, as in Fig. 13C. The random component is best visualized as the random variation from the line of equality, as depicted in Fig. 14B.

Probit Analysis

The relationship between the proportion of positive samples in replicate determinations and different input concentrations of microbes can be examined and characterized by probability unit (probit) analysis as a model for nonlinear regression. Using a regression curve determined by probit

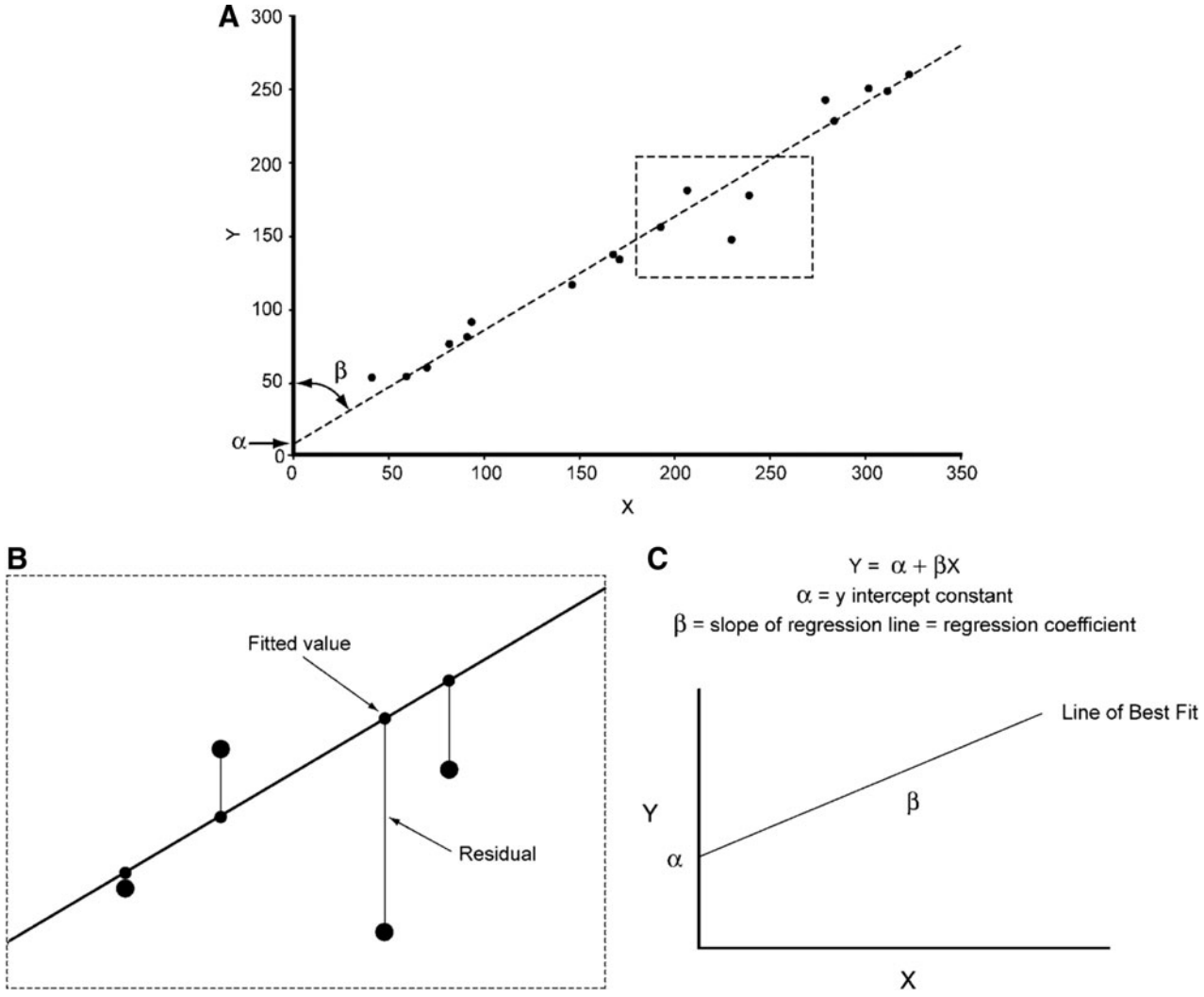


FIGURE 14 (A) An *x*-*y* graph with regression analysis: in the simplest case, there are two variables, one explanatory (*x*) and one response variable (*y*), i.e., a change in *x* causes a change in *y*. It is always worth viewing your data (if possible) before performing regressions to get an idea of the type of relationship (e.g., whether it is best described by a straight line or a curve). By looking at this scatter plot, it can be seen that variables *x* and *y* have a close relationship that may be reasonably represented by a straight line. This would be represented mathematically as $y = a + bx + e$, where *a* describes where the line crosses the *y* axis, *b* describes the slope of the line, and *e* is an error term that describes the variation of the real data above and below the line. Simple linear regression attempts to find a straight line that best fits the data, where the variation of the real data above and below the line is minimized. (B) Enlarged section of panel A, with residuals and fitted values shown. Assuming that variation in *y* is explained by variation of *x*, we can begin our regression. This output will tell us several things: the equation of the fitted line, formal information regarding the association of the variables, and how well the fitted line describes the data. There are two values: R^2 (the coefficient of determination) and *r* (the correlation coefficient). R^2 indicates the *proportion* of variance in one variable that can be explained by the variance in the other variable. If $R^2 = 0.98$, 98% of the change in *y* can be predicted by the changes in *x*. For the parameter, *r* is a measure of linear association (the degree of the relationship) for data with a normal distribution. For perfect positive linear correlations, $r = 1.0$; for a perfect negative linear correlation, $r = -1.0$ and the line slopes from the top right of the graph to the bottom left. Since not all relationships are linear, one must examine the data to assess if a straight line is the best fit for the data; in most laboratory evaluations, this will be the case. (C) The regression equation and its graphical depiction of the line of equality and the *y* intercept. The *y* intercept represents systematic bias of *x* to *y* quantitative comparison.

analysis, the LOD can be determined with 95% confidence. The relationship between the proportion positive from each replicate of 10 to 12 and the corresponding log concentrations of microbes can be examined by using the probit regression analysis function from a variety of statistical software programs, including SPSS for Windows 10.0 and Excel 97–2007. Using the probit model, two molecular tests can be compared, the median detection concentration of the microbe can be estimated, and the concentrations corresponding to probits of 0.01 to 0.99 can be calculated. Proportions can also be tested, with a *P* value of 0.05 defined as statistically significant for two-tailed analysis.

This concept proposed transforming the percentage killed into a probit, which was defined arbitrarily as equal to 0 for 0.0001 and 10 for 0.9999. The positive percentages can be plotted against the logarithm of the dose to obtain a more or less straight line. Such a probit model approach is justified if the response variation can be characterized as a log-normal distribution of tolerances among sample concentrations, where the tolerance of a particular sample is the dose that is just sufficient for the predetermined response of interest, such as the 95% LOD.

SELECTION OF STATISTICAL TESTS

Although we describe a few statistical test methods here, there are many others which are beyond the scope of this chapter and are often used in clinical research studies. The type of statistical test selected for analysis is determined according to the type of data collected, the types of variables, and the number of data categories. Statistical selection can be straightforward or difficult, depending on the experimental design. Some common statistical choices are depicted in Table 3. We are often asked which types of statistical analysis are preferred for molecular method assessment. Therefore, we have created a table summary to assist your understanding of the statistical methods, based on guidance from EP Evaluator from Dr. David Rhoads (Appendix I).

SUMMATION AND CONCLUSIONS

CLIA has clear guidelines on the verification and validation of LDTs and other non-FDA-approved/cleared assays. Choices of statistical methods and methods for drawing conclusions on accuracy are less well defined. For answers to these questions, several guidance documents are available through organizations such as CLSI, the American Society for Microbiology, and the Association for Molecular Pathology, which can further assist with the verification process (Tables 1, 2, and 3). However, it is ultimately the responsibility of the clinical laboratory directors and consultants to determine the basic question, "How good is good enough?" Each consultant will certainly have his or her own version of accuracy and utility required to introduce the method into the laboratory. To that end, the consultant will summarize the laboratory data and experimental conclusions and determine whether or not a method will be introduced.

Despite the confusion that can occur around method verification and LDTs, several themes prevail. When performing such verifications, it is important to have test design and test performance mimic reality as much as possible. Not only should statistical analysis be performed to characterize the performance of the assay, but it should also be ongoing as part of assay validation. Continuous error assessment of an assay is critical. In some cases this may entail testing your current LDT, which may have been used for years, against new assays on the market. Defining assay limitations and performing continual assay quality assurance can be key to maintaining a successful assay. Analysis of method verification data may require collaboration with a statistician or use of software specifically designed for CLIA method verification such as EP Evaluator (34, 35).

APPENDIX: SPECIFIC EXPERIMENTS FOR COMPARATIVE STATISTICAL ANALYSIS DURING ASSAY VERIFICATION

Note: Some examples are excerpted from David G. Rhoads's EP Evaluator.

Qualitative Methods

Analytical sensitivity (LOD) and specificity

Analytical sensitivity is used to determine the LOD of a new assay. The LOD may be termed LOD-100% (i.e., the microbial density at which 100% of the spiked samples yield positive results), LOD-95%, etc. It is common to test spiked specimens at 1 to 3 log microbial densities above the lower LOD (LLOD) (i.e., the lowest known density to produce any positive results during preliminary testing or as determined in the published literature) in replicates of at least 10, to determine the LOD. *Note:* Determine the number of replicates required by starting with the level of certainty required by a positive sample. For example, testing 20 spiked positive samples and finding 19 of them positive would define that microbial density as the LOD-95% (also known as 19/20 positive). If only 10 replicates are used, then only one false-negative will drop the LOD to 90%.

For qualitative methods, it is also common to include parameters such as analytical sensitivity and specificity in

TABLE 3 Overview of association methods

Dependent variable	Independent variable	Statistical method	Example
Categorical	Categorical	Relative risk (CI)	Epidemiology, (disease and exposure)
Categorical	Categorical	For independent data, chi-square test For paired data, McNemar's test, such as split samples	Positive/negative, 1+–4+
Dichotomous	Continuous	Logistic regression	Yes/no
Continuous	Continuous	Pearson correlation or linear regression	*x* predicts *y*
Continuous	Categorical	If variance unknown, one-sample *t* test If paired independent samples, paired *t* test If >3 categories, analysis of variance	CI by method

the form of a 2 × 2 table, inserting performance data from results of known spiked samples, converting them into either positive or negative results for the 2 × 2 table. In addition, if one calculates proportions from the numbers in a 2 × 2 table (e.g., % positive = number of positive/total number of samples tested), then one can use comparative statistics, such as those listed in Table 3, to compare the assay performances.

Comparison of a new method to a reference standard method

Number of methods per comparative experiment ≥ 2.

Number of replicates for each specimen = 1 or 2.

Number of specimens used = 40 to 1,000, across the assay's reportable range.

Note: Comparison of multiple instruments at the critical assay points across the reportable range requires up to 30 data points and must perform within "allowable error" parameters.

For qualitative methods, it is common to include parameters such as clinical sensitivity and specificity in the form of a 2 × 2 table, inserting performance data from results of known clinical samples that have been tested with a reference standard method or in some cases a clinical definition of disease as defined by one or more clinical laboratory and symptomatic parameters. In addition, if one calculates proportions from the numbers in a 2 × 2 table (e.g., % positive = number of positive/total number of samples tested), then one can use comparative statistics, such as those listed in Table 3, to compare the assay performances.

Percent carryover (% contamination)

Percent carryover is defined as the percentage of "no template" and negative controls that yield positive results in the verification period due to template or amplicon contamination of samples. It is common to perform a "checkerboard experiment," in which extraction methods are challenged by placing high-positive samples (in the expected range of positive samples with high microbial density) to be tested in the same run with negative samples, alternating positive and negative samples, to ensure there is no template carryover. There should be no carryover or contamination during the verification period.

Quantitative Methods

General accuracy (recovery) experiments

- Accuracy experiments are used for quantitative measures such as viral loads.
- Test a minimum *n* of 1 sample times three known concentrations (at least one each of low, middle, and high concentrations).
- For better assessment, test five or six concentrations in two to four replicates.
- It is practical to use serial dilutions and double up this function with the linearity experiment.

To calculate % recovery, defined by the following formula: (mean of assay/true mean) × 100:

- Determine the data mean and plot a graph that includes all data points and the mean.
- Identify outliers by predefined limits of variation from the mean (e.g., 95% CI, 3 SD, etc.).

Note: An accuracy experiment at the assay's LOD requires more replicates (n = 3 to 20); accuracy of the results will be diminished near the assay's LOD. Accuracy is related to the LOD by way of analytical sensitivity at a given concentration, where accuracy = (number of positive results by new test)/(all positive results by the reference method test). A data set passes the accuracy test if the mean is within the allowable systematic error, defined by the manufacturer or published laboratory standards.

Simple linearity experiments using serial dilutions

Simple linearity experiments indicate precision at a range of microbial densities. These experiments define the assay's reportable range (based on the limits of reliable quantitation).

1. One can use serial dilutions and combine data analysis with accuracy experiments and also with PCR efficiency experiments.
2. Estimate the linearity (for qualitative and quantitative assays). Use a minimum of three known microbial densities (in duplicate or triplicate) and eyeball the linearity by preparing a scatter plot with a trend line, derived by plotting all results in an *x*-*y* scatter plot. Test densities of at least low, medium, and high levels. This estimate will determine a microbial density range in which the actual linearity cutoff for quantitative measurements will lie (see discussion of lower LOQ below). Example: For a qualitative assay one can estimate the detection range by eyeballing a linear range (for example, 100 to 10^8 organisms/reaction) by plotting the C_T values of a real-time PCR method against their known microbial densities or copy numbers if known. One can calculate residuals and eyeball residuals to check for the "edge of uniform scatter" (Fig. 1) to help confirm the clinical linearity of a method, that is, the area under the curve for which residuals are markedly increased. This is more of a measure to determine the density at which the LOD will fall, because at low densities, the precision will widen and some drop-outs, also known as false-negative results, will occur and begin to affect the LOD.
3. For quantitative assays, fully define the linearity and define an assay lower LOQ. It is best if linearity is analyzed with a regression analysis, with a trend line depicting a direct positive or negative relationship of the x (reference method) and the y (new method) values. For regression analysis, it is best if five or six microbial densities are used and two to four replicates at each density are performed. In that way a regression line with error bars can be created. For analysis, include all data points. Do not exclude outliers; rather, determine the residuals (i.e., the mean y value observed at $x - y$ of regression line at x value). This approach confirms linearity, more than simple linearity, because it looks at residuals. So with a residual plot analysis, a data set will pass a clinical linearity test if the mean residual for *each* specimen does not exceed the allowable error assigned for the particular microbial density. For example, with HIV quantitative loads, a long-time acceptable variation has been established at 0.5 log. For other assays a common option for variability is <10% CV.
4. For FDA-approved assays, laboratories must verify the manufacturer's reportable range.

PCR efficiency experiments

1. One can use serial dilutions and combine data analysis with accuracy experiments and also with linearity experiments.
2. Use serial dilutions, with at least two to four replicates at each microbial density (refer to Fig. 1).
3. Note: the PCR efficiency equation differs for different real-time PCR instruments, but in each case the slope of the line in an *x*-*y* scatter plot will determine the PCR efficiency. Check with the manufacturer of the instrument to determine the correct equation in which to insert your *x* and *y* values in a typical equation.
4. If your PCR conditions are optimal, typical PCR efficiency for microbes or plasmids in molecular-grade laboratory water should exceed 85 to 94%. Efficiency in sample matrix may vary from 50 to 90%, with lower values representing matrices which are inhibitory to PCR amplification or extraction efficiency.

LOQ experiment

The LOQ is the "functional LOD," or the lowest concentration of analyte in which an acceptable CV (defined as acceptable either from the manufacturer or clinical laboratory community or via 95% CI) is maintained within a period of weeks to months or statistically by ≥28 independent runs (8).

Quantitative experiments that require statistical comparison of the mean

For quantitative experiments that require statistical comparison of the mean (viral load, mean C_T, etc.):

1. Calculate the mean and report "standard measures of variance" for the assay means. For intra- and interassay precision, one can use the SD or standard error of the mean.
2. Compare means with inferential statistics: One can use the 95% CI (in which 95% of similar tests should fall), a *t* test, Dunnett's test, or a Tukey-Kramer honestly significant difference test to compare means; acceptable power and sample size should be preselected.
3. Identify outliers (outliers are typically defined as data that lie more than 2 or 3 SD or >95% CI from the mean).

Precision experiment (simple reproducibility)

Intra- and interassay precision requires a minimum of two or three specimens with ≥10 replicates and values representing the range of low and high, as well as any medical decision point(s) that may be critical to the assay interpretation. A better assessment would be the use of 2 or 3 specimens and 10 to 20 replicates, if time and resources allow.

Precision experiment (complex precision)

Test a minimum of two replicates per day for 3 days or eight runs; it is better to perform two replicates/day for ≥20 days.

Reference interval

The reference values are normal values and are based on values observed in a nondisease population, i.e., one that does not have the disease or the infection that the assay is detecting. In the hospital setting, these subjects may have disease but not the disease the assay is aimed to assess. To assess the normal values, test at least 20 individuals that represent a typical nondisease patient population. According to CLSI, if two samples or fewer fall outside the manufacturer's recommended limits, the values are verified. More samples are required to establish a reference interval for which no prior information is known.

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Molecular Microbiology Test Quality Assurance and Monitoring

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51

This chapter will provide a discussion on test quality assurance and the ongoing process of monitoring test performance, including documentation, instrumentation, and testing personnel. Historically, the definition of either validation or verification has been complicated by the different verbiage used by the different regulatory organizations. For example, the College of American Pathologists (CAP) defines validation as "a defined process by which a laboratory confirms that a laboratory-developed or modified FDA-cleared/approved test performs as intended or claimed." The ISO 9000 definition states that validation is the "confirmation through the provision of objective evidence, that requirements for a specific intended use or application have been fulfilled" and includes the determination of performance characteristics such as accuracy, precision, sensitivity, and specificity. Likewise, CAP defines verification as "the process by which a laboratory determines that an FDA-cleared/approved test performs according to the specifications set forth by the manufacturer." The ISO 9000 definition states that verification is the "confirmation through the provision of objective evidence that specified requirements have been fulfilled" and that it is "the ongoing process that confirms specified requirements (predetermined by validation) are fulfilled." For purposes of this chapter, the discussion of the ongoing process of assuring that the expected performance of a test (or assay) is consistently met in the testing of clinical specimens will be called verification/validation. A recent article by Halling et al. brings the issue to light with verification and validation (1).

The approach used for the assurance of test performance encompasses multiple components, including preanalytical, analytical, and postanalytical phases of the testing process. Test (or assay) validation/verification in the analytical phase is an integral part of quality assessment/assurance (QA), which also includes quality control, reagent and instrumentation control, and quality improvement. QA encompasses routine quality control, proficiency testing (PT), technical staff requirements, training and competency, instrument calibration, and clinical correlation. Quality control measures and verifies/validates the correct performance of a test as determined by the manufacturer or clinical laboratory. QA is a comprehensive process used to ensure correct test results and encompasses all phases of the testing process (i.e., preanalytical, analytical, and postanalytical).

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If the test is FDA cleared or approved for *in vitro* diagnostic use, the clinical laboratory is required by both CLIA and CAP to verify/validate the manufacturer's claims (2). Likewise, tests developed by the laboratory (i.e., laboratory-developed tests) need to meet the stated test performance as specified by the laboratory test verification/validation document. Ideally, analyte-specific reagents, which are under more strict control by the manufacturer in terms of reagent production, should be used whenever possible in any laboratory-developed test. This latter category also includes "off-label" use of an FDA-cleared or -approved testing method, which must be supported by a laboratory-outlined test verification/validation protocol.

Method verification/validation is required by the 1988 and 1992 CLIA regulations and was further defined by changes in each nonwaived test system introduced on or after 24 April 2003 (3, 4). Likewise, test verification/validation is specified in the CAP checklist and labeled as a phase II deficiency if it is not met. The reader is referred to the CLSI document summary listed in Table 1. Specific documents provide detailed information on the validation/verification process and QA for molecular testing. The *Cumitech* series published by the American Society for Microbiology also provides a very useful and comprehensive resource (Table 2). Other resources found in specific book chapters dedicated to molecular microbiology and infectious disease are listed in the reference section (5–11).

MONITORING MOLECULAR TESTING

Quality management of molecular testing begins with the preanalytical test request and continues through specimen collection, transport, and processing, moves into the analytical phase, and culminates with the postanalytical result generation, result review, test interpretation, and reporting phases. Each stage of the process is important and needs to be under the careful control of guidelines and standards.

The preanalytical phase includes an appropriate test request from a qualified health care provider clearly indicating the desired test(s), diagnosis code, and other pertinent information in paper or electronic form. The collection, handling, and transport of the specimen must fully satisfy the written clinical laboratory protocol/instructions. This includes accurate patient identifiers on both the specimen test request form and the specimen label. Any deviation from this protocol may compromise the test result and

TABLE 1 Guideline documents from the CLSI[a]

Document number	Date	Document title and description
General laboratory		
GP27A2	02/22/07	Using Proficiency Testing to Improve the Clinical Laboratory; Approved Guideline, 2nd ed.
GP29A2	08/29/08	Assessment of Laboratory Tests When Proficiency Testing Is Not Available; Approved Guideline, 2nd ed.
GP31A	08/22/12	Laboratory Instrument Implementation, Verification, and Maintenance; Approved Guideline
QMS03A3	05/02/09	Training and Competence Assessment; Approved Guideline, 3rd ed.
QMS12A	12/29/10	Development and Use of Quality Indicators for Process Improvement and Monitoring of Laboratory Quality; Approved Guideline
Method evaluation		
EP14A3	08/15/14	Evaluation of Commutability of Processed Samples; Approved Guideline, 3rd ed.
EP15A3	09/11/14	User Verification of Performance for Precision and Trueness; Approved Guideline
EP23A	10/25/11	Laboratory Quality Control Based on Risk Management; Approved Guideline
EP25A	09/23/09	Evaluation of Stability of In Vitro Diagnostic Reagents; Approved Guideline
EP26A	09/30/13	User Evaluation of Between-Reagent Lot Variation; Approved Guideline
Microbiology		
M53A	06/30/11	Criteria for Laboratory Testing and Diagnosis of Human Immunodeficiency Virus Infection; Approved Guideline
Molecular methods		
MM03A2	02/17/06	Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline, 2nd ed.
MM06A2	11/30/10	Quantitative Molecular Methods for Infectious Diseases; Approved Guideline, 2nd ed.
MM09A2	02/28/14	Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline, 2nd ed.
MM10A	02/17/06	Genotyping for Infectious Diseases: Identification and Characterization; Approved Guideline
MM12A	05/30/06	Diagnostic Nucleic Acid Microarrays; Approved Guideline
MM13A	01/06/06	Collection, Transport, Preparation, and Storage of Specimens for Molecular Methods; Approved Guideline
MM14A2	05/23/13	Design of Molecular Proficiency Testing/External Quality Assessment; Approved Guideline, 2nd ed.
MM17A	03/21/08	Verification and Validation of Multiplex Nucleic Acid Assays; Approved Guideline
MM18A	04/28/08	Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline
MM19A	11/30/11	Establishing Molecular Testing in Clinical Laboratory Environments; Approved Guideline
MM22A	02/27/14	Microarrays for Diagnosis and Monitoring of Infectious Diseases; Approved Guideline

[a]Clinical and Laboratory Standards Institute (CLSI), Wayne, PA, http://clsi.org.

possibly result in patient harm. The reader is again referred to Tables 1 and 2 for specific details on this preanalytical phase.

Most of the discussion in this chapter will focus on the analytical phase of testing. This phase includes monitoring of reagents and test kits, quality control, instruments, and testing area considerations. QA also includes technical staff requirements, training and competency, and laboratory safety. The types of molecular testing in this discussion will include mostly qualitative, quantitative, and multiplex with some discussion of nucleic acid sequencing.

TABLE 2 *Cumitech*—Cumulative Techniques and Procedures in Clinical Microbiology[a]

Document no.	Date	Document title and description
3B	2005	Quality Systems in the Clinical Microbiology Laboratory
31A	2009	Verification and Validation of Procedures in the Clinical Microbiology Laboratory
39	2003	Competency Assessment in the Clinical Microbiology Laboratory

[a]American Society for Microbiology (ASM), Washington, DC, http://www.asm.org.

Nucleic acid detection (RNA or DNA) can be direct or amplified. Furthermore, the amplified product can be qualitative or quantitative or even be used in nucleic acid sequencing. Amplified testing can be target or signal based. For example, PCR, transcription-mediated amplification, and strand displacement amplification are all target based. Verification/validation of qualitative testing is typically the least complex compared to quantitative and multiplex testing, which are the most highly complex (i.e., especially with the software component).

The analytical part of molecular testing usually begins with the extraction and purification of nucleic acid (DNA or RNA). Manual, stand-alone automated, or fully automated methods may be involved in this process. The newly

introduced and more rapid methods combine the nucleic acid extraction with real-time amplification/detection into an automated series of steps. The stand-alone nucleic acid extraction step is then followed by amplification and target detection. Amplification can occur in a serial (i.e., amplification followed by detection) or parallel (i.e., real-time PCR with amplification and detection occurring simultaneously) fashion. Most testing today consists of the real-time format. In either case, this last phase leads to the generation of a test result. Detection formats are numerous, ranging from agarose gel electrophoresis, luminescence-based assays, capillary electrophoresis, mass spectrometry, and oligonucleotide microarrays to next-generation sequencing. The reader is referred to Table 1 for more specific details.

QUALITATIVE MOLECULAR TESTING

Qualitative molecular testing consists of a positive or negative test result, rather than a quantifiable value. In some cases, the specimen may be inhibitory, and a valid test result cannot be generated. Results may consist of a specific nucleic acid band present on a gel, a specific nucleotide base(s) detected in nucleic acid sequencing, a numeric value (e.g., crossing point [Ct]) or other indicators translated into a positive, negative, or inhibitory result by software or human intervention. Control of the testing needs to be incorporated into the process from the beginning nucleic acid extraction step followed by either a direct nucleic acid hybridization or amplification and ending in product detection.

Controls are incorporated into each run of an assay/test and may be internal, external, endogenous, or exogenous. Any of these controls can be used to detect assay inhibition, but an internal or endogenous control is preferred, because it lessens the chance of contamination by a "spiking" process and uses less of the specimen volume. Controls should be included at the nucleic acid extraction step and processed with the specimen throughout the entire testing sequence. In general, if the loss of gel band intensity is greater than ±1 or the Ct value is greater than ±3 Ct, test inhibition is probably present and the test should be repeated to ensure that the limit of detection (LOD) was not compromised (Table 3). External controls consist of positive (low and high positive are ideal), negative, and ideally, an external control without nucleic acid ("no template control").

Controls should simulate actual specimens, with nucleic acid being present in the same matrix as the specimen (e.g., urine, cerebrospinal fluid, or respiratory secretion). The low-positive control should be close to the test cutoff, and the high-positive control should approximate the midrange of the test. If only a single positive control is used in qualitative testing, the control should be within approximately 10-fold of the LOD. Extremely high-positive controls are strongly discouraged due to the possibility of carryover contamination, which could potentially generate false-positive results in the patient specimens. Three external controls (low, medium, and high) may even be used if desired and where it is not cost prohibitive. The negative control used in the test may consist of human nucleic acid or an intact microbe that is phylogenetically close to the actual gene target or represents a microorganism that could be present in the specific specimen type. This could be another pathogen for that site or a member of the normal flora. It is important that the negative control consist of the same matrix as is present in the actual patient specimen.

Controls are to be used on each test run, and it is also recommended that replicate controls be used on large-batch test runs (i.e., ≥20 to 30 specimens/run). The internal control (IC) can be excluded, but only if it can be shown that the specimen type rarely if ever contains inhibitors (i.e., less than 1% inhibition). Real-time PCR testing melting curves should also be inspected and accepted according to defined criteria in the procedures/instructions for the test. For example, the ±1°C melting point temperature range may be used as an acceptable range for lack of test inhibition.

QUANTITATIVE MOLECULAR TESTING

Quantitative molecular testing consists of numeric values with defined units in the test result. A dynamic range needs to be clearly defined based on the test verification/validation and should be challenged on each test run to

TABLE 3 Molecular microbiology and infectious disease testing controls and calibrators

Molecular test type	Controls and calibrators	Comments
Qualitative	Internal control (IC)	IC in same tube as specimen or spiked into a second specimen tube
	Positive (low, LP)	LP close to the test cutoff value
	Positive (high, HP)	HP in the test midrange
	Negative control (NC)	NC contains nonspecific nucleic acid
	No nucleic acid control (NNA)	NNA with buffer in place of nucleic acid
Quantitative	Internal control (IC)	IC, LP, HP, and NC similar to qualitative test
	Positive (low, LP)	CAL consist of at least 3 defined samples covering the dynamic range of the test
	Positive (high, HP)	
	Negative control (NC)	
	Calibrators (CAL)	
Multiplex microarray	Internal control (IC)	IC and NC similar to qualitative test
	Multiple positive controls (PC)	All positive controls should be included by a user-defined test frequency
	Negative control (NC)	
Sequencing	Positive control	The positive control should consist of a known DNA sequence that is verified/validated on each test run
	Negative control	The negative control is critical for contamination control

demonstrate reproducibility in performance. Unlike qualitative testing, it is mandatory that the quantitative molecular test has at least three controls on each test run (negative, low positive, and high positive) and should include an IC to control for test inhibition. However, unlike qualitative molecular testing, verification/validation of the quantitative linearity range should be demonstrated on each test run. However, if linearity can be demonstrated simply on the basis of external controls under stringent interpretative guidelines, then the use of a stored calibration curve may be acceptable. This is accomplished by including calibrators that are precisely defined by in-house methods or detailed in a certificate of analysis from a commercial source (Table 4). The raw data should also be reviewed from each run to ensure that the calculated value in the final test result is accurate.

Each quantitative molecular test is required to undergo calibration verification/validation at least every 6 months according to the CAP checklist. This may have to be verified earlier if an exception exists. Examples of such exceptions include a major system component change, changes in lots of chemically or physically active reagents, recommendations made by the manufacturer, or a pattern of quality control failure.

Validation of the reportable test range is a CAP requirement. CAP refers to the reportable test range as the

TABLE 4 Molecular microbiology and infectious disease testing quality control sources

Infectious disease agent	Source[a]							
	AM	BR	EG	MM	SC	QU	WHO	ZM
Bacteria								
Chlamydia trachomatis		X			X			X
Clostridium difficile					X	X		X
Mycobacterium tuberculosis				X				X
Mycoplasma pneumoniae								X
Neisseria gonorrhoeae		X			X			X
Bordetella pertussis, *Bordetella parapertussis*			X			X		
Staphylococcus aureus								X
Streptococcus group A and B						**X**		
Viruses								
BK virus	X		X					
Cytomegalovirus			X				X	X
Herpes simplex virus						**X**		X
Varicella-zoster virus						X		X
Epstein-Barr virus	X		X					X
Hepatitis B virus	X				X		X	X
Hepatitis C virus					X		X	X
HIV type 1	X				X		X	X
Human papillomavirus		X			X		X	
Influenza virus types 1 and 2						X		X
Parvovirus B-19							X	X
West Nile virus					X			
Adenovirus			X			X		
Enterovirus			X					
Parasites								
Entamoeba histolytica								X
Multiple ID agents and resistance genes								
Blood (sepsis panel)				X				
Carbapenem				X				
M. tuberculosis resistance genes				X				
Gastrointestinal (GI panel)				X				X
Ebola virus (control panel)				X				
Respiratory pathogens (RP panel)				X				X
Negative molecular control								
Blood and/or specific matrix control	X	X			X			

[a]Abbreviations: AM, AcroMetrix, Inc. (Life Technologies), Benicia, CA, http://www.lifetechnologies.com; BR, Bio-Rad Laboratories, Irvine, CA, http://www.bio-rad.com; EG, ELITech Group, Princeton, NJ, http://www.elitechgroup.com; MM, Maine Molecular Quality Controls, Inc., Scarborough, ME, http://mmqci.com; SC, SeraCare Life Sciences, Inc., West Bridgewater, MA, http://www.seracare.com; QU, Quidel Corporation, San Diego, CA, http://www.quidel.com; WHO, World Health Organization, Geneva, Switzerland, http://www.who.int/bloodproducts/ref_materials/en/; ZM, ZeptoMetrix Corporation, Buffalo, NY, http://www.zeptometrix.com

"analytical measurement range" (AMR) for verification/validation purposes. The test manufacturer may recommend a procedure for validating the AMR, or the clinical molecular laboratory may develop a procedure. The samples used for validation should be clearly defined and consist of undiluted, nonconcentrated and unaltered samples. Likewise, the target analyte should challenge the entire range (including LOD and low, middle, and high analyte levels). It should be noted that the limit of quantification is different from the LOD. Materials used in the validation must consist of an appropriate matrix (for example, whole blood, plasma, or cerebrospinal fluid). The sample source may be actual specimen, control material, or appropriate proficiency testing samples. The AMR has to be validated at least on a biannual basis. This can be satisfied using purchased commercial panels (Table 4) or by using a clearly defined panel produced by the laboratory. Whatever approach is used, it must fully satisfy the AMR for the specific test (12).

MULTIPLEX AND MICROARRAY MOLECULAR TESTING

Test verification/validation of multiplex assays and microarrays is more complex and difficult to perform than single target assays. Ideally, the test verification/validation should include a positive control for all nucleic acid targets in each run of the assay. In reality, this is neither practical nor cost-effective for assays with a large number of targets (e.g., gastrointestinal panel consisting of 22 targets). Therefore, smaller numbers of representative controls are often used on a run. Coverage of all nucleic acid targets can then be accomplished on a rotation schedule over a defined time period. For example, if five targets are detected by an assay, each may be used daily, with all five challenged once per week. In such cases, the controls may also consist of a single tube with multiple nucleic acid targets in the appropriate matrix to conserve costs. In the previous example, a single external control sample might include all five targets. The approach to control selection and type is also similar to the section above on qualitative molecular testing. On the other hand, microarray testing should include a more detailed verification/validation to adequately challenge array quality on a run-to-run basis.

MOLECULAR SEQUENCING

Sequencing by either gel or capillary electrophoresis usually relies on multiple primers and PCR amplification. Even though the LOD is usually higher than most other amplification assays, the chance for contamination is likely and should be under strict control. Since the LOD is higher, the amplicon template should be observed as a band on a gel before proceeding to the actual sequencing stage. A positive control of a known sequence and a negative (no nucleic acid template) control are required on each sequence run (Table 3).

The procedural/instructional manual should explicitly detail the acceptability and interpretative criteria for analyzing the sequence test results. Appropriate databases that have been critically verified/validated should only be used for obtaining test results. Likewise, the genotypic sequencing data should always be verified/validated with the available phenotypic data for accuracy. Lastly, the interpretative software is critical to the generation of a valid and accurate identification and should be extensively verified/validated before use on clinical isolates.

MOLECULAR INSTRUMENT CALIBRATION AND MONITORING

Instrument calibration should be performed on a biannual basis or according to the manufacturer's recommendations, whichever is the shorter time period. If multiple instruments of the same type are in use, the process consists of verifying/validating the instruments against each other with an adequate number of representative patient test samples. For example, a large test run that contains an appropriate number of positive and negative specimens could be used for this purpose. The decision on the actual number is usually made by the laboratory director.

MOLECULAR REAGENT VERIFICATION/VALIDATION

Reagent verification/validation includes commercial reagents, those produced in-house, those commercially purchased as a single reagent (e.g., an analyte-specific reagent such as oligonucleotides included in a primer set), and those included in test kits under a controlled lot number from the manufacturer (i.e., *in vitro* diagnostics). Reagent verification/validation should be performed on all new reagent lots before they are placed into clinical use. These should also be cross-checked with the reagents currently in use before being approved for clinical testing purposes.

Qualitative assays require testing new reagents or kits (shipments or lots) with the necessary controls and parallel testing of at least one negative and one positive patient previously tested with the current reagents. The laboratory should establish acceptability limits for result lot-to-lot parallel testing. For example, a real-time PCR test may include accepting the new reagent/test kit only if Ct results were within a 3-Ct range (approximately a 10-fold range in detectable target concentration). If the new reagent/test kit Ct is within the stated range, this would indicate that the LOD was most likely not appreciably compromised, even without performing a full-scale statistical analysis.

Quantitative assays require more extensive testing to ensure that newly introduced reagents are appropriately verified/validated. Quantitative testing should include at least three patient specimens at different target concentration levels. In addition, at least one of these samples should be a weak positive and close to the assay LOD, and at least one negative control needs to be included in the testing. Reference materials can be produced in-house or commercially purchased (Table 4). In any case, the reference material should contain the appropriate matrix for checking new controls or calibrators (13).

As with all other aspects of testing, it is necessary to include appropriate documentation and instructions related to QA practices in the molecular test procedure/instruction manual. This procedure should list precise step-by-step directions on verification/validation testing (e.g., frequency and methods), criteria for acceptability, and instructions for reagent or test kit implementation. Reagent or test kit documentation should always include information on the manufacturer, type of reagent, Materials Safety Data Sheet (MSDS), lot number with known stability (expiration date) and acceptable shelf life, storage and handling conditions, and lot-to-lot variation data for trending purposes.

QA, PROFICIENCY TESTING (PT), AND QUALITY CONTROL

QA includes quality control, quality improvement, and method verification/validation. To ensure the quality of testing on a routine basis, PT is required for each test offered in the clinical laboratory. PT is covered more extensively elsewhere in another chapter of this book. Therefore, only a brief discussion will be provided in this chapter. Frequent challenges from external CAP, CAP-approved alternative PT programs, or other external PT providers are sources of PT samples. The use of other PT sources, such as interlaboratory programs or split specimens sent to an external reference laboratory, is allowed if no external programs are available. The use of split specimens with clearly outlined criteria (i.e., documented in a procedural manual) and implemented without bias in specimen selection or grading can be developed in-house. This can be accomplished by placing the internal program under the direction of a QA department or similar unbiased regulatory area/department associated with the clinical laboratory. An example of the type of form that can be used is shown in Fig. 1. The internal program should be handled in the same fashion as a CAP PT program. The samples in the PT challenge should be handled exactly like specimens received for patient testing, without any deviations. As with any PT challenge, communication or discussion of the test results between laboratories or between the molecular testing laboratory and the QA department cannot occur until the PT report is finalized.

Any unacceptable PT challenges should be promptly reviewed and investigated, especially if the reason for that performance is unclear. If an investigation is required, it should consist of a structured approach and offer corrective action and preventative measures to avoid future occurrences. As a part of the procedure/instruction manual, the steps should be clearly listed as outlined in Fig. 2. Proficiency testing leads to quality improvement, which is an important part of QA.

The use of controls in clinical testing should consist of simulated patient samples using the same matrix as the real specimen. An obvious source for such material is a manufacturer of quality control reagents, ideally one with FDA-cleared/approved reagents (Table 4). However, this can be costly, and many types of quality control materials may not be commercially available. Therefore, a laboratory may have to rely on acquiring these control specimens internally or externally from other clinical laboratories (14). The acquisition of control specimens internally may be accomplished by simply using de-identified patient specimens that would usually be discarded. Whatever the source may be, the control material acts as a surrogate for actual patient samples in the testing process. Important considerations in the selection of controls include commonality of the matrix with patient specimens, use of undiluted samples (pooled specimens are acceptable), stability of the control material, absence of inhibitors, handling and storage requirements that coincide with those of actual patient samples, and absence of contamination (as might be demonstrated by microbial growth). A large lot of control material (sufficient for at least 1 month of testing), frozen at −80°C, not frozen/thawed more than three times, and with a known stability would be ideal. Verification/validation of controls to assess stability is necessary if a single lot is used for extensive time periods (e.g., 6 to 12 months).

All controls (low positive, high positive, and negative), whether used for qualitative or quantitative molecular testing, should be monitored for trends. Trend analysis is a means of ensuring that there is an absence of bias or changes in the controls used for patient testing. Depending on the test, a standard deviation or coefficient of variation statistical approach should be used to establish limits. In general, the standard deviation should fall within ±3 stan-

INTERNAL PROFICIENCY TESTING (I-PT)

DEPARTMENT: _____

TEST: _____

Date Tested	I-PT ID No.	Original Lab Result*	Reference Lab No.*	I-PT Test Result*	INTERPRETATION	Comments
					__ Accept __ Reject	
					__ Accept __ Reject	
					__ Accept __ Reject	
					__ Accept __ Reject	
					__ Accept __ Reject	

* Fill in the original lab result after testing by the Departmental Supervisor. Fill in a coded reference lab ID or internal test No.

CORRECTIVE ACTION:

SUPERVISOR REVIEW		NAME	Signature	DATE	
Laboratory Supervisor					
Laboratory Director					
QA Director					

FIGURE 1 Internal proficiency testing (I-PT) documentation report for PT when PT samples are not available from a qualified commercial source.

INVESTIGATION AND CORRECTIVE ACTION

Summary of Investigation

Conclusion for the Cause of the Unacceptable Result(s)

Corrective Action

Evidence for the Successful Correction of the Unacceptable Result(s)

Reason for the Unacceptable Result(s)
Methodologic _____
Technical _____
Clerical _____
Problem with PT Material _____
Other _____
No explaination following the investigation _____
Investigation form completed by: _____ **Date:** _____

FIGURE 2 Proficiency testing investigation and corrective action report for failed testing.

dard deviation and the coefficient of variation less than 25% (note that a value near the LOD may exhibit a higher coefficient of variation). In real-time PCR testing, the Ct should remain within the ±3-Ct range. The laboratory director or designee should perform at least a monthly review of all quality control documentation for all clinical tests performed in the laboratory. Repeated unacceptable controls, out-of-range test trending, and recurrent quality control occurrences should be resolved promptly by the laboratory director or designee. In addition, an action plan should be developed to prevent any future problems.

MOLECULAR TEST RESULTING, INTERPRETATION, AND CLINICAL CORRELATION

The results of molecular testing often require careful and thorough data analysis before interpretation. FDA test kits usually have sophisticated software to accomplish this task. Even so, results need to be carefully reviewed before the final reporting of patient results. However, more manual or complex tests such as gel electrophoresis with restriction endonuclease analysis, nucleic acid sequencing, and real-time PCR require more attention and review. For example, a lower trend in a control concentration (i.e., higher Ct) for a real-time PCR test may indicate control or reagent deterioration. This should be noted quickly and addressed, because it may affect the test performance and result in a higher LOD.

Test interpretation is based on an acceptable test run (i.e., acceptable controls), lack of inhibition in the patient specimen, and no evidence of contamination in the test run. In real-time PCR testing, the results of a melting curve analysis can also be used to confirm that the correct target is detected. It should also be noted that a single base change (polymorphism) in the true target can shift the melting point curve and result in a need for further testing to ensure specificity of the amplified product. In some cases, the presence of inhibition in a particular patient specimen may not be very obvious. In this case, and to ensure lack of inhibition, the patient IC result should be compared to the IC result in the negative control. A clearly defined range of acceptability as described in the procedural manual should then be used to interpret the presence or absence of inhibition in the specimen.

It is important to correlate the test/assay result with the clinical findings to maximize the clinical usefulness of the test result as a part of the verification/validation process (15). In qualitative testing, the detection of the infectious agent should correlate with other laboratory findings. This is especially true of real-time PCR testing for herpes simplex virus type 1 or type 2 in cerebrospinal fluid. The absence of pleocytosis or the predominance of monocytes rather than lymphocytes should raise suspicion of a possible nonviral etiology. A notable exception is in early aseptic meningitis with enterovirus disease, which may reveal an early increase in monocytes with a later shift to lymphocytes.

The clinical correlation of quantitative molecular test results should involve a review of the test history on a given patient. An abrupt increase or decrease over a short time period may suggest erroneous test results rather than a change in status of the infectious agent in the patient's disease. If unchecked, this could have serious consequences on both therapy and prognosis. Correlation with clinical findings should always be advised in such cases, and if mismatching with laboratory findings is found, retesting may be clinically indicated.

TECHNICAL STAFF REQUIREMENTS, TRAINING, COMPETENCY, AND SAFETY

One of the most important aspects of laboratory testing is to ensure that the laboratory personnel are qualified and competent in test performance. As with other aspects of

TABLE 5 Guide to verification/validation of test performance for various levels of FDA categories[a]

ID	FDA test description[b]	Analytical sensitivity	Analytical specificity	Clinical sensitivity[c]	Clinical specificity[c]	Accuracy	Precision[d]	Interfering substances
F1	FDA-cleared/approved nonamplification method	NA	NA	Test at least 20 known positive samples, which represent all specimen types	Test at least 50 known negative specimens (some with known nontarget pathogens)	Test at least 20 positive specimens and at least 40 negative specimens over 3 days by at least two different technologists	Testing includes positive (≥2) and negative (≥2) specimens and controls tested over 3–5 days by at least two different technologists	NA
F2	FDA-cleared/approved amplification method	NA	NA	Test at least 20 known positive samples, which represent all specimen types	Test at least 50 known negative specimens (some with known nontarget pathogens)	**Qualitative:** Test at least 20 positive specimens and at least 40 negative specimens over 3 days by at least two different technologists	**Qualitative:** Testing includes positive (≥2) and negative (≥2) specimens and controls tested over 3–5 days by at least two different technologists	NA
F3 F4 F5	FDA-cleared/approved amplification method using a different specimen type, collection device, or a modification of a test component other than the cleared/approved entities					**Multiplex[e]:** Testing includes all targets **Quantitative:** Test at least 20 positive specimens in duplicate and at least 40 negative specimens over 3 days (i.e., bracketing to determine linearity) by at least two different technologists	**Multiplex[e]:** Testing includes all targets **Quantitative:** Testing includes three replicates tested at three concentrations (i.e., high, medium, low covering the dynamic range, LLQ, and LOD) over 3–5 days by at least two different technologists	
F6	Laboratory-developed test	Test a defined number of titered or spiked specimens to verify/validate analytical sensitivity (i.e., defined as the "limit of detection")	Test a specimen known not to contain the target microbe or spike to rule out cross reactivity (e.g., human genomic DNA, similar microorganisms or pathogens, normal flora, or genotypically related microorganisms)	Test at least 50 known positive specimens, which represent all specimen types	Test at least 100 known negative specimens (some with known nontarget pathogens)	**Qualitative:** Test at least 20 positive specimens and at least 40 negative specimens over 3 days by at least two different technologists **Multiplex[e]:** Testing includes all targets **Quantitative:** Test at least 40 positive specimens in duplicate and at least 80 negative specimens over 3 days (i.e., bracketing to determine linearity) by at least two different technologists	**Qualitative:** Testing includes positive (≥2) and negative (≥2) specimens and controls tested over 3–5 days by at least two different technologists. **Multiplex[e]:** Testing includes all targets **Quantitative:** Testing includes three replicates tested at three concentrations (i.e., high, medium, low covering the dynamic range, LLQ, and LOD) in replicate for 3–5 days by at least two different technologists	Depends on the specimen source used in the test (e.g., hemoglobin, mucus or any other known interfering substance)

[a]Abbreviations: NA, not applicable; LLQ, lower limit of quantification; LOD, lower limit of detection.
[b]Categories F1 to F6 include the reference interval (normal values), reportable range, and linear range (only for quantitative tests).
[c]Test results should be equal to or superior to the comparative test method.
[d]Precision for a qualitative test is defined as reproducibility (i.e., intrarun and interrun).
[e]Multiplex testing consists of two or more targets. Rare targets can be tested when encountered and included in the verification/validation document as an addendum. Testing a large number of targets may be difficult initially, but all targets should be tested at least annually.

QA, this monitoring occurs in the preanalytical, analytical, and postanalytical phases of testing. Verification/validation in the analytical phase not only includes ensuring adequate and acceptable training on the test, but it also relies on evaluation by observation of the technologist performing the test on a recurrent basis (i.e., operator competency assessment). This should include verification/validation of unaltered and exact adherence to the procedural manual, biosafety training adherence, patient confidentiality, result interpretation, reporting, and quality control documentation. Additionally, laboratory personnel should have documented evidence of continuing education and active licensure.

EQUIPMENT AND THE PHYSICAL TESTING AREA

Equipment and instruments require daily or as-needed functional verification/validation (e.g., evaluation of a heating block temperature and precise monitoring of centrifuge speed), scheduled calibration, and scheduled preventative maintenance. The individual wells of thermal cyclers should be checked for both temperature precision and the ability to efficiently amplify the target in a PCR test. Testing of the thermal cycler using a thermocouple device will efficiently show temperature precision. Rotation of positive controls to cover all wells as defined in an established schedule will ensure that each well has the capability of amplifying target efficiently.

The physical testing area is an important consideration in the verification/validation process. It is an issue that may compromise the work if it is not adequately controlled. An area in disarray may not only affect efficient workflow, but may also contribute to clerical errors due to the inability to properly organize paperwork and review test results. It may even contribute to an actual safety concern for the staff or be the underlying cause of nucleic acid carryover contamination. The physical dimensions of the testing area(s) at the very least need to take into account the footprint of instruments, together with adequate workspace. Ideally, two separate molecular testing areas should exist: specimen processing (i.e., nucleic acid extraction) in a positive pressure room and amplification/detection in a negative pressure room. Molecular reagents should be handled in a clean area, which may be incorporated into either another room or prepared under a "dead-air" hood in the positive pressure room. This room separation may not necessarily apply if only "sample to answer" systems are in use.

SUMMARY AND CONCLUSIONS

Test verification/validation is an ongoing process that requires a comprehensive approach to ensure QA in test performance. The use of quality controls, proficiency testing, and monitoring technical staff training and competency and equipment and instrument performance are all essential parts of this process. The introduction of new testing or modification of existing FDA-cleared/approved testing will require verification/validation. This process is discussed elsewhere in this book, but a recommended approach to such testing is outlined in Table 5. The suggested number of samples/specimens tested should serve as a guide and be supportive of statistical significance. The actual number used in the testing will depend upon several variables, including the cost of the assay and the actual availability of the samples/specimens (e.g., rarely encountered infectious agents). Probit analysis for LOD determination may also be included in the verification/validation process. However, the reader is referred elsewhere in this book for specific details on this type of analysis. In conclusion, the laboratory or medical director holds the ultimate responsibility for assurance that all clinical testing is appropriately verified/validated before the test is used on patient samples. Adherence to the recommendations outlined in this chapter should provide the clinical laboratory with the necessary tools to support QA in the molecular microbiology and infectious disease laboratory.

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Proficiency Testing and External Quality Assessment for Molecular Microbiology

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52

The increased reliance on molecular test results for critical patient management decisions demands that those results be relevant, robust, and reliable from all laboratories that produce them. Commenting on the history of quality control (QC) and proficiency testing, F. William Sunderman, MD, a leading pioneer in clinical biochemistry laboratory standardization, stated, "There can be no more important task for the director of a clinical laboratory than to assess the precision and accuracy of the analytical procedures under his/her supervision. Maintenance of high standards of analysis not only serves as a scientific stimulus for the laboratory but is also of direct benefit to patients" (1).

Continued validation of the processes producing laboratory test results is critical in the overall quality management practices of the clinical laboratory. External quality assessment is one important tool in the continuing validation of the test methods, instruments, personnel, and procedures that generate patient results in the clinical molecular laboratory. The term "proficiency testing" is defined as "the evaluation of participant performance against preestablished criteria by means of interlaboratory comparisons" (2) and is heavily focused on the examination phase of clinical laboratory services. It is used interchangeably with the term "external quality assessment," which for some indicates a broader program, assessing pre- and postexamination phases as well (3). "Proficiency testing" (PT) will be used throughout this chapter describing requirements and strategies for implementing the discipline in the molecular microbiology laboratory. Because terminology in this area can be colloquial or standardized, depending on the region, the audience, and the time a document is written, Table 1 defines the terms selected for use in this chapter and the alternative or similar terms each represents.

The tradition of PT in clinical laboratories dates back further than the regulations that currently require it in the United States and the practice standards that dictate its use globally. Sunderman and his pathology colleagues instituted the practice of PT in the United States in the late 1940s after demonstrating "abysmal" results in a glucose challenge sent to their fellow biochemists in the Philadelphia area. Because of those results, laboratory professionals were motivated to improve their test procedures and processes. In addition, the need for standardized, universally available calibrators was also supported (1, 4). Though proficiency testing has evolved, the principles and justifications for this practice standard that began in the field of clinical chemistry over 60 years ago are still used with current technologies and laboratory processes. Proficiency testing is integral to clinical laboratory practice and is applicable to each new test method that the clinical laboratory employs.

Fifty years after those first glucose studies, the molecular infectious disease community experienced a similar phenomenon using multilaboratory blinded sample trials to evaluate the global robustness of molecular test results using the newly developed assays. For HIV and hepatitis C virus, therapeutic development and subsequent treatment decisions relied heavily on quantitative nucleic acid viral load results. Global and regional PT studies served as an indicator of the assays' collective sensitivity, specificity, and reproducibility and, for each laboratory, a barometer of proficiency (5–7). The recommendation to test quantitative serial samples for patients in a single laboratory with one method, the justification for international standard reference materials, and an influence on the adoption of standardized commercial assays for viral load measurement have been indirect outcomes of the work presented from molecular infectious disease PT and quality assessment studies (8–10).

THE REGULATORY BASIS FOR PT

PT has become an essential part of clinical laboratory operations. In some countries, including the United States, the practice is written into regulations governing clinical laboratories. Where PT may not be part of local regulation, globally, ISO 15189-accredited laboratories must incorporate PT as part of their quality management system. The following sections will discuss the requirements for PT in the United States under the Clinical Laboratory and Improvement Act regulations (CLIA 1988) and globally under ISO 15198. Table 2 provides a list of the most influential document resources for the regulation, administration, and implementation of PT in clinical laboratories.

PT Regulations: CLIA

In the United States, laboratories that perform tests on human specimens for the purpose of "providing information of the diagnosis, prevention or treatment of disease, or impairment of, or in the assessment of the health of human beings" are regulated under CLIA (11). Such testing

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TABLE 1 Terms used in this chapter[a]

Term	Definition as used in this chapter	Used in place of these similar terms
Analyte	A substance or constituent for which the laboratory conducts testing (11)	Measurand Target
Challenge	For quantitative tests, an assessment of the amount of substance or measurand present or measured in a sample For qualitative tests, the determination of the presence or the absence of a measurand, organism, or substance in a sample (11)	Sample Panel member
Clinical laboratory	Laboratory for the biological, microbiological, . . ., genetic, or other examination of materials derived from the human body for the purpose of providing information for the diagnosis, management, prevention, and treatment of disease in, or assessment of the health of, human beings (15)	Medical laboratory
Event	A single round of proficiency testing/external quality assessment testing, which may include more than one challenge (specimen or sample) (3)	
Laboratory-developed test	Test method—except when specifically referring to a noncommercial test and developed in the laboratory of use, or a commercial test altered by the laboratory	Laboratory-developed procedure Home-brew test In-house-developed assay
Laboratory director	The laboratory professional responsible for the activity described in the sentence (not necessarily a director)	All laboratory professionals in a clinical laboratory
Peer group	Participants in a proficiency testing survey that use the same type of platform, assay, technology, or category of test (e.g., laboratory-developed) whose data are analyzed and reported together (3)	
Proficiency testing	Evaluation of participant performance against pre-established criteria by means of interlaboratory comparisons (2)	External quality assessment
Proficiency testing sample	The material contained in a vial, on a slide, or other unit that contains material to be tested by proficiency testing program participants; when possible, samples are of human origin (11)	Panel member
Test method	The established process, sequence of operations, in a clinical laboratory by which a measurement is made on specimens	Measurement procedure Assay Laboratory test

[a]Note on terminology: Regulatory, traditional, standard, and colloquial terms are used in descriptions of laboratory tests, standardization, reference materials, and proficiency testing. Several uses of these terms depend on the country or region or the year a document was written. Those definitions without citations are the author's for the purpose of use in this chapter.

TABLE 2 Regulatory and guidance documents for PT

Service provider	CLIA 42CFR493	ISO documents	Guidance documents
Clinical laboratories	Subpart H: Participation in Proficiency Testing for Laboratories Performing Nonwaived Tests Subpart K 493.1256: Standard: Evaluation of Proficiency Testing Subpart R 493.1838: Training and Technical Assistance for Unsuccessful Participation in Proficiency Testing Subpart J 493.1105: Retention Requirements	ISO 15189: Medical Laboratories—Requirements for Quality and Competence. Annex C: Selection and Use of Proficiency Testing—in IS0/IEC 17043: Conformity Assessment—General Requirements for Proficiency Testing	CLSI GP27-A2 Using Proficiency Testing to Improve the Clinical Laboratory CLSI GP27-A2 Assessment of Laboratory Tests When Proficiency Testing Is Not Available
PT providers	Subpart I 492.901: Proficiency Testing Programs for Nonwaived Testing	IS0/IEC 17043: Conformity Assessment—General Requirements for Proficiency Testing. ISO 13528: Statistical Methods for Use in Proficiency Testing by Interlaboratory Comparisons	CLSI MM14-A2: Design of Molecular Proficiency Testing/ External Quality Assessment

laboratories must employ PT as an integral component of their quality assurance programs for all primary tests and procedures employed to produce patient results. CLIA regulations specifically stipulate the type of PT, analysis of results, and reporting responsibilities for a group of analytes that are designated as the "regulated analytes." These analytes and laboratory procedures had already been established when CLIA regulations were written; therefore, PT strategies could be specifically tailored for them. For the regulated analytes (e.g., glucose and cholesterol) and laboratory procedures (e.g., bacterial identification, antigen testing), there are specific requirements for the number of testing events (distributions per year) and the challenges (number of samples or activities) in each event. These analytes and processes and the specific PT programs are outlined in Subpart I of CLIA (12).

Molecular procedures were not specifically named in Subpart I because they were not in use at the time the regulations were written. However, for molecular infectious disease testing, the "analyte" is a microbiological organism, and the CLIA regulations do state requirements for microbial isolation and identification. Even so, laboratories performing molecular tests for analytes that are not in Subpart I must still assess the accuracy of their systems at least twice annually, even if they have to design their own PT programs. Because of this, molecular laboratory directors should have a working knowledge of what constitutes a good PT program from the provider's perspective, because they may need to assume that role when commercial PT is not available. When commercial PT is available, the selection and administration of the program also requires knowledgeable consideration of what is available to ensure that the program will meet the laboratory's regulatory and technical needs.

The requirements for the administration of a PT program in a CLIA-certified laboratory performing nonwaived testing are provided in several parts of the CLIA regulations, most specifically in Subpart H. According to the CLIA regulations in this subpart, for each of the specialties or subspecialties for which CLIA certification is sought, clinical laboratories must:

- Enroll in a Centers for Medicare and Medicaid Services (CMS)-approved PT program for specialties and subspecialties named in Subpart I (the regulated analytes) when an approved program exists.
- For those analytes which are not named in Subpart I, or those analytes named in Subpart I that have no CMS-approved PT programs, verify at least twice annually the accuracy of the test methods.
- If more than one PT program is used by the laboratory, the laboratory director must designate the one that shall be used to judge the fulfillment of the regulatory requirements for each test method (13).

Since specific regulations will certainly lag behind advancing technologies, the second point of the regulation paraphrased above provides the assurance that new test methods and analytes are always included within the quality management system of a laboratory, allowing for technological progress without compromising continued quality. This creates a challenge for the molecular laboratory, though; often a part of the testing menu is in the category of "no CMS-approved PT program available." In this case, the laboratory director must have a scientifically rational plan for the twice-yearly verification of these analytes and methods.

In addition to subscribing to or designing a PT program, CLIA regulations further stipulate the manner in which the testing of PT samples in the laboratory is conducted. The analysis must be performed in the same manner as patient samples, which means they are tested:

- In the same workload as patient samples
- With the personnel who routinely test patient samples
- With the same reagents and lot numbers that are used for routine patient testing
- The same number of times a patient sample is tested

Each step—receiving the PT samples, storage, preparation, testing, and reporting—should be documented. Entering the samples into the laboratory's order-entry and laboratory information system accomplishes a limited assessment of pre- and postexamination processes, because the test order should be visible within the laboratory information system and should be seen on pending and overdue work lists. Communication among other laboratories regarding the PT material is strictly prohibited until the results have been published. This includes intraorganizational discussion among laboratories with multiple sites. A laboratory may not refer the PT sample out for testing, even if the normal laboratory operating procedure is to refer the sample to a reference laboratory for further analysis or reflex testing. Laboratories should perform testing on PT samples only to the extent that is regularly done within their boundaries on patient samples, and then they should indicate in the results that further testing is not performed. Most PT providers have provisions for this in their results documentation. In addition, prior to the release of results by the provider, laboratories must not accept any PT samples to test from other laboratories and are required to report such activity if approached with this request. The last two stipulations are rigorously enforced, and laboratories can have their certifications revoked for violating them.

CLIA regulations also require that laboratories document the review of their PT performance. Explanations, corrections, or preventive action plans for any PT failures or unexpected results should be recorded with documentation of execution and verification of effectiveness where appropriate. All documents should be retained for a minimum of 2 years, and the laboratory must be able to provide its PT performance results to concerned parties if requested.

The CLIA regulations provide the minimum standards for clinical laboratories serving patients in the United States. CMS has published a brochure explaining the basic requirements of PT within the scope of CLIA regulations. It is available at https://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/downloads/CLIAbrochure8.pdf. Accrediting organizations and some states (even those organizations/states authorized to inspect for CLIA) may have additional requirements. CLSI document MM27-A2, *Using Proficiency Testing to Improve the Clinical Laboratory*, provides practical, best-practice guidance on the implementation and management of PT within the clinical laboratory as one part of the overall laboratory quality management program (14).

PT Providers and CLIA

For an organization to apply for CMS approval, it must offer a PT program that fulfills the requirements of those analytes and subspecialties listed in Subpart I of the CLIA regulations. These nonprofit organizations must demonstrate to CMS that they have the ability to provide suit-

able PT samples, prepared through good manufacturing processes, with the frequency and number of challenges specified for each subspecialty they serve. They must have an infrastructure to appropriately analyze and report the participants' results to PT-subscribing laboratories and to CMS as outlined in Subpart I of the regulation. In addition, they must have a QC system with processes to manage product or system nonconformances (12).

The scarcity of PT designed specifically for molecular targets and techniques is a constant challenge. Quality PT programs are sought by laboratory directors as part of ensuring the high standard of molecular testing they strive to provide for clinicians and patients. It benefits molecular test providers and the patients they ultimately serve if PT provider organizations which do not seek CMS approval for their programs abide by the quality management principles set forth in CLIA Subpart I, consider seeking accreditation of their programs to ISO/IEC 17043 *General Requirements for Proficiency Testing* (2), or follow CLSI MM14-A2 *Design of Molecular Proficiency Testing/External Quality Assessment* (3).

Global Standards for PT: ISO

In some areas of the world, PT is a long-standing tradition, integral to quality laboratory practice, and in other areas, it is relatively new. Though country-specific regulations for the certification of clinical laboratories exist, many jurisdictions recognize accreditation to the ISO standard, ISO 15189 *Medical Laboratories—Requirements for Quality and Competence*. Section 5.6.3 of this document ("Interlaboratory Comparisons") requires laboratories seeking ISO accreditation under the standard to participate in interlaboratory comparison programs that are appropriate to the analysis and interpretation of results produced by the organization. PT is named as an example of such a program.

The standard further states that:

- The selected PT program should fulfill the requirements of ISO/IEC 17043 *General Requirements for Proficiency Testing*.
- The laboratory should have documented procedures for PT participation.
- The laboratory must review/monitor the PT results and implement corrective action when the predetermined criteria of the PT are not met (15).

In addition to ISO 15189, Annex C of ISO/IEC 17043 provides guidance on selection of PT, implementation of a program, and use of the data by laboratories, regulators, and the public.

PT Providers and ISO

Globally, PT provider organizations can seek accreditation to ISO/IEC 17043. This standard addresses the total quality management system of the organization, including the design and execution of the programs, analysis of participant data, and reporting of the results. The annexes of this document contain classification of PT program (scheme) types, statistical analysis guidance, the selection of PT programs, and use of PT information (2).

As with CMS-approved providers, PT organizations accredited to ISO/IEC 17043 must be nonprofit organizations. Accreditation is through one of the many organizations which, in turn, are accredited to perform inspections and certifying assessments to this standard.

PT CONSIDERATIONS FOR THE MOLECULAR MICROBIOLOGY LABORATORY

PT has become a worldwide standard of assessment primarily for the analytical/examination phase of laboratory competence. Discussed in detail in the preceding section, the common elements in global requirements that can be applied to all laboratory disciplines are:

- Laboratories must participate in some type of periodic assessment program appropriate to their test menu.
- Laboratories must have written procedures and documentation regarding the implementation of this aspect of their quality system.
- Laboratories must document the review and use of the data from these programs as part of their quality management system.

Though proficiency testing is a standard applied to all laboratory test methods, application to molecular testing services may require some careful deliberation. Molecular laboratory directors are faced with fulfilling the requirements for the periodic assessment of each of the analytes in their test menu, whether or not PT programs are available. Since technologies, laboratory-developed tests, commercial tests, and PT programs continue to evolve, this can be challenging, because the director must know what programs exist, know if they are suitable to the laboratory's test menu, design PT assessments where none are suitable or do not exist, and constantly monitor the environment for new programs. (CLIA regulations and ISO 15189 both state that when CMS-approved/ISO 17043-accredited PT programs are available, the laboratory must [for CLIA] or should [for ISO 15189] use them.)

The most efficient way to fulfill the PT regulatory requirements is to subscribe to suitable programs provided by an approved/accredited organization as specified in the governing regulations or standards. Though PT programs for some molecular infectious disease methods have been offered for several years, assessing their suitability to the individual laboratory's needs is still important, especially for newly developed or approved tests. As technologies evolve, PT providers are continually challenged with producing PT samples that are compatible with new test methods, contain appropriate target analytes, are free of contaminants, and still mimic human samples as closely as possible. In addition, they must manufacture enough of the material for all participants, ensuring the stability of the product during storage and shipping. To address these challenges, there are necessary manipulations to the samples that constitute a testing event. Examples of issues that molecular laboratory directors and PT providers have faced and addressed are described below. These are presented because they could be reasons for problems with current or future PT events:

- PT samples may be prepared in matrices that are incompatible with one or more test methods in the field. Many molecular test methods are driven by enzymes. The sample matrix should not compromise the enzymes' function, or the test results will be skewed. For analytes for which the testing field has many diverse laboratory-developed tests and few commercial assays, this can be an even more serious problem because the PT organization will not have access to all of those procedures for the characterization of the PT samples prior to distribution.

- PT samples containing nucleic acid for only the target analyte, not the whole organism, can result in problems for some sample preparation methods which utilize lytic, probe capture, or size filtration strategies.
- PT samples containing partial genomes for analytes that are not the target region of detection for a test method can result in false negatives for that method.
- Some test methods require the presence of human sample genes to test sample integrity or internal test processes. If these necessary genes are not present in PT samples, participants may report invalid or negative results that can be scored incorrectly by virtue of this missing element.
- Negative PT samples containing minute quantities of the target can result in false positives for some laboratories or test methods with high/improved sensitivity. This can be a particularly evasive problem, since a very low level of contaminant will produce false-positive results in only a small number of laboratories. Sorting out whether the problem is with the laboratories or the PT samples usually requires a lengthy and potentially expensive investigation. Preparation and testing of molecular PT samples must be done with rigor. In QC release testing of the samples, replicate testing cannot be minimal and must be statistically defensible (e.g., testing one to three replicates of negative samples is not acceptable).
- Quantitative PT samples should be value-assigned in reference to international standards where they exist. Though an assignment with an international standard does not ensure identical commutability to patient samples for all methods, it can give greater substance to intra-peer group comparisons.

Commutability is the property of nonpatient materials (reference materials, calibrators, controls, PT samples) whereby those materials have been demonstrated to behave quantitatively the same as patient samples when assayed using different test methods. For example, if a PT sample and a patient sample have the same quantity of an analyte, their test results would be numerically equivalent. Further, if a patient sample result is numerically the same using two different test methods, a commutable PT sample would also give the same numeric result tested with both methods. In a situation where there is bias between methods, for example, method B consistently returns results 25% greater than method A for patient samples, the commutable PT sample results would reflect this bias. Commutability is established by testing a panel of representative patient samples with the methods in question, plotting the results of two methods against each other, drawing a regression line, and calculating the 95% prediction interval along the regression line of those patient samples. When tested with the same methods, results of commutable materials will fall within that 95% prediction interval. Results of noncommutable materials will fall outside the 95% prediction interval (4).

Demonstrating commutability is important in PT when value-assigned PT samples are used to evaluate the accuracy of results across quantitative test methods in relation to their behavior with patient samples. Unless commutability has been demonstrated, only within-method quantitative comparisons are appropriate, and inferences to patient sample behavior are not meaningful. For qualitative testing, noncommutable PT samples may potentially play a role in discordant results between test methods very near the analytic sensitivity of the methods. Missing a low-positive PT sample by one method may not reflect that test method's potential behavior with low-positive patient samples if the commutability of that PT sample is not established.

Many of these issues in providing PT programs for molecular infectious disease testing have been addressed in past testing events, but they remain considerations for those choosing new programs and troubleshooting test event results.

Choosing a Commercial PT Program

For CLIA-certified laboratories, each testing site within the organization must perform PT for each analyte tested using the primary test method. CLIA-certified laboratories should seek CMS-approved programs for their PT. A complete list of CMS-approved PT providers can be found at http://www.cms.gov/Regulations-and-Guidance/Legislation/CLIA/Downloads/ptlist.pdf. Lists of ISO/IEC 17043-accredited PT providers are less comprehensive because this accreditation is awarded through any of several notified bodies that are able to certify to the standard. The notified bodies may have lists of their accredited clients on their websites. The American Association for Laboratory Accreditation is one such organization (https://www.a2la.org/dirsearchnew/ptproviders.cfm). In addition to the list of American Association for Laboratory Accreditation-accredited PT organizations, a link to Eptis is provided on the website (http://www.eptis.bam.de/en/index.htm). Eptis is a comprehensive global PT database for many laboratory fields (e.g., health care, environmental) maintained by BAM (The German Federal Institute for Materials Research and Testing). Less formally, professional practice organizations such as the Association for Molecular Pathology (www.amp.org) and the American Society for Microbiology (www.asm.org) are also very useful to consult. The members of these organizations are willing to share their knowledge of existing programs and pragmatic approaches to PT for analytes when commercial PT programs are absent.

In addition to the regulatory criteria, there are other factors to consider when evaluating a PT program to implement or when constructing one for use in the laboratory. Table 3 lists some considerations that will be applicable to many molecular laboratories. Some of the more significant considerations are discussed further.

The PT samples in the testing event should consist of organisms at concentrations that are clinically pertinent. When more than one genotype of an organism is clinically relevant, those genotypes should be included as well. Other organisms that constitute normal or applicable environmental flora should be incorporated. In the case of organisms with virulence or resistance genes/mutations, the organism without those genes should be present in some samples (e.g., for multidrug-resistant *Staphylococcus aureus* screening, challenges might include multidrug-resistant *S. aureus*, methicillin-susceptible *S. aureus*, and *Staphylococcus epidermidis*).

The concentrations of the organisms in the samples should be challenging but not below the currently acceptable sensitivity limits of approved *in vitro* diagnostic tests. If the commutability of the PT samples is not experimentally established, their behavior in different test methods may not be exactly the same as that of patient specimens. For qualitative events, this will be most apparent in low

TABLE 3 Considerations in choosing commercial PT programs

For CLIA-certified laboratories: Are there CMS-approved programs that offer PT for your analyte or method?
For ISO 15189-accredited laboratories: Are there ISO 17043-accredited PT offerings for your analyte or method?
For laboratories accredited/certified by other organizations: Does the program meet your regulatory requirements?
If the PT program is not approved/accredited, does the frequency of test events/number of challenges per event meet with your laboratory's regulatory requirements?
Does the PT organization provide information on the details of design of PT programs, establishment of values/correct result, statistical analysis, and individual and summary reports?
Are PT samples prepared with good manufacturing processes/statistically valid quality control for release and distribution?
How experienced is the organization with molecular PT?
Has the program been used with your test method? How long?
Are the PT samples provided as the specimen type tested for in your laboratory?
Does the organization serve your region (shipping, sample preservation requirements)?
How are the PT samples shipped (frozen, preserved, lyophilized, infectious, noninfectious)?
Must you apply for transport permits to receive the specimens?
Do PT samples contain all the elements needed for valid results (sample integrity genes, alternative genes, concentrations in the range of your test method)?
Is online reporting available?
How is participant confidentiality maintained?
How are complaints/customer inquiries, communications handled by the organization? How are investigations conducted, resolved, communicated?
Are summary results publicly available?
Are there published reports in peer-reviewed journals using the data from the program?
Are there education/learning programs associated with the program?
What are the cost and value for the cost?

concentrations. PT samples with concentrations close to sensitivity limits should be prepared judiciously with this consideration in mind.

If a provider is using recombinant organisms, genomes, or constructs to manufacture PT samples, all areas of the genes employed by the test methods of the participants served, or at least those in approved commercial *in vitro* diagnostic tests, should be represented. If specific genes must be present in a patient sample to produce valid results, then they must be present in the PT samples for a valid test event (i.e., human cellular genes for sample integrity). Importantly, negative PT samples must be produced and tested to be verifiably molecularly negative, and they cannot contain minute amounts of the target nucleic acid. The investigation of negative PT samples with miniscule amounts of target nucleic acid can require burdensome resources from the participating laboratory, the manufacturer, and the PT provider. Statistically appropriate testing of negative samples before distribution can alleviate this.

For quantitative testing (i.e., viral load testing), the range of concentrations in the challenges should span the clinically relevant range. It is useful to incorporate repeat samples (to demonstrate reproducibility) within the same testing event or in a subsequent one. It is also useful to include concentrations or pairs of concentrations that test for the type of information needed for making clinical decisions (e.g., concentrations surrounding a decision threshold or paired viral load levels demonstrating a 2 $\log_{10}$ or 3 $\log_{10}$ difference).

The sample type is important in choosing PT. When possible, the PT sample should be consistent with that type validated by the laboratory for patient testing (e.g., Does the laboratory test for the organism in urine, serum, whole blood, CSF?). To source enough material for distribution to a growing number of participants, PT providers may make modifications to human samples or manufacture synthetic samples to mimic human specimens. These manipulations can involve pooling specimens, diluting specimens, or spiking organisms or natural samples into large-volume sample matrices or pools. Sample matrices may be natural, modified natural, or manufactured, and depending on the distribution requirements may have preservative or other treatments to render them noninfectious. These necessary operations produce samples which are suitable for PT, but their commutability to the behavior of patient samples for all available test methods may be unknown.

Ideal PT samples are commutable (meaning the PT sample would have the same numerical relationship between different testing methods as is observed for patient samples) and, for quantitative testing, are assigned a value in reference to a certified reference material or an international standard. This is difficult in molecular PT, for which there are few international standards and no recognized reference methods for quantification. Even for traditional (nonmolecular) PT programs, it has been observed that only about half of the PT samples for analytes tested have been found to be commutable (4). Demonstrating the commutability of PT samples is beyond the scope of this chapter, but Miller et al. provide an excellent discussion of the subject including the types of inferences that can be made with PT data using samples with demonstrated commutability and those with unknown commutability (4). These considerations are important when using PT data to evaluate different test methods.

In the absence of suitable PT specifically designed for molecular test methods, laboratories have sometimes chosen to use PT programs offered for classic microbiological techniques by approved or certified organizations. This can be a practical option because it would be assumed that the whole organisms would contain the genomes detected in the molecular test method. When doing this, one should consider the preparation and delivery of the testing event. Some manipulations for whole-organism preparations may render the organisms nonoptimal for molecular sample preparation methods (e.g., more resistant to lysis or altering the nucleic acid with respect to recognition by a capture probe). Another consideration is the negative samples in the testing event. PT samples designed for microbiological growth may not have been prepared to be free of minute traces of extraneous nucleic acids and will not necessarily be QC-tested for that purpose. A third consideration is to beware of choosing PT programs designed for antigen detection of microbiological organisms. These PT samples may have only the antigenic proteins in them and not the whole organisms. The positive PT samples in these testing events will not be detected by molecular test methods.

Attempting to list the growing number of organizations that provide PT designed specifically for molecular microbiology is beyond the scope of this discussion. Laboratory directors can consult the websites provided earlier in this section for their specific needs. Two organizations, CAP (College of American Pathologists, www.cap.org) and QCMD (Quality Control for Molecular Diagnostics, www.qcmd.org), have reliably served the molecular infectious disease community with clinically relevant molecular microbiology PT programs for over a decade and currently have the most comprehensive program offerings. CAP, a CMS-approved organization, and QCMD, with programs accredited to ISO 17043, are organizations with the wide range of services (design, production, and distribution of PT events, analysis, reporting, and troubleshooting) required of good PT providers.

Conducting the PT

When PT event materials are received in the laboratory, it is important to process them with the same timing and operations as used for patient samples. When opened, the packages should be inspected for the correct content and integrity of the samples. Deadlines for submission of results should be noted when the test event materials are received. The PT provider should be notified immediately if the contents or the sample integrity is not as stated or expected or if the instructions for use are confusing. The personnel who normally process and test patient samples should process and test the PT samples. Some laboratory directors assign PT to a rotating list of testing personnel to ensure that all those who test patient samples will have an opportunity to test at least one PT challenge during a specified period of time. When testing is complete, though the results are usually recorded in the laboratory's information system, they will be transcribed onto the forms required by the PT provider or, with increasing frequency, on the providers' online web-based forms. Attention to deadlines and to transcription is important because missing the deadline and making transcription errors are usually considered incorrect results in scoring.

Scoring and Reporting the Event Results

The analysis and scoring for individual PT participants depend on several factors. For CLIA-approved PT programs, the regulations state specific criteria for the challenge and the event scores. For ISO-accredited programs, a scientifically and statistically defensible rationale for the determination of correctness must be documented. Annex B of ISO/IEC 17043 provides guidance on measuring and reporting the performance of PT participants with statistical analysis and performance scoring strategies. Commercial PT providers supply well-characterized samples to subscribers; therefore, in most cases the PT samples will have been tested in referee laboratories by the predominant test methods in the field prior to distribution. Consequently, a quantitative target value or the presence/absence of the analyte will have been determined. Depending on the analyte, the constancy of the methods in the field, and the availability of a reference method or standard, referee group consensus may be used to determine the correct result. However, the field performance of an event can influence the final correct result (e.g., if field performance yielded statistically different results than the referee laboratories or if a problem with the PT samples was determined.)

For molecular viral load testing, often the correct quantitative result is determined by the mean of the participants' results (after the removal of statistical outliers). Depending on the analyte, and the history of the methods in the field, the mean may be an overall mean, separate means analyzed by broad technology peer groups (e.g., PCR, probe amplification), or separate means analyzed by test method peer groups (different commercial offerings and a category for laboratory-developed tests). Unless commutability has been established through formal experimentation, the PT samples should be considered as having unknown commutability, and participant results should be analyzed in peer groups. Predetermined criteria (e.g., ±2 SD [standard deviation] of the peer group mean or median) will define a "correct" result. Other criteria have included the demonstration of a clinically relevant difference between paired results or the reproducibility of two PT samples with the same concentration in the same event or a subsequent one. It is important for laboratory directors to understand the criteria by which their results will be judged for the programs used.

For qualitative tests, the correct answer may be based on the referee group if at least 80% of the participants have achieved the correct result. Peer groups may be broken out in a qualitative test report since different test methods can have different sensitivity levels. However, absolute comparison of test method sensitivity based on PT can only be meaningful with commutable PT samples. The biases in different test methods seen in the PT samples, particularly if the samples have been manipulated, may not necessarily reflect the biases that would be seen with actual patient samples. In qualitative test events where participants are penalized for detecting/identifying organisms that are supposed to be absent in the PT sample, it is again important to stress that PT samples should be prepared such that extraneous nucleic acids do not contaminate negative challenges and that, prior to distribution, they are tested with molecular methods in statistically relevant numbers to ensure their purity. When molecular contamination has been confirmed, the PT samples are usually rendered as nongraded challenges. This, however, can take months of investigative work.

The above are examples of the basic results analyses that may be performed by the PT provider. In addition to individual challenge (PT sample) scores, an overall score for the testing event is usually provided to the participant. Testing events can be a mix of samples that are graded and those used for educational purposes for the participants or the PT provider (ungraded). The overall score will incorporate only the samples that are determined to be graded. CLIA regulations specify successful passing scores for events in different subspecialties (usually 80%). Other accrediting agencies and regional regulatory bodies may have different scoring strategies and criteria for passing scores. Each laboratory director should know the scores needed to fulfill their certification or accreditation requirements and understand the challenge and event scoring criteria for the PT programs chosen.

PT Performance Report Review and Continuous Quality Improvement

Reviewing the individual laboratory and summary performance reports from the PT provider should be a high priority for the laboratory director and testing personnel. Challenges for which the laboratory provided an incorrect

response must be investigated to determine the root cause, even if the laboratory received a passing score for the overall event. Nongraded challenges can provide valuable information, and investigation into incorrect or unexpected results should be performed on those as well. CLSI's GP27-A2 describes a logical method with adaptable example forms to record the investigation of unacceptable PT results. The first step is to gather all the data surrounding the test event, QC data surrounding the testing day, interviews with the testing personnel, repeat testing of the PT sample if available, additional blinded sample or QC testing, and data from past events for comparison. The next steps are to classify the probable problem, narrow it down to the root cause, determine the impact on patient care, and institute immediate action if necessary. Longer-term corrective action should be planned and executed if warranted, and the effectiveness of those actions should be verified.

The CLSI guidance document leads the laboratory director through a systematic process of classifying problems into clerical errors, methodological problems, equipment problems, technical personnel errors, or problems with PT material (14). The classifications are applicable to all PT, including molecular microbiology. Some examples of molecular applications to these problem categories are described in the following paragraphs.

In the category of clerical errors, a particular issue for molecular microbiology methods is to recognize the type of units and measurements required by the PT provider. For quantitative molecular test methods, IU/ml and $\log_{10}$ IU/ml have become standard for some viral analytes. Other target analytes may still use copies/ml and genome equivalents/ml. Transformation to and from $\log_{10}$ units and selecting the appropriate units for the test method used is important.

For methodological problems, the laboratory might review the internal quantitation standard in quantitative test methods or the internal control on qualitative assays. Disintegration or shifting of the internal quantification standards or internal controls for amplification assays can result in biases for quantitative test methods; for qualitative test methods, invalid or negative results may occur. If the laboratory has a system of monitoring PT results over time, a shift or a trend in these components might be observed in the investigation.

Technical problems can be due to the special handling required for some molecular methods when using PT samples. Some PT events have alternative instructions for specific molecular test methods or for designated commercial assays. If these are not followed by testing personnel, difficulties with the PT sample or the results may occur.

In the category of problems with the PT material, several challenges to producing PT material for molecular diagnostics have been discussed. In a rapidly developing field like molecular diagnostics, one consequence is that for newer analytes, there may be too few participants in the PT events for adequate peer group analysis. If the predominant test methods are laboratory developed, the variability from laboratory to laboratory may contribute to difficulties in reaching consensus. It is important to work with the PT provider and report any investigations of incorrect results for which a root cause could not be determined within the laboratory organization. PT providers strive to distribute test events that are suited for their intended purpose. As part of their QC system, the PT provider will maintain records of participant contacts regarding problem test events. When complaints reach a specified threshold, the PT organization will begin an investigation. If a commercial test method was used, contact the manufacturer as well. Though PT providers and manufacturers maintain professional independence of one another, the manufacturer may have technical information that will help with the PT provider's investigation. Communications received by the laboratory from manufacturers and PT providers regarding failed PT challenges and events should be filed with the laboratory's investigation documents.

Classifying the problem usually does not establish the root cause but narrows the possibilities to help reveal it. For example, the problem with a certain failed PT challenge might have been that the special handling required for the PT sample was not performed. Investigating the root cause demands an answer to the question of why that happened. Were the instructions not clear? Were they not visible? Did the laboratory not have the materials to treat the sample as required? Did the testing personnel ignore the instructions? Or was the need for special handling not known by the PT organization at the time of the test event distribution? The last cause is not uncommon in rapidly developing areas of molecular diagnostics. Each of the questions leads down a different path of possible corrective actions. It is important to drill down as far as practically possible in determining the root cause so the most effective corrective action can be taken. The goal in PT, as with all quality assurance practices, is to find and correct possible technical or operational problems before they affect patient test results.

Using PT for Quality Improvement: Monitoring PT Results

In addition to reviewing reports for single test events, tracking performance over time can be useful, especially for quantitative test methods such as viral load determinations. Several suggested methods originating in the discipline of clinical chemistry are applicable to quantitative molecular methods. Since different sample concentrations are usually used for each event, a simple method is to compare the distance from the laboratory's result to the mean of the correct result (determined by referee labs or peer group analysis). An example is given in Fig. 1. The chart represents 2 years of PT results for a given viral load PT program which has three events per year and five challenges per event. In this hypothetical example, the laboratory's result is subtracted from the mean of the peer group and the SD is calculated. (This is sometimes provided in individual PT performance reports.) The SDs are then plotted in a Levey-Jennings type of chart. With this method, all the samples can be plotted on the same graph, even though they are at different concentrations. This type of tracking may provide insight to the biases in an individual laboratory compared to the peer group. Other methods are suggested in CLSI GP27-A2 and include dividing the laboratory's distance from the mean by the amount of difference allowed for PT sample, resulting in a percent of allowed difference (14). This may further normalize the impact of the magnitude of the large concentration ranges as seen in viral load test methods. However, where increasing development and standardization of test methods reduce the interlaboratory variability over time, a shrinking allowable difference due to the decreasing peer group variability could inflate a single laboratory's comparative error. When working with viral load quantitative assays, if results are in integer format, transformation to $\log_{10}$ units is usually performed before parametric statistical manipulations (16).

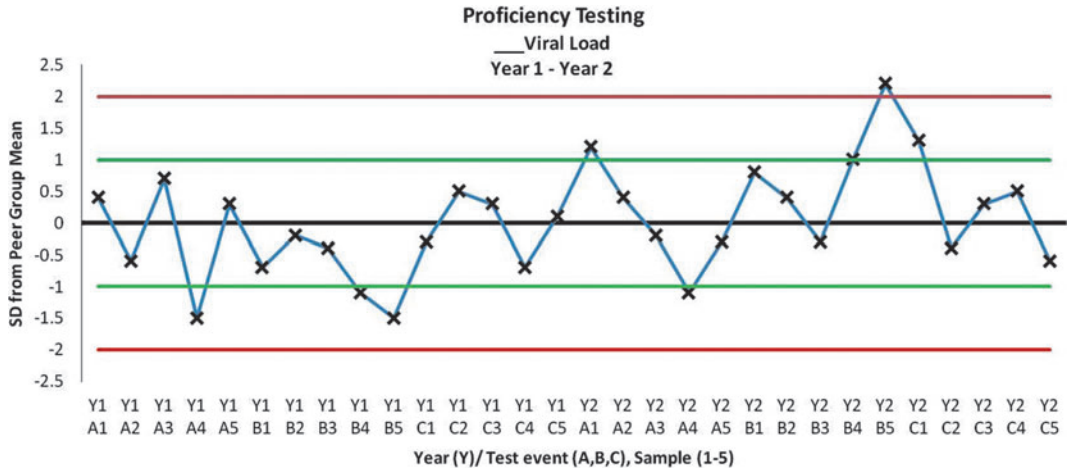

FIGURE 1 A Levey-Jennings chart representing a hypothetical 2 years of PT results for a given viral load PT program which has three events per year (A, B, C) and five challenges per event (1 to 5). For example, in the *x* axis, Y1, B2 is year 1, testing event B, challenge (PT specimen) #2. The laboratory's result for each challenge is subtracted from the mean of the peer group, and the SD is calculated, allowing plotting of challenges with different values. A shift quantitation with regard to the peer group can be observed in year 1 for the B testing event. A random failure is observed in year 2, event B, PT sample #4 because the laboratory result was above 2 SD from the peer group mean.

To monitor qualitative results over several test events, a simple percent of the correct number of positives and negatives detected may be useful.

Strategies for Alternative PT

The design and production of new PT programs cannot keep up with the breadth of molecular analytes and test methods continually introduced in the clinical laboratory. Therefore, it is expected that for some analytes and test methods, the laboratory director will have to find alternative proficiency testing or supplement commercial PT programs. According to CLIA regulations, the accuracy of test methods for analytes that are not named in Subpart I or for which no PT exists must be verified at least twice per year. Further, ISO 15189 requires that laboratories participate in interlaboratory comparison programs for all examination procedures (test methods). When no such programs can be found, the laboratory director must design and execute a practical and scientifically defensible method of assessment. The principles of implementing alternative PT are the same as those for commercial PT, and the issues and examples stated previously can be applied when designing in-house PT. Some additional considerations are discussed below.

Documentation for alternative PT may be more detailed and should include:

- The design of the program, including the frequency, sourcing of samples, the concentrations of the samples, the target organisms used for the analytes, and the rationales for each of these. For example, if the laboratory's test offering includes the detection of an agent to the species level and a clinically relevant genotype, then a panel of challenge samples for the testing event might include organisms of the same genus but different species, or those of nonrelevant genotypes if they are also expected to be in human samples. Quantitative testing PT samples should include the range expected in clinical samples, with attention paid to concentrations at medical decision points.
- Verification of the constitution and integrity of the samples used should be documented. (Examples of documentation may be literature regarding organism stability in the matrix used and temperature charts of the device storing the sample.)
- The determination of correct results for each sample in the testing event should be explained with the rationale. Determination of what constitutes a successful event score should be written before the testing and analysis.
- Comparator methods and statistics used should be outlined.
- The method of investigating unexpected results and failures should be described.

Investigations of unexpected results and failures may be more complex in these PT events because the reason for the failures may not be as clear. For example, when using patient samples maintained in the laboratory, the stability of those samples or possible changes during freeze-thaw may be unknown. Or, if using a single-test-prior-result for the sample as the target value for the PT challenge, the prior result may also be questionable.

Depending on the jurisdiction of the clinical laboratory, the use of human specimens and patient privacy regulations may also need to be considered. Whether the specimens are used in-house or shared with others may have a bearing on the types of permissions needed.

A common strategy for fulfilling alternative proficiency assessments for analytes not covered in commercial PT programs is by utilizing split-sample procedures. Aliquoting and anonymizing samples received in the laboratory for routine testing can be used to exchange with another laboratory or be used internally for retesting at a later time. Internal retesting is less desirable because systematic biases internal to the laboratory will not be apparent. In addition, for ISO 15189-certified laboratories, this will not fulfill the need for external comparison programs.

Professional organizations have been valuable in assisting laboratories with specific sample exchange needs to find

TABLE 4 Strategies when commercial PT programs are unavailable

Strategy	Implementation	Comments/considerations
Split samples within the laboratory	Preserve previously tested patient samples (usually aliquot in single use and freeze) Deidentify and redistribute within laboratory for PT testing at a later time	Useful if there are no peers with the same test method "Correct answer" may be established only by the initial test result Potential quantitative range of sample results is dependent on what the laboratory receives for testing Sample quantity and stability can be issues Best used in conjunction with another method (purchase controls, calibrators, or spiked-in samples) For quantitative assays, can provide insight to continued reproducibility of system System errors (incorrect or biased results) may perpetuate without detection if no external panels, calibrators, or samples are supplemented Does not fulfill the ISO requirements for external laboratory comparisons
Split samples with another laboratory or laboratories	Preserve previously tested patient samples (usually aliquot in single use and freeze) Deidentify Send to and/or receive from participating laboratory(s)	Potential quantitative range of sample results is greater than for within-laboratory split-sample testing Samples must be used in compliance with patient confidentiality policies and regulations "Correct answer" may be established only by the initial test result Sample quantity and stability can be issues Transport stability of specimens should be ensured Data collection, analysis, and acceptability criteria should be established prior to distribution Procedures to resolve discrepancies, outliers, and arbitrations should be agreed on before distribution Biases with regard to different test methods in participating laboratories should be understood
Split samples with another laboratory or laboratories, facilitated by professional organizations	Preserve previously tested patient samples Deidentify Contribute to organizer (Consult organizations; logistics and availability vary)	Alleviates many of the issues in self-managing a split-sample exchange Availability of analytes may depend on a required number of participants Data analysis, acceptability criteria, discrepancy analysis should be understood by participants Biases with regard to different test methods in participating laboratories and too few participants may be a factor in reporting
Spiked-in samples	Grow organism and spike into negative samples Or Dilute highly positive samples into negative samples	Time-consuming for the laboratory Cultivating organisms and diluting high-positive samples must be done in laboratory areas separate from molecular testing Samples must be tested several times for characterization, value-assignment prior to PT testing Pooling samples (diluting high-positive into negative) may induce matrix issues
Use of existing organism identification PT surveys	Purchase and test PT surveys intended for organism identification	Useful for qualitative assays Preservation/lyophilization of organisms could interfere with molecular assays If human sample nucleic assay needed for valid assay, it may not be present Laboratory's results might be combined in a heterogeneous "other" peer group for reporting Surveys for organism antigen detection should not be used
Purchased controls and panels	Purchase and preserve value-assigned/characterized assay controls or validation panels	Understand the vendors' methods for sample characterization (value assignment, positive/negative, genotype) Provides assessment with external well-characterized samples Matrix may be artificial Aliquoting and relabeling may be necessary
Manufactured calibrators as PT samples	Preserve test calibrators (aliquot in single use vials) Relabel and distribute for PT testing	Potential range for quantitative test should span the reportable range Provides assessment with external well-characterized samples Matrix will be artificial If laboratory assay uses calibrators, it is optimal to test with a different lot than that used for assay calibration Some calibrators may be assay and lot specific; these should not be used Does not fulfill the ISO requirements for external laboratory comparisons

each other. ASM (www.asm.org) and AMP (www.amp.org) each have active list serves where members can post their requirements. CAP has its Sample Exchange Registry for Alternative Assessment and serves as a facilitator for laboratories performing rare or infrequently used test methods. Laboratories register with CAP, and when there are three or more requesting the same analyte and methodology, CAP will facilitate a sample exchange and be the conduit through which result reporting and analysis are performed. Details on this program can be found at www.cap.org.

Another strategy for alternative PT is to purchase controls or validation panels from one of the many molecular control vendors. While not ISO or CMS approved for PT programs, several of these vendors have FDA approval for some of their products and/or ISO manufacturing certification. Their controls and panels are produced under good manufacturing processes. As with PT samples from commercial organizations, considerations must be made with regard to matrices, target content, compatibility with test methods, etc. Procedures in the laboratory should state how samples will be blinded (if necessary) and distributed and how the evaluation of the correct result will be conducted.

Molecular infectious disease testing is performed for a growing number of analytes in diverse settings using numerous test methods. Alternative PT approaches will necessarily be tailored to the needs and expertise of each laboratory. Table 4 outlines some traditionally and commonly used strategies, how they are implemented, and issues to consider for each. Laboratory directors should carefully research the suitability of different approaches for their needs.

The CLSI document GP29-A2 *Assessment of Laboratory Tests When Proficiency Testing Is Not Available* discusses several strategies for alternative assessment of implemented laboratory test methods, some beyond the traditional understanding of proficiency testing (17). Additionally, document MM14-A2, which was written primarily for PT provider organizations, has a section focused on individual laboratories acting as the PT provider for their own facility or others (3).

A newer strategy for alternative PT is method-based PT, assessing laboratory competence in the basic techniques of molecular testing: yield of DNA after sample preparation, quality of amplified products, and/or ability to design probes. The utility of method-based PT, particularly for sample preparation and extraction, sequencing, quantitation, and sequence reporting, continues to be explored. Often these studies have been grant-funded and short-termed projects (3 to 4 years), but the information published from them has been insightful and valuable. As currently implemented, this type of PT can be very resource intense because it can involve experimentation to choose appropriate markers for assessment, precise and rapid preparation of single human samples for distribution, assessment of the participant laboratories' product of nucleic acid extraction, in-depth assessment of success at each step of the process, specific review of individualized reports, and other customized assessments. Some programs have added educational training/workshop components for participants. In most cases, improvement in laboratories from the initial event to subsequent events was demonstrated (18–22).

SUMMARY

PT is an important part of clinical laboratory quality management practices. For most certified or accredited clinical laboratories, it is required. The purpose of PT is to assess the competence of clinical laboratories, but data from PT can convey evidence of the overall robustness of the test methods in a particular area of the field (e.g., viral load testing, *Mycobacterium tuberculosis* screening). For the individual laboratory, monitoring PT may provide insight to potential problems before they become manifest. However, caution should be used in the direct translation of PT performance to patient samples when the PT samples do not have demonstrated commutability.

Challenges exist in finding PT programs for new and developing molecular test methods. Because of this, molecular microbiology laboratory directors must be able to evaluate the continually developing program offerings in the field for suitability to their laboratories' test methods. In addition, they may be in the position of designing their own laboratory's PT for some analytes or test methods for which no commercial PT exists.

PT providers, molecular laboratory professionals, and manufacturers are working together to design, distribute, and analyze the most relevant PT that is practical. Further challenges are the incorporation of pre- and post-examination phase assessments and attempting to stay abreast of areas where the technologies are developing. The common goal of all stakeholders is to produce the highest quality molecular microbiology test results for practitioners who rely on them in the care of their patients.

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Practices of Sequencing Quality Assurance

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53

While the breakthrough discovery of DNA structure was in 1953, many innovations still needed to occur before labs could generate the sequential order of nucleotides in genetic material. Results began to materialize 15 years later in 1968, when Wu and Kaiser performed "cohesive end" sequencing on phage DNA using radiolabeled nucleotides and *Escherichia coli* DNA polymerase (1). This method produced reads of 10 to 20 bases, and fragments were separated by two-dimensional chromatography.

Between 1975 and 1977, DNA sequencing rapidly evolved into a method that would dominate for the next 30 years. The basis of this method is the random termination of DNA polymerization to yield fragments ending at every possible nucleotide in the template (see Fig. 1). Electrophoresis is then used to order the fragments by length, and migration speed correlates with fragment size. Base reading (base-calling) is conducted by writing the sequence from the smallest fragments to the largest (2). Two main approaches predominated at the time, with Frederick Sanger developing sequencing methods using dideoxy nucleotide-mediated chain termination, and Maxim and Gilbert using chemical cleavage. Both methods used ^{32}P-labeled fragments electrophoresed through polyacrylamide slab gels and imaged on x-ray film. This manual sequencing method produced the first genome sequences from phi X174 virus (2).

The Sanger dideoxy method eventually became the dominant approach when it was coupled with single-stranded DNA cloning into phage M13 in a protocol developed by Messing and collaborators (3). In the late 1980s, Leroy Hood, in collaboration with Applied Biosystems, replaced the radioactive isotope labels with fluorescent dyes and developed an automated electrophoresis and sequence data collection instrument (4). Incremental improvements such as capillary electrophoresis (CE), more robust sequencing polymerases, and brighter dye chemistry culminated in the primary method used today, which is interchangeably called Sanger sequencing or CE sequencing. An additional technology surfaced in 1996, when Ronaghi et al. (5) described pyrosequencing, employing photon-based detection and now forming part of the Qiagen Pyromark system (5). Ultimately, beginning with DNA structure in 1953, decades of technological advances cumulated in the sequencing of the human genome almost 50 years later in 2001 (6).

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While the Human Genome Project cost upward of $1 billion and took over 10 years to complete, whole-genome sequencing in 2016 is in the $1,000 range and takes a matter of hours. This revolution began in 2005, when Jonathan Rothberg, at 454 Life Sciences, launched a new pyrosequencing-based system that surpassed the throughput of Sanger sequencing by 100-fold (7). Although error rates were higher than Sanger sequencing, the massively parallel nature of the instrument allowed generation of 40 reads covering every nucleotide, instead of a single read obtained with Sanger sequencing. Generation of a consensus sequence from the deeper read coverage allowed for acceptable error profiles. Solexa followed suit with its own technology, launching the Genome Analyzer in 2006. The Genome Analyzer leveraged fluorescently labeled terminator nucleotides in a manner similar to Sanger sequencing; however, in this case, millions of template clusters were fixed to a slide (flow cell), and incorporation of fluorescent nucleotides was measured by sophisticated optics, which captured an image following each incorporation event. Single-molecule sequencing from Pacific Biosciences then emerged, which facilitated longer read lengths and enhanced the ability to assemble complex genomes (8). The next major technology came with the advent of semiconductor-based detection of hydrogen ion release that occurs naturally during nucleotide incorporation (9). Ion Torrent sequencing is extremely fast due to elimination of the need to capture images after every nucleotide flow. Currently, the major players in next-generation sequencing (NGS) are Illumina (which bought Solexa), Ion Torrent (Thermo Fisher Scientific), Pacific Biosciences, and Roche/454 Life Sciences.

Sanger Sequencing versus NGS: What Applications Are Best Suited for Each Technology?

Sanger Sequencing

Different applications of Sanger sequencing and NGS are outlined in Table 1. Sanger sequencing is well suited for the detection and identification of microorganisms (e.g., through 16S rRNA gene sequencing), as well as for the discrimination of genetic targets that are highly polymorphic or have rapid mutation rates. HIV and hepatitis C virus (HCV) are important examples of viruses that show rapid drug-resistant variant outgrowth. Viral infections with low fidelity replication machinery, recombination, and reassortment result in the generation of genetically related quasi-species within one individual, some of which may be

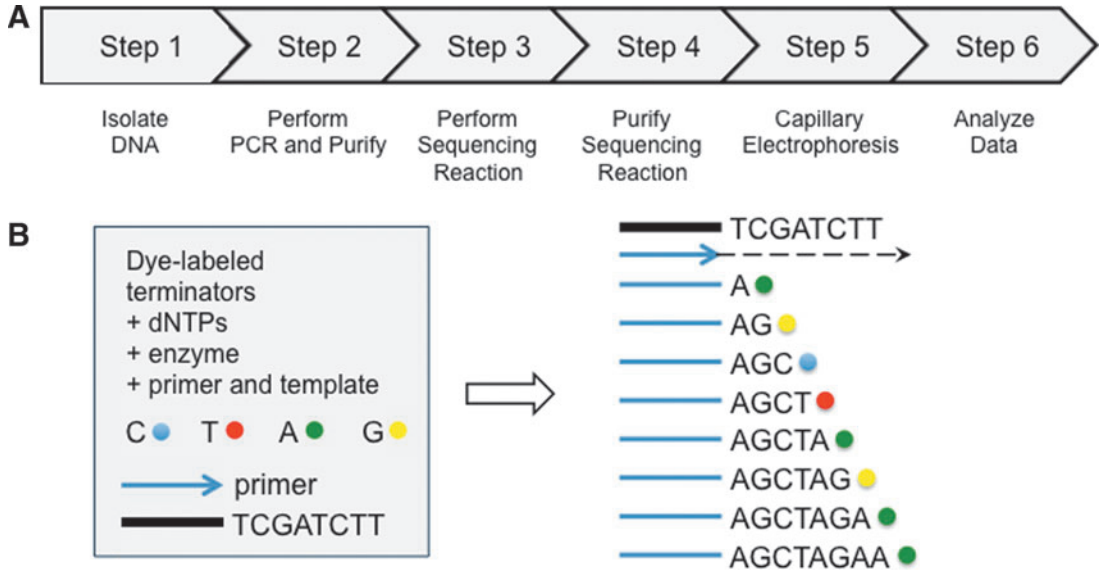

FIGURE 1 (A) Sanger sequencing workflow. (B) Terminator nucleotides prevent further polymerization and label each fragment with a dye corresponding to the final nucleotide identity.

drug-resistant (reviewed in reference 10). In this case, it is especially important to understand genotype-to-resistance phenotype relationships, which are highly dependent on well-curated databases. Because Sanger technology can produce contiguous reads up to 1,000 bases long, it is also useful for determining which genetic variations are in *cis* on the same molecule over the span of long genetic regions, but only if cloning is performed. Otherwise, many quasispecies may be amplified (e.g., with HIV) and a diverse population sequenced together to produce a consensus sequence. Sequencing in general is especially useful for organisms for which traditional culture methods are slow or challenging. In the case of *Mycobacterium tuberculosis*, culture methods to assess phenotype can take weeks, but sequencing directly from samples without the need for culture can accelerate resistance typing where mutations are known (11). Several examples also exist for fungal sequencing (12–14). Finally, high-volume applications requiring rapid turnaround times for sample prep and instrument workflow are well suited for Sanger sequencing.

The predominant instruments used for DNA sequencing were originally built by Applied Biosystems (now Thermo Fisher Scientific) in the 1990s. Current instruments are CE devices with integrated base-calling software. A wide range of consumables and sequencing reagents accompanies CE instruments, allowing for a large breadth of applications.

TABLE 1 Examples of sequencing technology applications

Sanger	NGS
Resistance typing (high-frequency variants)	Resistance typing (low-frequency variants clonal information)
Organism identification	Organism identification
De novo sequencing	*De novo* sequencing
16S metagenomics	16S metagenomics
Phasing long polymorphic regions	RNA-Seq
	Outbreak screening
	Mixed infections

For example, a research-use-only 16S rRNA sequencing assay is available for detection and identification of bacterial species. These CE systems can support processing of up to 1,000 samples per day. Several other platforms incorporate sequencing and electrophoresis with targeted applications. The OpenGene Sequencing System is a slab gel electrophoresis system with rapid run times but more limited throughput compared to the Thermo Fisher CE instruments. Siemens offers the Trugene HIV-1 genotyping assay on this system, which identifies and interprets antiretroviral resistance mutations for use in clinical diagnostics. The Amersham-GE MegaBace systems and SciEX GenomeLab GeXP genetic analysis systems from Beckman Coulter are research-use-only CE instruments that have the capacity for DNA sequencing and other molecular assays. Both of these instruments have a limited number of published applications with microbial DNA sequencing.

NGS

NGS infectious disease assays are currently for research use only, but instruments and assay providers are quickly moving toward *in vitro* diagnostic-labeled submission. While NGS is a new technology, there are a number of areas in which massively parallel sequencing has already changed the field. Like Sanger sequencing, NGS offers advantages in organism detection, especially for slow-growing organisms. Additionally, outbreak detection, organism identification in mixed infections, interrogation of and samples with unknown infectious etiology, and detection of drug resistance are all well suited for potential NGS applications (15–18). NGS diagnostics are expected to grow significantly over the next few years as *in vitro* diagnostic-labeled instruments become available, workflows improve, and reimbursement infrastructures are established.

One of the first applications of infectious disease NGS was in the area of pathogen outbreak investigation, when NGS identified the cause of a serious *E. coli* outbreak in Germany in 2011 (19–21). Whole-genome sequencing has also been used to craft a more nuanced understanding of disease transmission. In 2012, single nucleotide polymorphism tracking by NGS helped to reveal unexpected extended silent transmission in a carbapenem-resistant

Klebsiella pneumonia outbreak (22). More recently, den Bakker et al. (23) demonstrated that whole-genome sequencing offered improved *Salmonella enterica* outbreak resolution (23). Now, hospital-acquired infection NGS panels are beginning to be used to study a battery of pathogens, including *Staphylococcus aureus*, coagulase-negative *Staphylococcus*, *Acinetobacter baumannii*, *Enterococcus faecalis*, *Enterobacter cloacae*, *Enterobacter aerogenes*, *Enterococcus faecium*, *Klebsiella pneumoniae*, *E. coli*, *Clostridium difficile*, *Proteus mirabilis*, and *Pseudomonas aeruginosa* (http://www.bioinnova.ch/hai-bioinnova-kit.html). Overall, whole-genome sequencing by NGS is expected to replace standard bacterial multilocus sequence typing very soon (24, 25).

NGS has also shown promise for organism identification in idiopathic disease. While organism detection by PCR or Sanger sequencing is faster than culture, it can only detect up to 30 pathogens or targets in a single test and can potentially miss subspecies. Because of its high throughput and enhanced multiplexing, NGS is much more scalable to enable interrogation of hundreds of targets in parallel. Wilson et al. (26) recently described the use of unbiased NGS to identify *Leptospira* in a child's cerebrospinal fluid, when an extensive infectious disease workup was otherwise negative (26). Note that increased sensitivity needs to be associated with clinical validity, because detection of a pathogenic sequence does not necessarily correlate with disease causation. The potential for false-positive associations stresses the importance of current efforts to characterize and understand the microbial, viral, and fungal communities in healthy and diseased individuals (27–31).

In addition to organism identification, NGS can detect variants to as low as 1% frequency. For example, when a drug-resistant mutation emerges in a pathogen population, the mutation (mutant pathogen) can be detected at a level of 1 mutant relative to 99 wild-type pathogen genomes. This sensitivity allows enhanced detection of preexisting minority drug-resistant variants compared to Sanger sequencing. For example, evidence is accumulating that drug-resistant viral quasi-species may already be present in drug-naïve HCV-infected individuals at levels below the Sanger limit of detection (LOD) (32, 33).

A new technology presents new hopes but also new challenges in achieving reliable results. Unparalleled multiplexing ability, differential extraction characteristics, expanding sequence databases, and enhanced sensitivity each come with their own set of challenges. This chapter will review common practices in assessment of Sanger and NGS system performance with an emphasis on infectious disease, and will also review what kinds of controls are being implemented to ensure data quality. A complete quality assurance program also involves appropriate training, documentation, inventory management, equipment, etc.; however, this chapter will focus only on the technical aspects of assay development and process control. Since the field is evolving rapidly, this is not a prescriptive protocol but, rather, is intended to provide a general overview of sequencing quality optimization and assessment for every step of the workflow. Assay developers should always refer to current local or national guidelines.

SEQUENCING WORKFLOW OPTIMIZATION: KEY PARAMETERS

Specimen Handling and Extraction

Sample collection, preparation, concentration, and storage methods vary with the assay context; like other nucleic acid-based technologies, extraction kits for sequencing also differ depending on whether the assay is for body fluids, fixed or frozen tissue, or environmental samples. Multiple samplings will also provide more reliable and accurate results. If a mixed population of organisms is being measured, sample preparation choice and optimization become even more important, since optimal preparation conditions of one organism can differ from those of another organism (34). It is important to consider the false-negative rate that may occur due to inadequate extraction or, conversely, overextraction of subsets of a microorganism community. Ultimately, characterization of sample collection and nucleic acid extraction methods must be performed using known control organisms. This may be done by spiking known amounts of the organism into a negative sample and comparing yields of different procedures. Remarkably, a study by Muñoz-Cadavid et al. (35) found a 31% range in assay sensitivity across five different extraction protocols for formalin-fixed, paraffin-embedded tissue (35). Well-characterized, full process reference materials are essential for metagenomic assay optimization.

Sanger Sequencing Workflow Optimization

Template Preparation Methods

The Sanger sequencing workflow is illustrated in Fig. 1. The first step of Sanger sequencing usually involves PCR amplification of regions of interest directly from genomic DNA and can be completed in about 1 hour. This PCR step generates the target template for the assay; therefore, its optimization is critical for assay specificity and sensitivity. Optimized PCR buffers and polymerases are available for different sample types. Some kits enable running the PCR directly in lysed samples, eliminating the need for nucleic acid purification (36). Other PCR reagents allow rapid target amplification in under 30 minutes. Additionally, nested primers used in two rounds of PCR can increase sensitivity without losing specificity (37, 38). Once template is generated, purification of the amplified target is required to eliminate the remaining PCR primers and reagents that can impact the sequencing reaction.

Random Extension Termination and Electrophoresis

Next, cycle sequencing generates incremental-length copies of the template. In this step, a sequencing primer is annealed to the template in the presence of a mix of natural and fluorescently labeled dideoxynucleotides. During polymerization, random incorporation of a terminator dideoxynucleotide prevents further extension. This yields an incomplete template fragment, which is labeled with the dye corresponding to one of the four final terminator nucleotides (Fig. 1B). Multiple rounds of annealing and polymerization generate randomly terminated fragments of varying length.

Most instrument vendors provide instrument-specific reagents, labeled dideoxynucleotides, and polymerases. These components are optimized to yield uniform sequencing results from a variety of DNA templates. Because a critical parameter for the sequencing reaction is optimization of the proportion of template strand to sequencing primer and labeled terminators, terminator dilutions for cost savings can produce unreliable sequence due to low signal and inaccurate base-calling. On the other hand, limiting template can yield a predominance of short termination fragments, and signal toward the 3′ end of the template can become too low for reliable base-calling.

Once cycle sequencing is complete, the Sanger reactions must be purified to remove unincorporated terminators and other reaction components. Clean-up can be performed by alcohol precipitation, spin columns, or magnetic bead methods. Optimization of this step may be needed to ensure adequate yield and prevent loss of short termination fragments.

Finally, sequencing reactions are loaded onto the instrument and fragments are resolved by size. The electrophoretic matrix chemistry and run parameters have a major impact on maximum read length of the sequencing assay. Instrument manufacturers usually offer a selection of matrix gel or polymer types depending on the assay, run time, and region of interest relative to the sequencing primer site. Longer electrophoresis paths allow for better resolution of longer termination fragments and therefore longer sequence reads. Longer paths will also increase the overall electrophoresis run time, but this can be compensated to some degree by increasing the run voltage. Optimization of run parameters may be necessary depending on the distance between the target region of interest and the sequencing primer. If the polymorphic positions are near the sequencing primer site, short termination fragments are of most value.

Sequence Anomalies

Common sequence anomalies are generated during the termination reaction or during electrophoresis. During termination (as well as template generation), polymerases face an inherent nucleotide incorporation error rate, and some sequence motifs are more challenging than others. Homopolymer runs and microsatellite sequences can cause the polymerase to stutter during extension and termination. Additionally, GC-rich regions can form secondary structures in the template strand, which impede the polymerase and result in excessive terminations at a single base.

Sequence anomalies generated during the electrophoresis step can be a result of compression. In this case, secondary structures in the migrating fragments cause poor resolution (compression) and low-quality base-calling (39). In most cases such pitfalls can be avoided by performing sequencing reactions in both the forward and reverse directions on the same double-stranded template. Bidirectional sequencing should ultimately produce readable sequence in at least one direction.

Base-Calling Algorithms and Quality Metrics

The choice of base-calling algorithm can greatly affect sequence quality. Instrument manufacturers offer data collection and base-calling algorithms that convert signals collected during electrophoresis into the four bases of DNA. Each nucleotide call is accompanied by a quality score, which is a statistically based probability value describing the error rate at that position. These algorithms must plot collected signal data points versus migration time. It is essential to generate proper data point spacing to discriminate terminated fragments that differ by a single nucleotide. In instances where the terminated fragment signal or resolution becomes degraded, the algorithm assigns an ambiguous call, usually "N." It is also important that an algorithm can distinguish insertion/deletion errors from substitution errors. Besides instrument vendor-provided base-calling programs, the most common base-calling algorithm is PHRED, which has improved accuracy and incrementally increased read length (39, 40). Mixed base-calling algorithms are also available to assign IUB (International Union of Biochemistry and Molecular Biology) codes to heterozygous sequences where polymorphic positions are relatively infrequent (PolyPHRED). Some algorithms exist that attempt to make calls on mixed sample results (RipSeq and BCV).

Beyond base-calling and quality scores, other metrics used to assess sequencing quality are read length, signal values, and background. For any assay, such metrics and their acceptable limits must be verified with guard-banding studies and validated by blinded performance studies. Guard-banding studies test variation in multiple components of an assay to determine acceptable ranges for each component. Ideally, the range of variation is exaggerated during guard-banding to cause test failure, which can be correlated to specific output measurement values in the software. In the end, a comprehensive data analysis workflow must be developed that includes version-controlled software, documented instructions, and criteria for all possible results scenarios.

NGS Workflow Optimization

Figure 2 illustrates the general steps of an NGS workflow. Workflow optimization with known sample types and known organism or variant abundances helps to ensure reliable performance (41).

Library Preparation

To make a sequencing library, sample DNA fragments are generated and then are flanked by instrument-specific common adaptor sequences. Adaptor sequences are later used on the instrument to prime an enzyme-driven sequencing reaction. If multiplexing samples in one run is desired, a unique 10- to 20-nucleotide barcode sequence can be included with the adaptor during library preparation. It is recommended to rotate barcodes with each run to minimize cross-sample carryover.

Several library preparation methods are available for NGS, including fragmentation/shearing-adapter ligation (42–44), transposon-mediated (45, 46), direct PCR (Fig. 3A), fusion PCR (Fig. 3B), mate-pair (Fig. 3C), and other techniques (47–50). Whole-genome library preparations may use DNA shearing, for example, by enzymatic digestion or sonication, prior to library adapter ligation. Alternatively, transposon-mediated DNA fragmentation can be performed. Transposon-mediated library preparations for small genomes can be as short as 15 min of hands-on time with 90 min of total prep time.

When specific targets are needed, for example for viral or bacterial typing, direct PCR (Fig. 3A) or fusion PCR

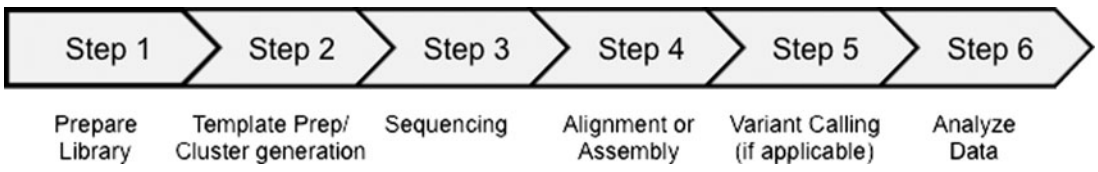

FIGURE 2 Next-generation sequencing workflow. See text for details.

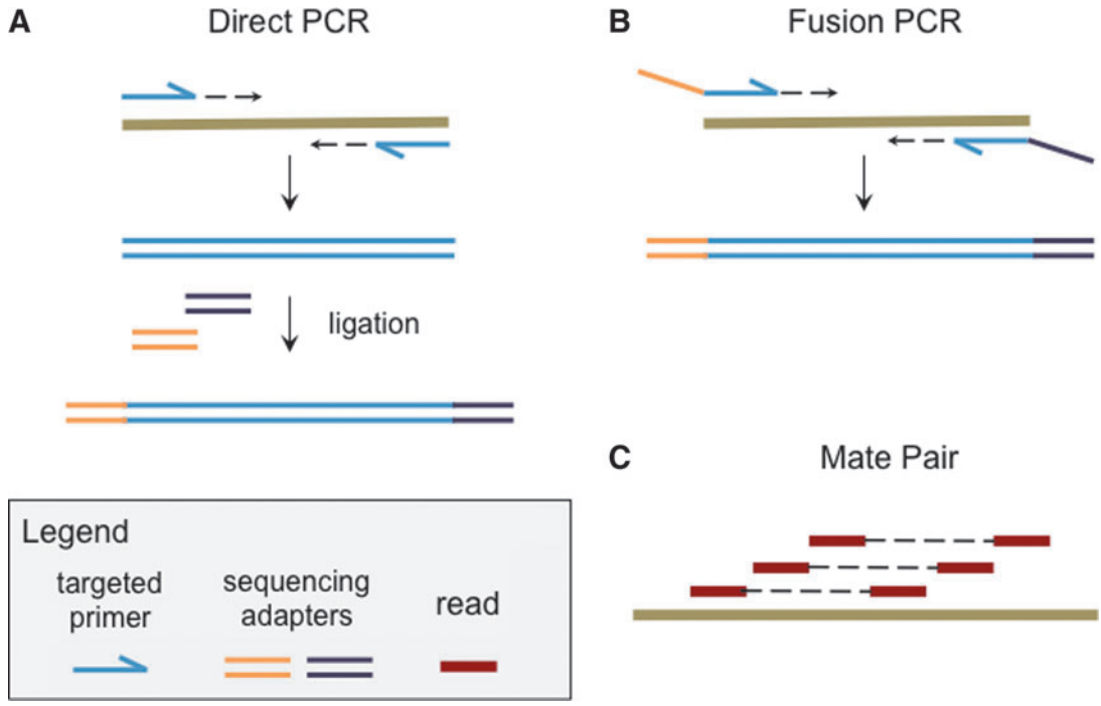

FIGURE 3 (A) Direct PCR. (B) Fusion PCR library preparation strategies. (C) Mate-paired reads are separated by a known intervening sequence length (dashed lines), allowing for accurate relative positional alignment. See text for details.

(Fig. 3B) may be performed. For example, some highly multiplex PCR library kits amplify regions of interest prior to library adapter ligation, allowing for very low DNA input amounts. Fusion PCR is a modified protocol in which library adapters are fused to the PCR primers during oligo synthesis. This eliminates the need for an adapter ligation step. With amplification technologies, uniform amplification efficiencies across the primer panel yield the best results. Primer design or PCR conditions may need modification in the case of nonuniform amplification, which can be caused, for example, by the presence of variants under primers, or GC content. Notably, GC content affects coverage uniformity to different extents depending on the library preparation method (51).

Another library preparation technique uses a modified library construction protocol to generate mate-pair libraries (Fig. 3C). Mate-pair libraries are used to align pairs of reads against a reference with a known intervening sequence distance between the two reads. With this strategy, large DNA fragments of known size (e.g., 4 kb) are isolated, and both ends are sequenced (e.g., 200 bp from each end) through a specialized library and sequencing protocol. Since the intervening distance between ends is known (3.6 kb in our example), the 5′ and 3′ sequence reads can be mapped relative to each other on a genomic scaffold. This helps with mapping repetitive sequences, where the location of a read is not always obvious due to limited diversity. For mate pair sequencing, protocols that ensure low rates of chimera artifacts are preferred.

In addition to target enrichment by PCR-based methods, other library preparation techniques can offer increased sensitivity and specificity as well as reduced target bias. For example, microbiome enrichment by a hybridization method for methyl-CpG-binding domain proteins has been used to separate human host DNA from prokaryotic sequences producing a higher yield of microbiome sequences (52). Another new technology employing magnetic beads has been used to capture fungal pathogens from blood (53).

In all library preparation types, care must be taken in library preparation design to ensure detection of low-abundance organisms within the context of a microbial community or in the detection of low-abundance variants within the context of a wild-type population. Small changes in library preparation methodology, such as whether adapters are fused to primers or ligated post-PCR, have been shown to skew the relative abundance of bacteria in a metagenomic control sample (54). The use of known, quantitated samples is highly valuable in extraction optimization.

Template Preparation

Once libraries are constructed, they can be loaded onto an automated template generation instrument, such as the Ion Chef, for Ion Torrent sequencing, or into a flow cell for Illumina sequencing. Library quality control is critical at this step. Loading too much library leads to reduced sequence output. If library input is too high, with Ion Torrent technology, polyclonal reads are generated, and with Illumina technology, clusters will overlap and prevent resolution of individual reads. To optimize this step, library inputs may be normalized with platform providers' kits, or libraries may be quantified by Qubit, qPCR, or digital PCR. Library size distribution may be verified with an instrument such as the Agilent Technologies Bioanalyzer or Tape Station. Primer dimers or other unexpected peaks indicate problems with the library prep.

Sequencing

Most NGS platforms employ sequencing-by-synthesis (SBS), in which a DNA polymerase drives a primer extension reaction based on the template. The key difference between SBS and the Sanger sequencing reaction is that nucleotide incorporation events are detected in real time

during the reaction. Most platforms use a flow cell/chip/plate with the capacity for millions of parallel sequencing reactions. Pyrosequencing detects the release of pyrophosphate by conversion to photons, and imaging takes place with a charge-coupled-device camera. Ion Torrent platforms detect polymerization-driven release of hydrogen ions; a change in pH in each well is converted to a change in voltage by a field effect transistor surface. Illumina uses a fluorescence-based SBS method with reversible terminator nucleotides. After each round of data collection by imaging, terminators are deblocked and fluorescent dyes removed in preparation for the next SBS reaction. Recent advances in Illumina technology have modified the nucleotide dye strategy. Pacific Biosciences uses fluorescently tagged nucleotides in single molecule reactions with the polymerase bound to the reaction surface. Each NGS method provides platform-specific base-calling algorithms that are optimized for the raw read data generated by each instrument. In a manner similar to Sanger sequencing, individual base quality scores are provided, which allow read trimming, discarding of questionable reads, and confident variant calling.

The choice of sequencing kit can influence data quality, and each application will work best with a specific sequencing kit. For example, *de novo* genome assembly benefits from longer read-generating kits. If more rapid turnaround times need to be achieved, and whole-genome assembly is not required, shorter read lengths combined with targeted sequencing protocols (e.g., direct PCR) are ideal.

Alignment and Data Analysis

With the abundance of data provided by an NGS run comes an equally complex bioinformatics challenge; therefore, pipeline automation will be of key importance for general clinical adoption (55). It must be emphasized that NGS data are extensive and have considerable storage requirements, so careful consideration should be taken in planning downstream assay maintenance.

The first standardized data output type shared among NGS platforms is the FASTQ file format, used to encode sequence data as produced by the instrument. In addition to the nucleotide sequence, FASTQ files contain data such as cluster location and nucleotide quality score.

Once encoded, nucleotide sequences stored in a FASTQ file may be aligned to a reference ("mapped"), or sequences may be assembled into scaffolds using software such as Velvet prior to alignment (56–58). Sequences can be aligned to a reference genome/metagenome database using platform-provided software, or users can choose from a variety of open-source aligners such as the Burrows-Wheeler Aligner (BWA) for incorporation into custom bioinformatics pipelines. FASTQ files may also be similarly compared to custom databases.

To optimize quality, questionable reads can be removed during alignment; however, analysis should be performed to determine the source of discarded or unmappable data. For example, a low percent on-target score may signal contamination and amplification of different sample type. Alternatively, low mapping may be related to nonspecificity in the assay or platform-specific sequencing anomalies. Trending analysis of the percentage of discarded reads and error profiles provides critical quality assurance data. Various other filters can be applied during alignment, and error correction methodology becomes very important when evaluating minor viral populations such as quasi-species in HCV infections (59).

Once reads are aligned, the depth of coverage metric describes the number of aligned reads that contribute to the base call at a given position of the reference. The recommended depth of coverage for most platforms and assays is above 30 reads but may be as high as 500 to 1,000 reads depending on the application.

Aligned reads can then be analyzed by a variant caller, which looks for nucleotides that differ from the reference genome. For example, one may evaluate single nucleotide substitutions leading to drug resistance. Both on-instrument and open-source variant caller parameters can be adjusted for more or less stringent filtering. For example, limiting variants to those that are supported by balanced numbers of forward and reverse reads (low strand bias) provides added specificity (59). Reference sequence selection can also greatly affect variant calling and is discussed below.

The choice of bioinformatic pipeline has a major impact on data quality. Independent software packages, such as CLC-Bio, can streamline analysis considerably but require significant expertise in setup. End-to-end analysis solutions that require no user intervention are highly preferred and can save a lab significant time and cost in setting up NGS. The SURPI software pipeline describes end-to-end process time as the time from sequence data input to pathogen report (60). Software providers that actively partner with instrument providers are ideal to ensure up-to-date solutions that evolve with the platform. Both custom and packaged pipelines may be tested on known samples to assess data quality. Overall, bioinformatic analysis remains one of the most expensive and challenging barriers to overcome when adopting NGS.

VERIFICATION AND VALIDATION

There are several guidelines, online resources, and articles outlining the assessment of sequencing and other nucleic acid detection assay performance, including but not limited to those listed in Table 2. Additionally, a detailed discussion of the differences between validation of tests for their intended use versus performance verification of a previously validated test can be found in Jennings (61). Generally, analytical validation studies conducted during assay development identify high-level assay strengths, limitations, and potential sources of technical variation. Experiments characterizing overall sequence run quality, reportable range, accuracy, LOD, interfering substances, precision, carryover, and database validity are all important in sequencing assay validation. Examples of how labs have approached these experiments can be found in Table 3. Note that this discussion does not account for biological sources of variation, i.e., clinical validation, and independent studies must be performed as required by relevant applicable regulatory organizations. The reader is recommended to always refer to current versions of published guidelines, because guidelines and regulations may change with this rapidly evolving and exciting field.

Prior to implementing a sequencing assay, it is important to appreciate the qualitative versus quantitative aspects of the assay. For Sanger sequencing, a result comprises a qualitative nucleotide readout (A, G, C, or T) and a ratio if applicable (percent mutation). NGS additionally provides a numerical absolute value for read depth of coverage (number of reads per nucleotide). Qualitative and quantitative assays are validated in different ways (61), so it is important to identify the desired assay measurements prior to

TABLE 2 Selected resources for sequencing assay development

Author	Resource
Clinical and Laboratory Standards Institute (CLSI)	MM-18-A: Interpretive Criteria for Identification of Bacteria and Fungi by DNA Target Sequencing; Approved Guideline
	MM-3-A2: Molecular Diagnostic Methods for Infectious Diseases; Approved Guideline
	MM-09-A2: Nucleic Acid Sequencing Methods in Diagnostic Laboratory Medicine; Approved Guideline
	MM-10-A: Genotyping for Infectious Diseases: Identification and Characterization; Approved Guideline
	EP-12-A: User Protocol for Evaluation of Qualitative Test Performance; Approved Guideline
	EP-17-A: Protocols for Determination of Limits of Detection and Limits of Quantitation; Approved Guideline
Jennings et al. (61)	Recommended Principles and Practices for Validating Clinical Molecular Pathology Tests
State of New York Department of Health (79)	http://www.wadsworth.org/labcert/TestApproval/forms/Microbiology_NAAT_Checklist.pdf
FDA Workshop (71)	Workshops Conferences (Medical Devices): Public Workshop—Advancing Regulatory Science for High Throughput Sequencing Devices for Microbial Identification and Detection of Antimicrobial Resistance Markers, April 1, 2014
College of American Pathologists	CAP Microbiology Checklist
Gargis et al. (65)	Next-Generation Sequencing: Standardization of Clinical Testing (Nex-StoCT) workgroup guidelines
Rehm et al. (124)	ACMG clinical laboratory standards for next-generation sequencing
Pont-Kingdon et al. (67)	Design and analytical validation of clinical DNA sequencing assays

designing validation experiments. Most sequencing assays are currently qualitative, yielding a sequence present/absent result. However, it would be extremely valuable if NGS could be used, for example, to quantitate viral load while concurrently assessing mutation status. Several variables must be characterized before this can come to light: for example, quantitative assays require sufficient quantitative precision to achieve reproducible results. How variable is read depth from run to run? Minimum process performance with a sigma value of 3 or more (a reflection of precision) has been recommended for other quantitative assays (62). Absolute quantitation of, e.g., viral load would also require assessment of measurement accuracy, analytical measurement range, and linearity using acceptable quantitative reference standards (e.g., reference 63). Similar experiments would also need to be conducted if mutation frequency was being evaluated in a quantitative manner. Does the software calculate frequency linearly over a range of low to high frequencies? As with all claims, quantitation is not proven until it is tested. Ultimately, the experiments used to assess assay performance should address the intended application of the assay.

Overall Sequence Quality Metrics

The overall quality of a Sanger sequencing run is measured on multiple levels, including instrument run parameter monitoring, base-calling quality, and successful alignment to reference database entries. First, for run parameter monitoring, sequencing instrumentation has startup parameter checks as well as in-run electrophoresis parameter tracking. Files containing these data are stored with every run to provide a history of run validity. Second, base-calling quality scores are generated by base-calling algorithms, which reflect signal values and electrophoresis anomalies arising from incorrect assay or run setup. In general, acceptable sequence quality scores are at or better than 1 error in 100 bases. This is equivalent to a PHRED score of Q20 ($1/10^2$ chance of error = Q20, $1/10^3$ = Q30). Third, software tools such as BLAST conduct sequence alignment to a reference. This process allows sample species and variant identification. For variant calling, sequence quality scores at the single nucleotide polymorphism positions become most important in assessing data confidence. At minimum, these tools provide the percent match to the reference sequence. More sophisticated programs allow users to align multiple output sequences, view quality scores, and review the potential impact of specific genotypes related to antibiotic resistance or clinical outcome (64).

For NGS, overall run quality metrics are established during assay development and serve as a benchmark for all future use of the NGS assay. Quality scores in NGS are similar to Sanger sequencing and reflect the probability of a nucleotide having the called identity. Overall run quality is monitored through depth of coverage (how many reads per target sequence), coverage uniformity across the target sequence, percent reads aligned to the intended target, percent of target sequence covered (e.g., at 50×, 100×, and 400×), total number of reads, and an overview of the read length distribution (65, 66). Each platform also has its own useful quality control metrics; for example, as mentioned above, too many polyclonal wells on the Ion Torrent PGM platform signals library overloading at the templating step, which will result in decreased overall throughput (chip loading efficiency is used as a metric in reference 43). Similar issues occur if the Illumina MiSeq is overloaded, yielding too many clusters. Average quality scores across the length of all reads also provide an idea of run quality; a poor run will show a wide distribution of quality scores, dipping much lower than 20 toward the 3′ end nucleotides, indicating degradation of sequence quality over the course of the run. Other metrics that can be monitored in every run include GC bias, human genome transition/transversion ratios, and DNA fingerprinting (66).

Reportable Range

The reportable range represents the breadth of the assay's detection capabilities. This includes all possible results that could be reported (61). In sequencing, assays are generally not quantitative, and the reportable range has been inter-

TABLE 3 Examples of analytical validation strategies

Metric	Examples[a]
Precision	
Interlaboratory	30 samples sent to 10 labs
	Specialized software for sequence analysis ensured common typing nomenclature (96, 98, 125)
	Same sample shared across labs (100)
Interoperator	4 replicates of 5 samples tested by 3 in-house assay runs and 1 TRUGENE run under different operators
	Expressed as concordant sequence identities ranging from 98.22% to 99.65% (97)
Intrarun	Sample tested in triplicate or quadruplicate (67, 79, 96)
Interrun	10 data sets from 10 different PCR amplifications (126)
	Samples tested in 3 different runs on different days with different reagents (67)
	4 technical replicates and 4 bioinformatic replicates of a known organism mixture (107)
	48 replicates testing RT-PCR, barcoding, library preparation and sequencing (43)
Interinstrument	Several samples tested in 3 runs on different days on different instruments (67)
Accuracy	
Sensitivity	100% × (TP)/(TP + FN) ± 95% confidence interval
	To characterize false negatives, known positives were tested that were characterized by a gold standard method (13, 72, 75–78, 80, 81, 83)
Specificity	100% × TN/(FP +TN) ± 95% confidence interval
	To characterize false positives, known negatives were tested that were characterized by a gold standard method (13, 72, 75–78, 81, 83)
	HIV: 8 nontarget viruses tested for false positives (43)
Limit of detection	
Organism input	Samples value assigned with gold standard method then diluted, e.g., 1:2, 1:4, 1:8,1:20
	Diluted samples value assigned based on the dilution factor, yielding a range of calculated IU or CFU
	Each sample tested for positivity (34, 83, 90, 92)
	HBV: 3 replicates over 4 days, 95% hit rate of 103.9 IU/ml (95% CI 80.0–173.3) (73)
	HIV: 5 levels, 7 extractions per level, amplifications performed in triplicate (94)
	Influenza virus B: serial dilutions 10^6 to 10 infectious units (127)
	HIV: 79 samples tested (43)
Coverage (NGS only)	4 read downsampling levels (107)
DNA input	3 DNA input levels (107)
Mutation frequency	Mutant samples mixed with wild type at increasing mutant:wild type frequencies, e.g., 10, 20, 30, 40, 50% mutant
	Each sample tested for positivity (73, 91)
	HIV: 8 levels (0%, 0.1%, 1%, 2%, 3%, 5%, 10%, and 100%) tested (43)
Run controls	
Negative	Negative control run in parallel with every 1 to 10 samples, e.g., extraction control from uninfected tissue, no-template control (12, 13, 34, 72, 86, 107, 121)
	Wipe tests (102)
Positive	Genomic DNA from bacteria (83)
	Known positive human or commercial sample (80, 86, 121)
Interference	Equal mixture of specimen DNA mixed with positive sample (13)
	Duplicate sample reaction including spike-in (35)
	Exogenous internal amplification control (12)
	Parallel amplification for human target (75, 86)
	Other internal controls (121)
Extraction	Examples included in "Positive" and "Negative" controls

[a]Abbreviations: FN, false negative; FP, false positive; TN, true negative; TP, true positive.

preted in different ways, depending on the study. Reportable range has been described as the genomic regions in which a variant may be called, the genomic sequence that can be reliably analyzed by the assay, or the range of species detected by an assay (65, 67, 68). In one study, Woo et al. (68) evaluated Sanger sequencing of the 16S gene using 409 species of medically important aerobic Gram-positive cocci and Gram-positive rods (68). They found the reportable range for species identification to be below 50% of the tested species. This is in contrast to earlier studies, in which 80 to 90% of the species were identified (69, 70). The differences in reportable ranges for species

identification were due to different analysis protocols and a lack of Gram-positive bacteria.

Accuracy

Accuracy is a measure of the concordance between a test result and the expected result for a given sample. For a quantitative test, accuracy is defined as how close an assay result is to an expected reference value or the "true" quantitative value of the sample, for example, a value-assigned WHO international reference standard (61, 63). In sequencing, which is a qualitative test, accuracy refers to the ability to detect true-positive samples (sensitivity) and true-negative samples (specificity). Sensitivity in this case is not to be confused with LOD, which may be termed *analytical sensitivity* in some reports.

To calculate sensitivity and specificity, expected test results are predetermined on a set of samples using a "gold standard"/reference method; for example, Sanger sequencing can be used as a gold standard against NGS for homogeneous samples or samples containing high-frequency mutant populations. A combination of comparator methods, e.g., several NGS platforms, could be used to verify a Sanger result. Additionally, targeted PCR may be appropriate for certain applications. The use of a well-curated database is also being considered as a comparator method (71). Other gold standards have been used to confirm sample identity, such as bacterial and fungal culture (72). Reference materials, such as WHO and National Institute of Standards and Technology (NIST) standards and commercial reference materials, are often characterized by several methods and have been used for initial accuracy estimates (73). It is important to note the intended use and origin of such materials to ensure that they are compatible with your assay.

Sensitivity is defined as percent positive agreement to a comparative method (sensitivity = 100% × [TP]/[TP+FN]), where TP = true positive and FN = false negative). On the other hand, specificity is defined as how often a test will be negative when the analyte is known to be absent (specificity = 100% × TN/[FP +TN], where FP = false positive) (74). In other words, specificity reflects the incidence of false-positive results. Finally, the 95% confidence interval around sensitivity and specificity metrics can be calculated (e.g., 72, 75) and is dependent on the sample size. Larger sample sizes give more precise confidence intervals. For discussions on confidence intervals, see references 76–78.

For sensitivity studies, it is recommended to test a range of positive sample concentrations, including low-positive samples (77–79). Specificity has been tested by sequencing known negatives, genetically related organism types and subtypes, nonmutated organisms, as well as organisms that yield similar symptoms or are commonly found in matrices of interest (e.g., Table 3). If related strains are not available, nucleic acid spike-ins may provide some supporting accuracy data (79).

Examples of sensitivity and specificity studies can be found across bacterial, viral, and fungal sequencing assays (13, 38, 39, 80–83). A test for *Mycobacterium* rifampin resistance showed a sensitivity of 38 out of 39 true positives detected (97.4%), with only one false-negative result (83). Specificity was 74 out of 75 true negatives correctly identified (98.7%), with one false-positive result (83). In a recent panfungal PCR-sequencing assay report, sequencing results for 34 samples were compared to culture and histology data confirming invasive fungal infection (81). Sensitivity was 100% and specificity was 62.5% on a patient level (81). While all positive individuals tested positive in at least one sample, on a per-sample level, sensitivity was only 69%. The authors propose that this discrepancy may have been due to some samples containing large areas of necrosis and lacking fungal nucleic acids. In another panfungal PCR-sequencing report, sensitivity was 93.6% (13). Samples from individuals with no invasive fungal disease were used as negative controls, and no false-positive results were obtained (13).

Interfering Substances

Interfering substances can introduce systematic data biases, affecting specificity and sensitivity, and usually stem from the original biological sample. In both Sanger sequencing and NGS, interference is most likely to occur at the initial PCR step of the workflow. For example, heparin contamination inhibits DNA amplification (84).

To test for assay sensitivity to inhibition, low-positive controls can be spiked into matrices ± inhibitors (61; Table 3). Note that plasmid spike-ins ordered directly from gene synthesis vendors can be highly concentrated, so laboratories should implement methods to prevent and monitor contamination. Purchase of prediluted synthetic nucleic acids helps to mitigate this risk. Inhibition controls can also be included in the normal workflow to ensure that isolated nucleic acids are free of amplification inhibitors (85). For example, Lau et al. (13) used an equal mixture of test sample DNA and *Candida parapsilosis*–positive sample as an inhibition control (13). An alternative spike-in method has been used, in which *E. coli* genomic DNA is spiked into a parallel replicate test sample reaction (34). Inhibition controls could be a sequence containing the same primer binding sites as the target, with a different insert sequence, or could be for a different target (79, 86). Yi et al. (75) recently used human beta globin as an internal control for a human papilloma virus NGS assay (75).

LOD

The LOD is the lowest level at which a positive result is reliably differentiated from a negative result (61). Usually, the LOD is calculated as the lowest concentration at which an analyte is detected 95% of the time, which can be formally determined by probit analysis (73). Extensive description of LOD calculations can be found in CLSI EP-17-A (87). To calculate the LOD in sequencing, it is essential to test samples for which the "truth" has been previously characterized using a gold standard reference method or an adequate comparator method.

Although organism burden cannot yet be accurately measured using current sequencing technologies, several LODs can still be calculated for sequencing assays, including but not limited to (i) sample input, (ii) mutant sequence frequency relative to wild type, and in the case of NGS, (iii) coverage (number of reads aligning to a region of interest). Once the LOD is established, this level may be confirmed by testing at least 20 samples at the LOD to verify that detection occurs in at least 95% of cases.

Sample input level required for a reliable result will depend heavily on the assay used (Table 4). To determine the sample input LOD, samples are first value-assigned at a high concentration using an independent quantitative assay. Quantitative real-time PCR or digital PCR can be used to value-assign samples, keeping in mind the limitations of each technology (88, 89). Next, samples are diluted to produce several lower concentration levels of the organism being tested. Once dilutions are made, each level

TABLE 4 Example nucleic acid input amounts across various platforms

Technology	Input amount
Life Technologies 3500	25–300 ng DNA
Ion AmpliSeq	10 ng DNA or RNA
Illumina Nextera DNA Sample Prep Kit	50 ng DNA
Illumina TruSeq DNA	1 μg DNA
Illumina TruSeq Nano DNA	100–200 ng DNA
Illumina TruSeq DNA PCR-Free	1–2 μg DNA
Pacific Biosciences	250 ng to 5 μg DNA

is then tested for detection rate. For example, Choe et al. (90) used 13 plasma samples to estimate the LOD for the HCV NS5B gene (90). Thirteen samples were value-assigned between 745 and 1,804 international units (IU)/ml, and then each sample was diluted 1:2, 1:4, 1:8, and 1:20. Diluted samples were value-assigned based on the dilution factor, yielding 78 samples with a range of calculated viral loads from 37 to 1,804 IU/ml. Each of the 78 samples was then tested for positivity. It was found that samples below 250 IU/ml were not always detected, while samples above this level were detected 100% of the time (90). In a similar study, Anton et al. (91) calculated the LOD for HCV NS34A (91). A total of 135 samples were tested, ranging in concentration from 400 to >10,000 IU/ml. It was found that a 95% detection rate occurred in samples above 1,978 IU/ml.

In bacterial sequencing, colony-forming units have been used to define LOD (34, 83, 92). For example, in the Mycobacterial IDentification and Drug Resistance Screen (MID-DRS) assay, Pérez-Osorio et al. (83) determined that 8 to 40 cells are needed for generation of all PCR products needed for sequencing (83). Related to sample input, LOD can also be calculated in terms of the quantity of extracted DNA or RNA that goes into the first amplification step of the sequencing workflow.

The LOD for mutation frequency is defined as the percentage of mutant (or polymorphism) that can be detected when mixed with a wild-type population. For mixed infections it is essential to test known titrations of different combinations of variants to identify potential sequencing bias from the entire assay. In general, most algorithms for identifying mixed sequences in Sanger data report a sensitivity of 15 to 20% mutant to wild-type ratio (93). For example, Anton et al. (91) tested detection of drug resistance mutations using mutant samples mixed with wild-type HCV at 10%, 20%, 30%, 40%, and 50% mutant frequency (91). A 20% or greater mutant mixture was detected 100% of the time, while mixtures at 10% frequency were only detected 75% of the time (n = 6 per level). Mutant/wild-type mixtures have also been used for HBV nucleotide analogue resistance mutations (73). Notably, higher viral input levels allow for a lower LOD for mutant frequency (94).

NGS applies the same principle to mutant frequency LOD. In NGS, the minimum coverage needed depends on the application. For mutations occurring at low frequencies (e.g., 5% mutant), coverage in excess of 1,500 reads (1,500×) has been used (66). Sensitivity depends largely on variant type, e.g., single nucleotide variant, insertion, or deletion. Even within one variant type, sensitivity depends on the sequence context. For example, a single nucleotide variant within a highly repetitive, high-GC content region will have a higher LOD than a variant within a complex GC-balanced sequence. Because of the large diversity of variant types and contexts in any given NGS assay, some organizations are considering a subset-based approach for analytical validation; however, this may miss some important outliers (95).

Precision

Several parameters may be measured to estimate assay consistency. Intraoperator, interoperator, intrainstrument (within run [repeatability] and between run), day-to-day, reagent lot-to-lot, and lab-to-lab reproducibility can all be measured in replicate, using well-characterized positive reference materials (61, 67, 79, 96, 97). For these experiments, it is recommended to include several challenging samples, such as positive samples near the LOD, or serial mixtures of variants (61, 79). For example, to test reproducibility, Gibson et al. (43) amplified four HIV samples in triplicate, tested each amplicon barcoding in quadruplicate, and ran duplicate libraries and duplicate sequencing runs to yield a total of 48 sequences per virus (43). Shafer et al. (98) included mixtures of HIV quasi-species in a study between two laboratories and found that discordance was likely due to PCR sampling variation rather than technical errors in the sequencing process itself (98). On the other hand, a recent paper by Campo et al. (99) looked at sampling reproducibility in NGS quasi-species data, yielding interesting results. Blood samples were taken 2 hours apart from each of 16 patients infected with HCV. It was found that greater than 93% of major quasi-species subpopulations (of 5% or greater frequency) were present in both the first and repeat samples. Viral subpopulations missing in the second sample were near the arbitrary frequency cutoff.

Lab-to-lab reproducibility analysis can help to uncover environmental contamination in NGS workflows (100). A recent analysis of data from six laboratories sequencing the same human sample using the same library preparation kits demonstrated a surprising 30-fold range in normalized contaminating bacterial reads (100).

Carryover and Environmental Contamination

As technologies increase in sensitivity, it becomes ever more important to characterize cross-sample contamination rates and to implement controls mitigating contamination (101). Operators may run a series of sequential positive and negative samples to measure the sample carryover rate. Additionally, positive and negative samples may be prepared side by side or run side by side to determine intra-preparation and intrainstrument carryover, respectively.

Implementation of a monitoring program using negative controls and/or wipe tests helps to ensure maintenance of best practices to prevent contamination (34, 102). Environmental contamination, for example, due to fungal spores in a pan-fungal assay, has also been monitored by including a negative control; for example, Lau et al. (13) processed each specimen alongside a negative control from DNA extraction to PCR amplification (13). Contaminants can also originate from reagents themselves, for example, as a by-product of recombinant enzyme preparations (85, 103). A series of surprising studies recently reported viral sequence contamination in extraction spin columns by NGS (104–106). Interestingly, data from extraction and no-template controls have been used to "decontaminate" experimental NGS sample data *in silico* (107). Use of wet bench and bioinformatic quality control methods is essential to ensure that contaminating sequences are adequately detected.

Mitigations to lower the risk of cross-sample contamination are extensively discussed in reference 108. Because closed systems have not yet been developed for sequencing technologies, special care should be taken at any step in the workflow that exposes amplified material to the environment. The highest-risk processes involve amplification of sample DNA sequences where a small amount of carryover can compete or even dominate the reaction. In Sanger workflows, this has been limited to the initial target amplification step using PCR. Uracyl-*N*-glygosylase is often used to reduce contamination from re-amplification of PCR products from previous Sanger runs (34, 73). In this case, amplification conditions are modified to incorporate dUTP nucleotides into product, which renders amplicons susceptible to uracyl-*N*-glygosylase (109).

Finally, physical separation of preamplification versus postamplification processes and equipment can lower the risk of amplicon contamination. Aerosol-resistant tips also help to prevent sample carryover. Unidirectional personnel workflow from template-free areas to areas of high amplicon potential helps to mitigate the risk of contamination (13, 34, 82).

For NGS workflows, the sample bar-coding step adds another level of carryover contamination potential. The clonal amplification step for template generation can also be a major source of carryover. While this varies by platform, the use of consumable reagents, vessels, and tubing is preferable. Additionally, stringent cleaning steps should be performed after each run. For highly variable organisms such as HIV, sequence data may be compared to all previous specimens in the lab's database or to all published sequences. Intrapatient diversity has been compared to interpatient diversity for this reason (43). If a sequence matches a previous data set too closely, and the data are not from the same patient, one may consider checking for contamination. Overall physical separation of pre-PCR and post-PCR activities, unidirectional personnel flow, and the use of disposable consumables continue to be the most effective methods to reduce carryover contamination.

Data Analysis and Interpretation

The most critical step in any lab workflow is data analysis and interpretation. Sequencing data has many advantages in this regard by providing discrete and comprehensive data on a given target. However, the additional data also bring increased complexity. Accurate assignment relies on linking phenotype information to a catalogue of genetic information. After base-calling and verification of run controls, typical data analysis procedures involve (i) assembly of redundant data from the same sample, (ii) resolution of any discrepancies between the redundant base calls, (iii) building of a consensus sequence for the target, (iv) query of the consensus against a database of known target sequences, (v) assignment of the sample sequence to a known species, and (vi) reporting of the result as either a species or variant identification.

Sequence Databases

Selection and validation of reference sequences are critical for accurate interpretation and reporting. Using multiple sources of sequence databases is common practice, especially upon imperfect matching of sample sequence to reference sequence. A major challenge to anticipate is disagreement in query results from multiple databases. For example, this can happen in public databases; although such databases have abundant sequences, some entries may have poor annotation or inaccuracies. Commercial databases have many advantages but require a fee. There is a vital need for unified curation and standards for medical microbiology sequences (64, 71). An FDA-sponsored project is under way to build a comprehensive, curated database of pathogens and other microbes at the University of Maryland School of Medicine (110). Other databases include the International Sequence Database Collaboration (www.insdc.org), which is a massive repository with annotation for most organisms. The Multi Locus Sequence Typing database (www.mlst.net) is a common database for medically important bacterial strains and provides tools for analysis and reporting. The MicroSeq 16S database (Life Technologies, part of ThermoFisher Scientific) is a curated database with traceable documentation and tools for phylogenetic tree building. The documentation and annotation required for traceable database entries include sample-specific information, metadata on laboratory and analysis methods, as well as sequencing data (111).

If following mutations for antibiotic resistance over time, reference sequences take on a different meaning. Kwong et al. (112) suggest that a patient's baseline pretreatment sequence can be used as a reference for longitudinal monitoring of HCV infection (112). It is worth noting that reference sequences originating from laboratory strains, such as those used for replicon generation, may differ from patient sequences in important ways. Replicons often have adaptive mutations that allow growth in culture but are not relevant to human infections (112).

Validation of the reference sequence database should be part of a comprehensive protocol defining the performance characteristics of the assay. The CLSI MM18-A guidance recommends a thorough understanding of the strengths and limitations of a database and its maintenance procedures. The guidance recommends selecting a database that is curated with periodic updates to nomenclature as well as documented and traceable improvements to sequence entries. High-quality databases should contain reference sequences from type strains that have been published in peer-reviewed journals. Multiple reference entries from different sources should be present for a given species. Ideally, all common variants of a species should be included. Finally, reference sequence length should be at least 300 bp and cover a region of the gene where variation is expected. Analytical validation of reference databases can vary depending on the target and assay but ultimately relies on blinded experimental studies using well-characterized controls (68, 113–115).

Analysis

Once a database is chosen, many analytical steps can be automated with software, but manual review is usually done in the reporting step. Often this is a completely objective process, but ambiguity can arise from incomplete matching of the sample sequence to the database sequences due to novel mutations or incomplete databases. Distance scoring or percent matching is used to define cutoff values for acceptance of the final result (116). The cutoff values may be predefined and based on inter- and intraspecies variation for a given target. CLSI MM-10 and other standards define cutoff values for common situations.

A study published on *Mycobacterium* detection by Hall et al. using 16S gene sequencing established reportable range values based on a distance score or percent sequence identity to the reference database (111). They tested a

commercial sequencing assay and a 16S rRNA gene reference database using 387 isolates that were previously characterized. Based on concordance with previous results, they set the distance score cutoff of ≤1% to reference database entries. Therefore, for a sample to be positively called, the sequence must have 99 to 100% similarity to an entry in the reference database. For resolution of more divergent novel sequences, querying multiple databases may produce a better match; however, database annotation quality and entry provenance become critical when leveraging multiple databases (68, 113).

Drug resistance assessment adds another level of complexity to the analysis pipeline compared to pathogen detection and identification. Resistance does not always correlate with the presence of a resistance mutation. For example, a drug resistance mutation may cooccur with other mutations that decrease viral fitness, essentially inactivating the virus. This may be overcome by complementary phenotypic assays (117).

Sanger Sequencing

Because Sanger sequencing produces targeted genetic analysis leading to a limited amount of data to combine and review, manual interpretation is typically the gold standard. Software tools can be used to combine data and provide an interface to view the trace data together with reference sequences. A reproducibility study for HIV resistance mutation detection using an optimized Sanger assay shows the nuances required in this analysis step (97). When mixed bases are present at a given nucleotide, defining a cutoff value for mutations is greatly aided by the web-based software tool RECall (Fig. 4). Most importantly, it is critical to establish a standardized analysis protocol for trained users, to ensure consistency in anomaly resolution and error mode recognition.

Sequences from mixed infections are extremely challenging for Sanger technology, and NGS has an advantage in these cases. However, some software such as RipSeq and BCV can deconvolute and perform base-calling on mixed samples with Sanger sequence data (93, 118). Such algorithms can allow detection and identification of microbial species without physically separating the target sequences (e.g., by variant-specific PCR primers or culturing and cloning). Data are published describing performance in mixed infections of HCV, HIV, HPV, and *Mycobacterium* from various target genes (93, 118, 119). These programs should be approached with caution and thoroughly validated with known mixing studies before deployment in the clinical setting.

NGS

Since NGS platforms produce significant data output, most analysis steps use automated algorithms. NGS data analysis and custom bioinformatic pipeline construction is an extensive topic, but generally these pipelines are validated with data sets replicating challenging assay situations similar to the wet bench part of assay validation. Analysis of metagenomic data generally includes removal of host sequences to yield the targets of interest. The complex data sets from these complex samples present an additional issue of analysis time. A recent article describes a sequence-based ultra-rapid pathogen identification (SURPI) tool and demonstrates its use on over 200 clinical samples (60). The major advantage of SURPI is the reduction in time for data analysis from weeks down to hours.

ONGOING SEQUENCING ASSAY QUALITY CONTROL

Ongoing quality control depends on reliable run controls, appropriate training, and user proficiency.

Run Controls

Independent daily quality control material is used to monitor test performance over time across reagent lots, operators, instruments, etc. and may signal to users when there are increases in systematic error. Including consistent controls in every run during assay optimization and validation also helps in establishing acceptable assay performance characteristics (61). Run controls with well-characterized analytes can also help to detect artifacts leading to false negatives or false positives. A recent paper looking at transcript detection found that RNA underwent recombination *in vitro* during the PCR step, creating fusion transcript artifacts (120). Reference materials with known truth are needed for detecting such unexpected results.

Importantly, bioinformatic analysis remains one of the more challenging steps in the NGS workflow. Controls with known expected results also help to monitor these "dry bench" procedures. For example, is the software calling variants properly? Is genome assembly functional and consistent? Has the bioinformatics workflow been updated or parameters adjusted, which might affect result generation?

Run Control Content and Characterization

Control material characteristics are normally established using a gold standard method. For sequencing, it is of utmost importance to know the truth of the sample. Orthogonal confirmation using multiple methods is ideal.

Several types of controls exist, including interference/internal (discussed above), negative, no-template, positive, and full process/extraction controls (e.g., 13, 75, 121). Labs obtain controls from commercial providers, leftover samples, sample exchanges, GetRM, and other sources (66).

Negative controls and no-template controls are especially important for monitoring cross-sample and environmental contamination. Negative controls do not contain any sequences expected to be present in a test sample. No-template controls are also expected to yield a negative result, but they contain no DNA and, depending on the platform, may be checked directly after PCR by gel electrophoresis to ensure that no amplification occurred.

Positive controls used near the LOD help to indicate system issues (79). For sequencing, the nature of positive controls will depend on the application. This is straightforward when looking at whole-organism identification, whether it is a single organism Sanger sequencing control or a panel of organisms to control for a highly multiplex NGS workflow. Controls for infectious disease testing (e.g., HIV, HBV, and HCV) can be obtained from commercial providers such as AcroMetrix, ATCC, ZeptoMetrix, and SeraCare.

When characterizing resistance mutations, which might occur at very low frequencies, a heterozygous positive run control may be made with a predetermined mix of wild-type and mutant sequence. We have observed lab-to-lab and run-to-run variability in variant calling in NGS assays, stressing the importance of heterozygous run controls (M. Shahbazian, N. Nataraj, P. Chiang, and K.L Norman, unpublished). Some variants are detected more consistently than others.

In choosing positive controls for polymorphism detection, users sometimes choose representative mutation classes (i.e., single nucleotide variants [SNVs], insertions, deletions),

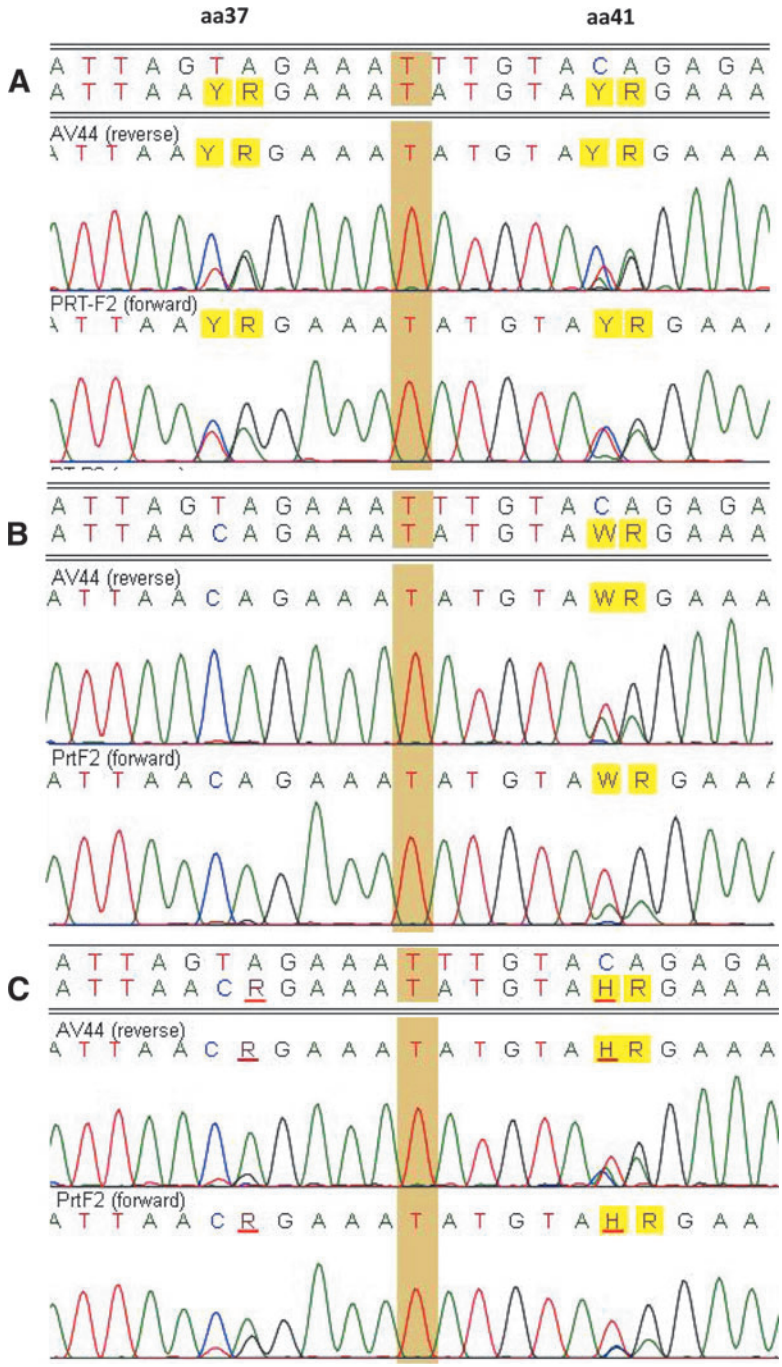

FIGURE 4 Image of mixed base traces viewed in RECall software showing examples of mixed base variation in HIV Sanger assay at two codon positions: amino acids 37 (aa37) and 41 (aa41). Each panel consists of sequencing traces from the forward sequencing primer (PRT-F2) and the reverse sequencing primer (AV44). Mixed base positions are identified by the software and labeled with IUB codes: Y = T + C, R = A + G, W = A + T, H = C + T. The RECall software aligns all sequences to a reference at the top of each panel and shows the composite base call below the reference. The highlighted T position across all the panels is a vertical alignment position between the panels. Panel A shows 2 codons (37 and 41 of RT) with nucleotide base-calling of AYR; panel B shows the AWR at codon 41 (the second peaks at codon 37 were not detected in this replicate); panel C shows ACR at codon 37 (minor T was not called by RECall at the cutoff of 15%) and AHR at codon 41 (almost equal height of second and third peak at the second position) (97).

because each class generally falls into similar categories of sequencing difficulty. However, since surrounding sequence affects performance, positive controls are more robust if they reflect the sequence contexts being tested. Controls for resistance mutations are most useful if they represent the true-positive sequence being tested. Additionally, for targeted NGS assays using many amplicons, users often choose to have at least one mutant control per amplicon.

Extraction controls mimic test samples and are considered full process controls because they can be used to monitor the entire workflow from sample to result. For example, a methicillin-resistant *S. aureus* control has been used to monitor run quality from extraction to data analysis (122). Such controls may be sourced and pooled from multiple human donors or may be recombinant microorganisms that contain target sequences of interest. Both positive and negative extraction controls are useful, because negative extraction controls help to monitor contamination in extraction reagents. For example, Kühn et al. (121) used blood from healthy individuals for negative extraction controls. Diluent matrix may affect performance, so full process reference materials are also essential tools during assay development.

Robust control material characterization helps to ensure predictable performance, rendering the control more sensitive to system issues. NIST is currently developing a microbial genomic reference material (123). This DNA reference material contains genomes from four microbes: *S. enterica* LT2, *S. aureus*, *P. aeruginosa* (high percent GC), and *Clostridium sporogenes* (low percent GC). This reference material will be sequenced on the Pacific Biosciences RSII, Illumina MiSeq, and Ion Torrent Personal Genome Machine. Incorporating a mixture of read lengths and platform types will allow for full characterization of material homogeneity, purity, stability, genome structure, and sequence identity. The methods used for characterization can be applied to future microbial reference materials.

Run Control Consistency

Perhaps most importantly, reference materials used for assay optimization and regular quality control monitoring should be of the highest quality, so that variability in control material does not confound data analysis. Run control materials, in particular, must be of high enough quality and consistency to achieve the purpose of monitoring performance over hundreds or even thousands of assay runs. Home-brew controls made from leftover materials can become problematic over time, because they may not be characterized for analyte stability and may be of limited availability. Moreover, replenishing home-brew controls requires recharacterization and inventory management.

Commercially available control materials offer a reliable source of consistent material over time. Demonstrated lot-to-lot consistency, open-vial stability, freeze-thaw stability, and real-time stability provide assurance that control performance will not change over time. Additionally, products can be made using standardized validated protocols and validated quality control release testing procedures, following current good manufacturing practices. Altogether, consistent controls allow users to understand assay system variability without having to factor in potential sources of control material variability.

To aid in lot-to-lot consistency, manufacturers may also follow the International Organization for Standardization guideline ISO 15711. Much like the international meter that is used to calibrate length, international biological reference standards can be used to calibrate control materials. Organizations such as the WHO and NIST create a limited stock of reference material for each analyte of interest, which is then characterized by labs around the world. The combined data are then used either to assign a value to the material (e.g., a viral titer) or to assign a consensus sequence (e.g., NIST bacterial standard). As manufacturers build derivative control materials, every new lot can be calibrated following ISO 17511 by comparing product performance to the international standard.

User Proficiency

As technologies become more complex, appropriate user training, proficiency testing/external quality assessment, and peer-to-peer comparison become an ever-important part of a lab's quality assurance program. In a recent inter-lab study using a standardized AcroMetrix NGS reference material containing 500+ variants, we observed significant differences in lab variant reporting, even within the same assay and platform (M. Shahbazian, N. Nataraj, P. Chiang, and K.L. Norman, unpublished). One lab that performed very poorly had inadvertently used incorrect settings in its bioinformatic analysis. Another lab that missed many variants was using an uncommon bioinformatics pipeline. Use of platform-agnostic, independent reference materials in proficiency/external quality assessment programs helps labs to ensure that their performance is in line with their peers.

THE FUTURE

Sequencing has been used for many years to identify specific organisms as well as to find genetic sources of antimicrobial resistance. Major challenges for the technology center on data analysis, cost, and ease of workflow; however, much like viral quasi-species in an infection, sequencing is currently in an accelerated state of evolution. NGS platforms and technologies are evolving rapidly to accommodate clinical needs. While NGS is currently a qualitative assay, the use of calibrators could potentially allow for incorporation of microbial identification, resistance, and quantification in a single assay. Undergoing equally rapid growth is our understanding of mechanisms of antimicrobial resistance and host-microbe interactions, especially as they relate to the microbiome. Ultimately, the clash of technology and medicine promises to have an important impact on microbial diagnostics and therapy.

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Verification and Validation of Matrix-Assisted Laser Desorption Ionization Time of Flight Mass Spectrometry-Based Protocols

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54

Over the past decade, matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) has moved from the research laboratory into the clinical microbiology laboratory to aid in the identification of microorganisms. This technology uses nonfragmenting or "soft" ionization to generate a mass spectrum profile of an analyte. For bacterial isolates, this profile is composed primarily of ribosomal proteins. The spectrum generated from a given isolate is compared to a reference database, or spectral library, and the instrument reports a list of organisms that best match the query spectrum (1, 2). These results are reported with a confidence score, providing a measure of the reliability of the identification. Numerous studies have demonstrated that MALDI-TOF MS greatly reduces the time and cost of bacterial identification and allows for further automation of the clinical laboratory (3–5). However, prior to the implementation of MALDI-TOF MS for patient testing, the system and any alternative methods used must be verified by the laboratory.

In accordance with the *Clinical Laboratory Improvement Amendments* (CLIA '88), *Code of Federal Regulations, Title 42, part 493, Subpart K, 493.1253, Standard: Establishment of Verification Performance Specifications*, it is required that all laboratory tests undergo method verification and validation to establish specific performance characteristics prior to reporting of clinical results (6, 7). Method verification ensures that the new method is acceptable for clinical use by assessing several performance specifications. Specification requirements are dependent upon whether or not the test is FDA-cleared. In general, the required performance characteristics of the test are precision, accuracy, reportable range, reference range, analytic sensitivity, and analytic specificity (8, 9). Once verified, the laboratory director, using both federal (CLIA '88) and accrediting organization guidelines, determines if the new test meets the quality standards to be implemented into the laboratory for clinical use.

Verification of MALDI-TOF MS can be challenging because of the large number of organisms that can be identified using this technology. The output of potential organisms can create difficulties for the laboratory such as determining the number of organisms and/or which organisms to test for as well as which gold standards are necessary for method comparison studies. Further, current CLIA amendments are not specific for MALDI-TOF MS and do not address these questions. This chapter focuses on the aspects of MALDI-TOF MS verification, discussing the requirements for each performance characteristic and providing sample methods. In addition, this chapter will cover the ongoing validation that is required after the assay is verified.

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VERIFICATION AND VALIDATION

The terms verification and validation have often been used interchangeably although they are two separate processes. The definitions change depending upon the governing body. CLIA '88 and the American Society for Microbiology (ASM) define "verification" as a one-time process performed to determine or to characterize and compare the diagnostic accuracy of a new method to that of a reference method, which is accepted by the laboratory community as standard of care for a particular analyte or disease (CLIA '88). In other words, verification asks the question "Is the new method better or worse than the current standard of care?" "Validation" is an ongoing process of monitoring the procedure, method, and staff to ensure that the test continually performs as expected (10). Validation asks the question "Does the test still work?"

The College of American Pathologists (CAP) and CLSI define verification as an abbreviated process to demonstrate that a test performs in substantial compliance to previously established claims (11). CAP and CLSI define validation as provision of objective evidence through a defined process that a test performs as intended (12). The difference is that CAP and CLSI use the terms to distinguish the introduction of FDA-cleared tests (verification) and laboratory-developed tests (LDTs) (validation). The differentiation was likely instituted because there are different requirements for approving the use of FDA-cleared tests and LDTs. These differences will be discussed in detail throughout the chapter. For clarification, this chapter will use the definitions originally established by CLIA '88 and ASM.

GENERAL VERIFICATION REQUIREMENTS

FDA-Cleared Systems

Before a manufacturer can bring to market a MALDI-TOF MS system for bacterial identification, the system must obtain FDA clearance through a 510(k) premarket notification. The 510(k) submission consists of rigorous testing and providing valid scientific evidence, and it demonstrates that the device is substantially equivalent to a similar device currently on the market. For multiple analyte tests, the FDA requires 510(k) testing on each organism the system claims to identify. Finally, a package insert is approved by the FDA containing the summarized data from the 510(k) submission, including the intended use, specimen collection and transportation, storage recommendations, warnings and limitations, results and their interpretations, recommendations for controls, and the system's performance characteristics (13).

At the time this chapter was written, there were two FDA-cleared MALDI-TOF MS systems: Vitek MS (bioMérieux, Marcy l'Etoile, France) and the MALDI-Biotyper Clinical Applications (Bruker, Bremen, Germany). Each of these systems currently has FDA clearance for only a small subset of organisms that can be identified by the respective system, and the specific organisms claimed by each system are different. The manufacturers continue to expand the number of claimed organisms through subsequent 510(k) submissions. The package insert will contain a list of claimed organisms. Any organism(s) the laboratory intends to report clinically that are not claimed by the system will be considered "off package insert" and will be subjected to LDT requirements.

Introduction of an FDA-cleared system provides several benefits for the clinical laboratory. One benefit is that the 510(k) submission helps supply important information about the sensitivity and specificity of the system, calculated from a large and diverse patient population. The laboratory then performs verification to demonstrate that the test performs to the manufacturer's performance specifications. Specifications required for FDA-cleared tests are precision, accuracy, reportable range, and reference range (PARR) (9). Because MALDI-TOF MS is a qualitative clinical test, both the reportable range and reference range are not required for verification (8). Specific requirements for assay verification can be found in section 493.1253 of the CLIA regulations (9). The purpose of this additional testing is to verify that the predicted performance of the system operates as intended in the laboratory's patient population. What each of these specifications means and suggestions on how to measure each specification are discussed in detail later in this chapter. Overall, the process of verification of FDA-cleared tests is less rigorous than non-FDA-cleared tests.

Laboratory-Developed Tests

The laboratory can choose to use a spectral library(s) containing organisms not cleared by the FDA. For example, there are libraries to detect mycobacteria or fungal species (14). The benefit is that the laboratory has a larger selection of systems that may be incorporated into its workflow better than other systems; however, a larger, more comprehensive study is required to verify non-FDA-cleared libraries. Using a non-FDA-cleared system defines the test as an LDT. Similar to FDA-cleared tests, PARR is measured (8, 9). In addition, the laboratory must determine the analytic sensitivity and analytic specificity as well as any other characteristic deemed necessary by the laboratory prior to clinical use. A summary of the required specifications for both FDA-cleared systems and LDTs can be found in Table 1.

TABLE 1 Requirements for verification of MALDI-TOF MS assays[a]

Category of verification requirement	FDA-cleared	LDT/FDA-modified
Precision	R	R
Accuracy	R	R
Reference range	C	R
Reportable range	C	C
Analytic sensitivity	NR	R
Analytic specificity	NR	R
Other	C	C

[a]R, required; NR, not required; C, conditionally required.

When researching options for MALDI-TOF MS systems, it is important to note that laboratories cannot verify systems labeled as devices for research use only (RUO) (13). These products are essentially unregulated by the FDA and are considered to be in the research phase of development. RUO devices cannot have intended clinical use (13). Any system that is defined as an RUO device is required to be clearly labeled on package inserts (11).

Laboratories that have verified FDA-cleared MALDI-TOF MS systems may decide to modify the package insert procedure. Any changes from the package insert methods will negate the FDA-cleared status of the test, and verification must follow LDT requirements. A few examples of possible modification include the reporting of organism not claimed by the manufacturer, performing testing using growth media not found in the package insert, or developing methods to test directly from patient specimens. Each of these methods will require additional verification, following the CLIA '88 guidelines for LDTs.

Once the laboratory has determined if the system will be FDA cleared or LDT, a written procedure can be created to measure the required specifications. In summary, FDA-cleared tests must verify PARR and require fewer samples when testing accuracy. LDTs require verification of PARR along with additional testing of analytic sensitivity and analytic specificity. The following sections will describe what each of these performance specifications are and examples of how to perform the study, obtain samples, and determine if the new test meets CLIA and CAP requirements.

ESTABLISHMENT OF PERFORMANCE SPECIFICATION: PRECISION

Precision Definition

The term precision is defined as the statistical closeness of agreement between repeated test procedures of the same sample under stipulated conditions (15). Precision studies are also referred to as reproducibility studies or replicate studies. Measuring precision is important because it will determine how the system will perform through the random error caused by changing day-to-day factors (time from spotting onto the test plate, temperature, reaction with the matrix, operator techniques, etc.). Precision itself

is not a numerical value and is reported as high, medium, or low (8). In order to report a numerical value, the term imprecision is used. Imprecision is defined as the dispersion of results of measurements obtained under specified conditions (8). Imprecision is expressed as either the standard deviation (SD) or the coefficient of variation (CV).

Precision Study Design

To ensure a robust estimate of precision, reproducibility studies should include testing of identical specimens in the same run (within-run) and repeated testing over several days (between-run). Multiple testing in the same run will calculate the within-run precision, defining the variation in a single run. The repeated testing over multiple days will calculate the within-laboratory precision, defining the system's variation from day-to-day factors. The amount of within-run specimens tested and the days tested can vary between laboratories. Testing specimens in triplicate will allow the use of statistical analysis.

Factors that may alter protein profiles, such as high salt concentrations or growth on various media types, may hinder confidence scores reducing overall precision (16). One study compared the ability of the MALDI Biotyper (Bruker Daltonics, Billerica, MA) to correctly identify known bacterial samples that were grown on various agar sources (17). The researchers found that growth on various selective and differential media yielded varied confidence scores when whole bacteria were utilized. Variability could be decreased and identification scores increased through the use of an extraction process. When performing the reproducibility study it is important to use the same strains under the same conditions for all of the testing to eliminate external variables while testing.

For optimal consistency, a single plate per organism should be subcultured out for the week's testing. Each day, the technologist should perform the day's testing from these streaked plates to ensure consistency in specimens. In addition, it is good practice to utilize different operators to smear the reproducibility strains to account for variation between the technicians' techniques (18). Strains should only be re-subcultured after 5 days or if plates were stored at <4°C because there is evidence demonstrating significant effects on confidence scores under these conditions (19, 20). This procedure will help reduce the day-to-day variation and allow closer precision.

An issue when designing a reproducibility study for MALDI-TOF MS is that the system identifies a large number of analytes. These analytes consist of several different bacterial genera and species and contain Gram-negative and Gram-positive bacteria and yeast. For example, the Vitek MS system is cleared for 197 organisms, and it is unreasonable for a laboratory to calculate the precision of each organism. Currently there are no specific regulations for MALDI-TOF MS precision study requirements; therefore, it is the responsibility of the laboratory director to determine which organisms should be tested for verification studies. If verifying an FDA-cleared test, the laboratory can replicate the reproducibility study found in the package insert. Alternatively, the reproducibility study should test at least one representative from each broad class of microorganisms that will be reported (21). Additional strains can be tested based on various types of organisms, such as anaerobic or fastidious organisms (22, 23). An example of the workflow of a suggested reproducibility study can be found in Fig. 1.

Depending on the workflow of the laboratory, additional reproducibility studies measuring the contribution of other source variation may be required. As previously mentioned, different variables may affect protein profiles and reduce confidence score. A full list of variables is beyond the scope of this chapter, but some common factors to consider are growth media, culture storage conditions, and exposure to oxygen (anaerobic cultures) (24). For efficiency, the laboratory can perform this additional replicate testing in combination with the package insert protocol being verified. Any significant difference calculated between the package insert protocol and the variable tested (growth media, storage, etc.) should be noted in the verification packet. Variables that cause significant differences to confidence scores will require either additional standard values for quality control testing, or further verification studies under those conditions prior to patient testing. For example, growth on Hektoen enteric agar (HE) has been shown to lower confidence scores (17). When using HE for patient testing, quality control organisms will have different pass/fail parameters than quality control organisms grown on blood agar plates. Each run containing bacteria grown on HE will require controls, specific for HE testing. It is important to note that for verification of FDA-cleared tests, only variables claimed by the manufacturer need to be verified. The package insert contains all media and growth conditions that are FDA cleared. Any testing not following the claims in the package insert will define the test as an LDT and is subject to LDT requirements.

Calculating precision for LDTs or a modified FDA-cleared test is similar to FDA-cleared tests. Multiple organisms covering different groups are selected and tested in duplicate over multiple days. There are no regulations suggesting more rigorous testing to be performed for calculating precision; however, performing the modified experiment over a minimum of 20 days is recommended to ensure that the various factors of error are adequately measured (25). If the method being verified is testing direct patient specimens, samples should be spiked into a negative matrix (blood, urine) and tested similarly to patient samples (26–28).

Precision Sample Source

There are several types of samples that will fulfill the requirements for reproducibility studies. These sample types include characterized patient samples (prospectively or retrospectively collected), assay standards, quality control materials, or specimens purchased from third parties. All specimens need to be treated as patient samples, meaning that samples follow the planned laboratory workflow and all specimens are adequately stored after plates are subcultured for testing (−20°C to −70°C) to ensure stability of the samples over the course of the study (29, 30).

Precision Data Analysis

The data that are collected and used for measuring precision are dependent upon the system that is being verified. All MALDI-TOF systems compare the test spectrum against a database created from spectra of characterized strains, but each system's algorithm used to report confidence scores is different and may affect how the data should be collected and analyzed. The Vitek MS system's algorithm assigns weights to peaks for various species, and the software compares the obtained spectrum to claimed

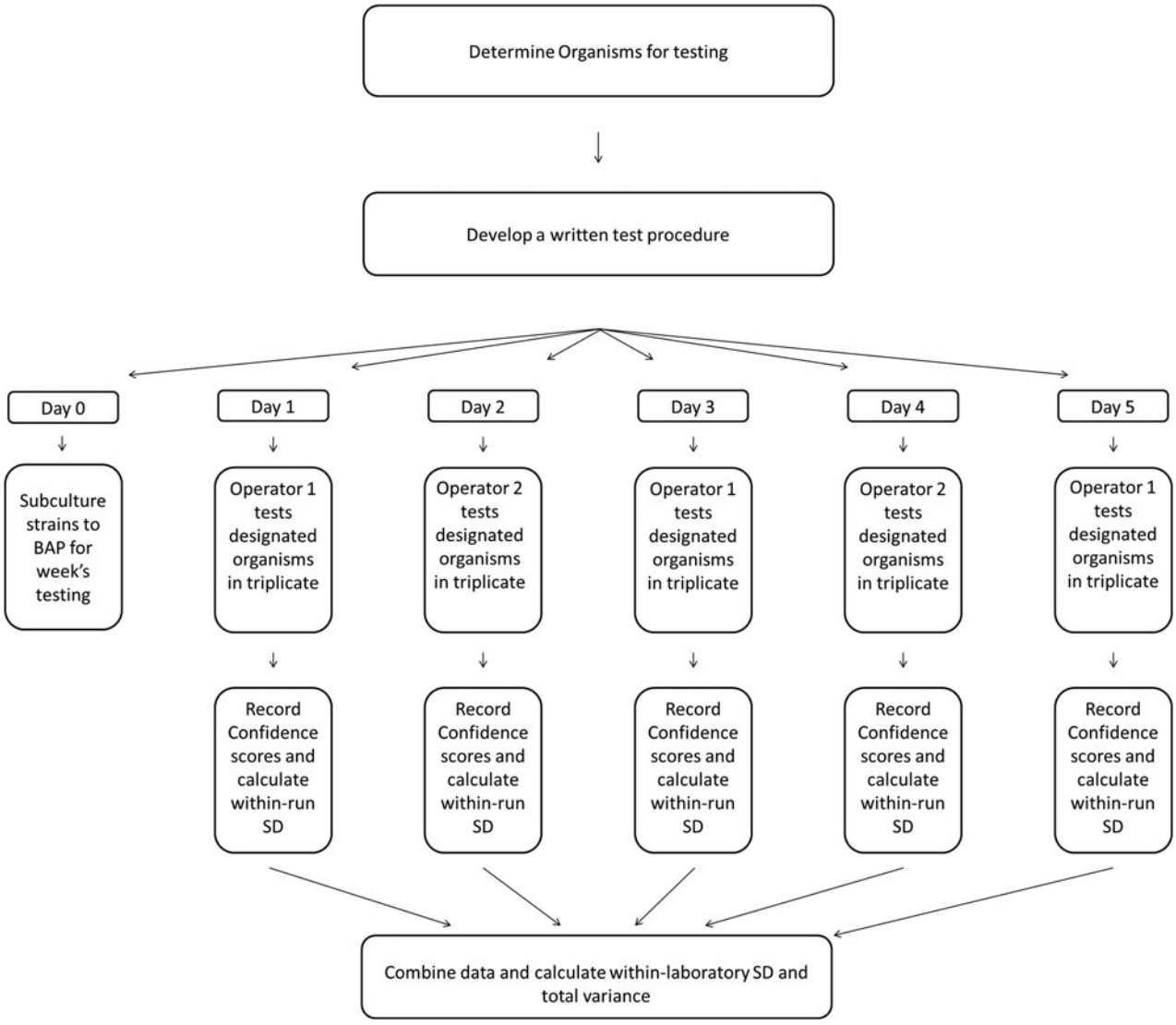

FIGURE 1 Example of reproducibility study flow chart. Prior to conducting testing, the laboratory should determine which organisms will be utilized for reproducibility testing. Guidance can be found in the package insert of the MALDI-TOF MS system. The laboratory should then design the test procedure, addressing how to store, test, record, and analyze the data. The laboratory staff should subculture out a fresh specimen to test over the next 5 days. If testing occurs over 5 days, a new subculture from the original frozen strain should be made. At least two different operators should perform the testing. After testing is complete, the total variance or percent agreement should be calculated to determine if the test precision is acceptable.

organisms. The system reports a confidence value ranging from 0% to 100% confidence and can report up to four possible species per sample. The Bruker Biotyper system reports a confidence score between 0 and 3. This system's algorithm is a correlation between the acquired spectrum and the reference spectrum, with the identification confidence reported as a logarithmic score value. The difference between algorithms creates some debate on whether the MALDI-TOF MS confidence scores are qualitative data of spectral similarity or quantitative. The reporting values from MALDI-TOF MS have been used as both qualitative (19) and quantitative (17) in the literature. Several studies have demonstrated the value of using the Bruker Biotyper confidence score as quantitative data, demonstrating that a 10% difference in confidence scores between matches can increase species identification (31, 32).

If the system is defined as a qualitative assay, the final identification should be collected and compared to the true result. The precision of the system is measured by calculating the percent agreement between the MALDI-TOF MS result and the true species identification. For example, if *Klebsiella pneumoniae* is tested 20 times and is correctly identified 19 out of 20 times, the percent agreement would be 95%. For FDA-cleared assays overall agreement should be at least 90%, while overall agreement should be 95% for LDTs (10).

When using the confidence score as a quantitative representation of spectral similarity a statistical measurement of imprecision, represented as either SD or CV, should be determined (8). The benefit of performing these calculations is that variation that affects the spectra but does not reduce the confidence score below reporting range can be

observed. Small changes in spectra may not affect identification of common bacteria but may result in lower identification rates of rare bacteria for which the database may have fewer spectra for comparison. Standard deviation can be calculated using the confidence score as these data represent the variability of the algorithm used by the system (33). An analysis of variance (ANOVA) will calculate the CV taking the following into the formulae: the number of replicates per run, the number of runs per day, the number of days the reproducibility study is performed, and the number of instruments used along with several other variables used in the calculation (8). Many statistical programs can assist in calculating the CV for a study (8). For FDA-cleared tests, calculating the total variance (SD_{total}) is sufficient for verifying reproducibility claims demonstrated by the manufacturer.

Separate calculations are necessary if various factors are being tested, such as age of isolate or different growth conditions. Since each calculation is different for each experimental design, it is not possible to generalize the formulae required to account for each variation. When necessary, the laboratory should use the worksheets provided in the CLSI document EP5-A2 for guidance (8). In addition, there are several resources that can be used to help guide laboratories through the statistical analysis required to calculate total variance (18).

With the calculated method imprecision determined, the laboratory can conclude if the calculated variation is acceptable for patient testing. Calculating the precision for reproducibility studies is well defined by regulating guidelines; however, determining if the calculated variation is acceptable is not well defined. For verification of FDA-cleared tests, the package insert contains information regarding the acceptable range of error. The mean values of the study should fall within two or three SDs of the package inserts claims and have similar SD values (8). For LDTs and modified FDA-cleared tests there is FDA guidance available (34). In general, the guidelines state that the range of error should not exceed 15% of the CV. The only exception is when methods use variable concentrations of analyte (direct sample test with LDTs). When a lower limit of quantitation (LLOQ) is required, the precision should not exceed 20% of the CV when tested at the LLOQ (34).

ESTABLISHMENT OF PERFORMANCE SPECIFICATION: ACCURACY

Accuracy Definition

Accuracy is a broad term and is defined as the statistical closeness of agreement between a measured value and the true value (15, 35). The term accuracy has also been synonymous with trueness in describing the closeness of agreement between the average values obtained from a large series of measurements and the true or accepted reference value (36). Numerically, accuracy is expressed as the lack of agreement between the test under verification and the gold standard test. Because accuracy will measure how often the MALDI-TOF MS system will misidentify an organism, the method comparison is the cornerstone to method verification.

Accuracy Study Design

Two different approaches can be used to evaluate accuracy. The first and recommended approach is to perform a methods comparison study. A methods comparison study consists of testing specimens on both the MALDI-TOF MS system being verified and the current gold standard (15, 29, 37, 38). Because MALDI-TOF MS has a wide range of organisms that it can identify, multiple gold standards of testing may be required. All gold standard tests need to be verified and validated tests. Gold standard tests can comprise other multi-analyte tests, such as Vitek2 (bioMérieux, Marcy l'Etoile, France), Phoenix (BD, Franklin Lakes, NJ), DNA sequencing, or other biochemical testing. Several publications have performed comparison testing of various MALDI-TOF MS platforms that can guide the clinical laboratory (3, 39, 40). All specimens need to be tested on both the MALDI-TOF MS system and the gold standard. In addition, proper quality control testing with each test is essential to confirm that both the MALDI TOF MS and the gold standard method are functioning correctly.

An alternative method used to measure the accuracy of the system is to perform a recovery study. A recovery study utilizes proficiency testing samples and compares the results from the method undergoing verification to the expected reference value (41). This approach is normally reserved for new tests that do not have a comparison method, but may also be used when verifying rare isolates (42). A laboratory using a recovery study should aim to test any samples under expected clinical conditions. Samples that are not tested following normal patient samples can result in less accurate testing because possible factors in clinical isolates may affect the protein profiles and reduce the confidence score. This is more important for assays that test directly from a specimen and is likely not applicable to assays that use plate cultures.

Because there are no specific regulations for MALDI-TOF MS, the laboratory can follow the minimum requirements for automated, multi-analyte tests (10). For FDA-cleared tests, a minimum of 20 isolates representing a wide range of clinically relevant organisms for the institution are required (10). Large institutions should increase this number to accurately test the wide array of isolates that the laboratory encounters. Examples of comparison studies are numerous throughout the literature and can act as a guide for identification of specific bacterial species (5, 43–45).

Unlike precision, requirements for verification differ between FDA-cleared tests and LDTs (or modified FDA-cleared tests). For LDTs and modified FDA-cleared tests, it is recommended that comparison testing be performed with a minimum of 200 isolates (46). If the new method is targeting a specific analyte, the laboratory should at minimum perform comparative analysis of 50 samples positive for the analyte and 100 samples that lack the target analyte. For example, if the method is to identify *Escherichia coli* directly from urine specimens, the laboratory should perform testing on 50 urine samples containing *E. coli* and 100 negative samples. For methods testing directly from plate and identifying a variety of organisms not claimed by the manufacturer, 200 isolates consisting of multiple target analytes should be tested. Current literature can guide study design. One study compared the ability of the Vitek MS v2.0 to identify fastidious Gram-negative bacteria (22). The comparison tested 226 specimens consisting of 9 genera and 15 species consisting of both common pathogens and rare isolates.

The current guidelines define a minimum of samples needing to be tested but lack guidance on how to properly verify the wide range of analytes that MALDI-TOF MS

can detect. In the remainder of this section we propose one possible method for performing verification of FDA-cleared MALDI-TOF MS systems. This proposed method is not required by any governing body but will provide an all-encompassing study to compare the method to the laboratory's current gold standard on the wide variety of organisms identified on a yearly basis. Because it is important to determine how the system will perform in the laboratory's patient population, calculating the past year's prevalence will create a list of targets that the laboratory either frequently encounters or encounters more rarely. From this list the laboratory may aim to test 10% of the total species encountered within the year to a maximum of 20 per species and a minimum of 5 per species. For example, if a laboratory identified 1,000 *Staphylococcus aureus* isolates and 5 *Nocardia farcinca*, it would perform comparison testing on 20 *S. aureus* isolates and 5 *N. farcinca* isolates. The laboratory would perform this calculation for each isolate it has identified from the previous year and test the appropriate number of isolates. A flow chart describing this proposed testing can be found in Fig. 2.

In almost all method comparison studies, there are samples that will have discrepant results between the new test and the current gold standard. During the planning stages of verification it is important to determine how the laboratory will resolve any discrepancies. Any type of disagreement between the new method and gold standard needs to be scrutinized because misidentification is the most serious error for a MALDI-TOF MS system (10). The ideal method for discrepant resolution is nucleic acid sequence analysis of a genetic sequence target(s) appropriate for the organism. However, for small laboratories or when there are large amounts of discrepant results, the cost of sequencing may not be feasible. Alternatively, a second method for detection can be utilized, such as a molecular assay or another multi-analyte test system not already used for verification. Interpretation of discrepant results can create bias in the results because only discrepant specimens are tested, and it is possible that additional testing may match incorrect results by chance (47–51). In the ideal study, all specimens tested would be sequenced to remove any bias that might be created by sequencing only discrepant results.

Accuracy Sample Source

When possible, the laboratory should use residual patient specimens that have been previously tested by the laboratory's standard of care method. Residual patient specimens are ideal because these most accurately test the patient population and test the variability of clinical samples compared to known controls. When detecting uncommon organisms, it is more practical for the laboratory to acquire samples from other laboratories or commercial sources. However, these sources may not be representative of the patient population (52). Any testing of commercial specimens should be documented, with the potential limitations stated in the assay verification report.

All specimens should be collected and handled in accordance with current laboratory practices. If testing requires either storing samples between testing or using retrospective samples, it is essential that these specimens are stored appropriately to ensure strain stability. Any testing with improperly stored specimens may lead to inaccurate comparison that is not reflective of the method's true performance.

Accuracy Data Analysis

For MALDI-TOF MS systems, there needs to be at least 90% agreement between the reference method and the new system before it is considered verified (10, 38). The following equation should be used to calculate the percent agreement:

$$\frac{\text{No. correct results}}{\text{Total no. of specimens tested}} \times 100$$

If the accuracy does not meet the 90% agreement, then the new method cannot be verified and corrective measures are required by either the manufacturer or the laboratory staff (10, 38, 53). Once corrective measures are taken, the laboratory can decide to perform a new method comparison study to determine if there is acceptable agreement. The 90% agreement minimum applies for both FDA-cleared tests and LDTs or modified FDA-cleared tests.

Another important statistic to calculate is the clinical sensitivity and specificity of an assay. Sensitivity is also known as the true positive rate and is the ability of the test to identify the correct condition. For example, to calculate the sensitivity of MALDI-TOF MS to identify *S. aureus*, the laboratory would compare the number of true positives (TP), the number of isolates correctly identified by both MALDI-TOF MS and the gold standard method, to the total number of *S. aureus* tested, TP plus false negative (FN), which is the total positives identified by the gold standard result (54).

$$\text{Sensitivity} = \frac{\text{TP}}{(\text{TP} + \text{FN})} \times 100$$

Specificity relates to the ability of the test to correctly detect negative effects. Specificity is calculated by taking the number of true negatives (TN), the number of isolates that tested negative for *S. aureus* on both tests, and dividing by the total number of negatives tested, TN plus false positives (FP), or the total negatives identified by the gold standard (54). Knowing the clinical sensitivity and specificity will aid in identifying the test's limitations (55).

$$\text{Specificity} = \frac{\text{TN}}{(\text{TN} + \text{FP})} \times 100$$

It is recommended that the results are separated by the confidence score and by organism (an example of this data organization can be found in Table 2). Organizing the data this way is helpful because it allows assessment of accuracy for different organisms and the accuracy for organisms reported with various confidence scores. Separating the data will help determine if the new method has problems differentiating specific organisms. In addition, the data will aid in defining the laboratory protocols for the technician's workflow. For instance, due to species similarity, *Burkholderia* is difficult to differentiate into species using MALDI-TOF MS (56). In this instance, the laboratory could add a note for the technician stating the need to perform extra testing if differentiation is required for any *Burkholderia* identifications.

ESTABLISHMENT OF PERFORMANCE SPECIFICATION: REFERENCE RANGE

The reference range or reference interval is the measurement of variation in healthy individuals. As mentioned above, the reference range is not necessary for MALDI-TOF MS verification because the test is qualitative. However, it

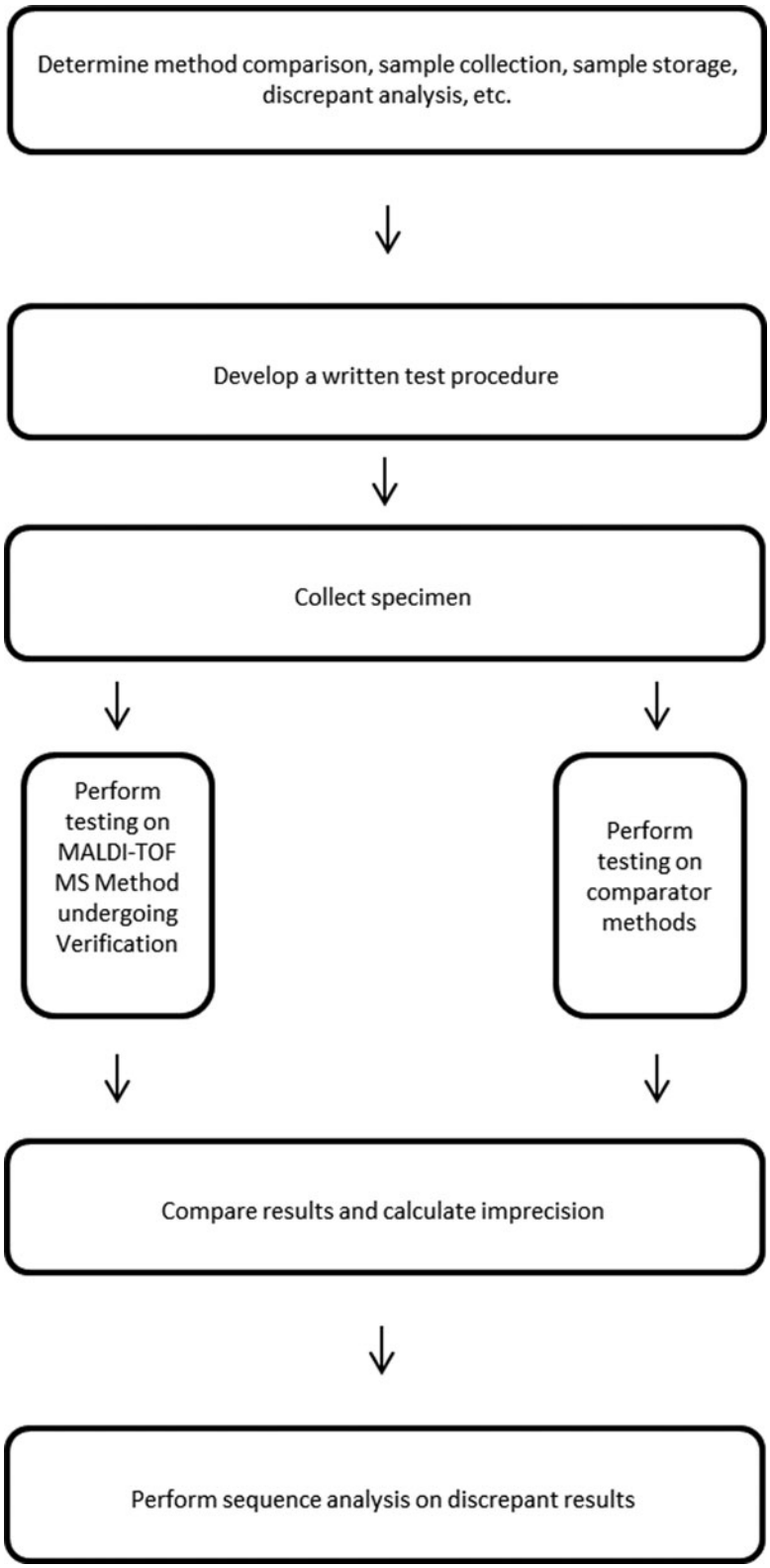

FIGURE 2 Example of a method comparison studies for MALDI-TOF MS verification. Prior to testing, a planned procedure describing how the laboratory will collect and store specimens, along with what comparator testing will be performed and how discrepant results will be characterized if needed. Collected specimens are tested on both the method under verification and the gold standard comparator test. The results of the method under verification are compared to the results from the gold standard to determine the accuracy of the test.

TABLE 2 Example of accuracy report for Gram-positive organisms

Organism	No. tested	Confidence score values			Discrepant identifications
		<1.7	1.7–1.99	>1.99	
Bacillus cereus	20	1	5	14	1 isolate called *Paenibacillus timonensis* by MALDI that used to be in the *Bacillus* group
Lactobacillus delbrueckii	20	0	2	18	2 isolates identified to different species name than what had been called on culture
Listeria monocytogenes	1	0	0	1	
Staphylococcus aureus	20	0	1	19	
Total	61	1	8	52	
Percentage		1.6	13.1	85.2	
% Correct to genus		0.0	100	100	
% Correct to species		NA	NA	96.1	

should be noted that in the event that the method is testing specimens that can be sterile, the verification report should state that, "based on literature review, no reference interval study was performed" (25). Examples of these methods can include LDTs that are testing directly from specimens, such as urine (57, 58). For these types of tests the reference range of not detected would be appropriate.

ESTABLISHMENT OF PERFORMANCE SPECIFICATION: REPORTABLE RANGE

The reportable range refers to the test result values where the assay can accurately measure the analyte of interest (25). Similar to the reference range, defining the reportable range is not required for MALDI-TOF MS because the test is qualitative. The package insert for the system being verified will define the reportable range. For LDTs the laboratory would use these suggested reportable ranges for identification.

ESTABLISHMENT OF PERFORMANCE SPECIFICATION: ANALYTICAL SENSITIVITY

Analytical Sensitivity Definition

The analytical sensitivity of an assay is defined as the ability of the assay to detect very low concentrations of a given substance in a biological specimen (59). More commonly, analytical sensitivity is known as the limit of detection (LOD). For any FDA-cleared application using a spot method of MALDI-TOF MS it is not necessary to verify the LOD. However, the LOD is required for any LDTs and modified FDA-cleared tests that are used for direct detection from usually sterile clinical samples. For instance, verifying detection of organisms directly from cerebrospinal fluid will require the laboratory to define the LOD before the method can be used on patient samples. For MALDI-TOF MS, the LOD for each analyte that the laboratory is aiming to detect directly from patient samples must individually be determined.

Analytic Sensitivity Study Design and Sample Source

Because the LOD is expressed as the minimum amount of bacteria the instrument can detect, it is essential that well-characterized samples with known quantities are used in all testing. These samples can be either previously characterized patient specimens or control strains spiked into known negative patient specimens. Known concentrations of these well-characterized isolates should then be spiked into an appropriate matrix ranging from low to high concentration. Various concentrations can be created by starting with a McFarland standard followed by serial dilutions. Plating of serial dilutions for colony counts will quantitate the bacteria per milliliter in each dilution. Initial studies should be performed using high, medium, and low concentrations to determine the probable LOD, or the concentration that is not detected by the method. Furthermore, multiple negative matrices should be used rather than a single negative sample to account for variability between patient specimens (60). To determine the LOD, spiked specimens should be tested above and below the probable LOD.

Analytical Sensitivity Analysis

The most common analysis used to determine LOD is probit analysis (25, 61). A probit analysis is a type of regression analysis used to analyze binomial response variables. The probit analysis will transform the concentration response curve to a straight line that can then be analyzed by regression. Once the data are fit to a regression line, the 95% endpoint can be determined. The 95% endpoint represents the concentration at which 95% of the samples containing that concentration of analyte will report a positive result. Many statistical packages are available to perform a probit analysis, including Analyse-it statistical analysis software addendum for Microsoft Excel (Analyse-it Software Ltd., Leeds, United Kingdom) or SPSS (Statistical Package for Social Sciences; IBM, Chicago, IL). It is up to the laboratory director's discretion to determine if the calculated LOD is sufficient for the method's intended purpose.

ESTABLISHMENT OF PERFORMANCE SPECIFICATION: ANALYTICAL SPECIFICITY

Analytical Specificity Definition

Similar to analytical sensitivity, analytical specificity is only required for LDTs and modified FDA-cleared tests (8, 9). Analytic specificity refers to the ability of the test to detect the analyte in the presence of interfering substances. "Interfering substances" is a general term for any substance that may be in a clinical specimen that will

disrupt detection of the analyte. MALDI-TOF MS uses protein profiles to generate spectra; anything that disrupts these protein profiles can disrupt discriminatory power of the system (19). The literature has demonstrated several substances that can disrupt identification. Substances such as high salt content, different growth media, growth at various temperatures, ion suppression, and prolonged age of a culture can modify protein profiles (17, 19, 20, 62, 63). LDTs that are detecting organisms directly from the specimen may encounter other interfering substances such as whole blood or mucus from patient specimens, which may affect spectra generation. It is impossible within the scope of this chapter to create a specific list of interfering substances because possible substances are dependent upon the methods being tested. CLSI does supply a list of common substances that can be used to aid in performing an analytical specificity study (64).

Analytical Specificity Study Design and Sample Source

Before testing begins, a list of potential interfering substances should be compiled, specific to the method being verified. Spot method assays should consider testing various growth mediums (various plates or broths) along with growth conditions (growth temperature, culture age). Current literature comparing the effects of these factors perform at least duplicate testing using various strains (39, 65). In these studies either the generated peaks and confidence scores or percent concordance were compared to a standard package insert protocol (16, 17, 31, 66). Statistical analysis (*t* test) comparing these variables will calculate if there is any significant difference between the spectra and confidence score. Significant differences should be noted in the verification report.

Direct sample preparation studies should follow similar protocols to LOD studies. Spiked samples with a known concentration that contain both the analyte and the potentially interfering substances should be tested using the method's planned standard of care procedure. The recommended concentration for each interfering substance can also be found in the CLSI guidelines (64). All testing should use analyte concentrations at the medical decision limit of the assay, which is the lowest concentration of analyte that the test can report as a positive test result. Using this low concentration ensures that the interfering substance will not change the clinical decision. Furthermore, each test should also have a positive control that does not contain any interfering substance. The positive control is necessary to compare the effects of the interfering substances to the assay's performance. Currently, there is no recommended minimum number of samples required; however, current literature measuring difference in spectra compare a variety of bacterial species and perform testing in triplicate (17, 19).

Analytical Specificity Data Analysis

Similar to data analysis of reproducibility studies, the system that is used and how the confidence score is defined affect the methods for specificity data analysis. When defining the confidence score as quantitative, a paired *t* test should be performed to determine if the specific substance affects the confidence score. The paired *t* test will compare the means of the control confidence scores without interfering substances, with the means of the confidence scores containing interfering substances. The resulting *P* value is used to determine if the interfering substance has any significant effect on the test's performance ($P < 0.05$). Most statistical programs including Microsoft Excel (Redmond, WA) can perform a paired *t* test. If the laboratory defines the confidence score as qualitative then the identification result is reported. The results of the test with the interfering substance are compared to those of the test without the substance to determine if there is a loss in identification.

VERIFICATION STRATEGY

The initial planning and design of a verification strategy are important for timely and efficient introduction of MALDI-TOF MS into the laboratory. Prior to testing the acceptance criteria should be defined (10). Defining the acceptance criteria allows the laboratory to perform proper error assessment and determine the system's limitations. There are several approaches to defining the acceptance criteria; however, in general the criteria should be constant with industry standards or standards published in peer-reviewed publications (7, 9). It is suggested that a protocol be planned and written before actual verification testing is performed. The protocol should address which samples are used, how they will be stored, the test procedure, the statistical methodology used to determine if the new test meets the acceptance criteria, and how discordant results will be evaluated. In addition, all testing should be performed to mimic reality as closely as possible.

For each new assay or method introduced in the laboratory, a verification document should be written. This document serves as an overview of the method's performance and summarizes the assay describing when, why, where, and how the assay was verified and by whom. In accordance with CAP guidelines the document should include a statement approving the test, a summary of the verification data addressing PARR and the clinical claims regarding the test (67). LDT reports should also address analytical sensitivity, analytical specificity, and any other parameters that are considered important such as specimen stability and linearity (67). Finally, the recommended policies and procedures for the method should be written into the document. The laboratory must maintain these records for both internal and external review.

MALDI-TOF MS VALIDATION

The process of verification either confirms or denies that the laboratory can meet the test performance characteristics suitable for clinical use. However, verification does not provide quality assurance that the test continues to perform well over its lifespan in the laboratory. Test validation is essential to the clinical laboratory because it provides necessary quality assurance. All components of the test validation process are addressed by CLIA regulations (68). These regulations consist of both daily quality control (quality control organisms, system calibration) and semiannual quality control (proficiency testing, personnel competency). The remainder of this chapter will discuss the key concepts required to validate MALDI-TOF MS systems.

DAILY VALIDATION PROCEDURES

MALDI-TOF MS Quality Control Organisms

All FDA-cleared tests contain a list of quality control organisms that must be tested with each run. This list can be

found in the package insert. The package insert is a minimum requirement for the test; additional controls can be added by the laboratory. For LDTs it is the laboratory's responsibility to determine which quality control organisms are necessary for the test. It is recommended that quality control organisms represent the diversity of analytes being tested such that at least one Gram-negative and one Gram-positive bacterium and one yeast be tested daily (21). Assays that require extra processing of specimens, such as identification of *Mycobacterium* species, should contain controls specific to the organism and processing (69, 70).

Quality control organisms should come from a reputable source, such as ATCC (Manassas, VA) or National Collection of Type Cultures (NCTC; Salisbury, UK), to ensure accurate testing of the system's performance. Furthermore, all isolates must be maintained at −70°C, and fresh isolates should be subcultured and stored on slants on a monthly basis to maintain strain stability (10). If any quality control testing fails, a new subculture from the freezer stock should be created, and quality control testing must be repeated before patient tests can be reported. The laboratory can decide to use nonreference strains, but documentation must be maintained that comprises the characterization, storage, and recovery from storage of the organism (10).

Daily Testing

The CAP microbiology checklist list contains the required quality control testing that is necessary for MALDI-TOF MS systems performing organism identification (21). These guidelines require two separate tests to be performed: a system calibration and within-test controls. The system calibration is performed with a bacterial test standard (BTS). BTSs are composed of a reference organism that has a characteristic peptide and protein profile used to calibrate the MALDI-TOF MS system. The BTS should be performed each time a target plate is changed (21). This calibration ensures that the system is generating the expected m/z ratio peaks.

CAP guidelines also require control organisms to be tested for each plate used for patient testing. These controls should contain positive controls comprising representatives of possible analytes (Gram-positive, Gram-negative, yeast, etc.) and a negative control that does not contain any organisms (21). The negative control is used to demonstrate that the plate is clean, which is represented by a lack of peaks. If any of the controls have confidence scores that fall out of the acceptable ranges (as determined by verification) the entire plate's results must be discarded and retesting must be done. Furthermore, if any extractions are performed, an extraction control using quality control organism is required.

SEMIANNUAL VALIDATION

Personnel Competency

For each laboratory employee that will perform testing, written documentation must be provided that establishes competency for the employee. To aid laboratories in developing a competency program, CLIA has developed a list of six assessment areas. These six areas are (i) direct observations of performance of routine patient tests, (ii) monitoring of the recording and reporting of results, (iii) review of intermediate test results or quality control records, (iv) direct observation of performance of instrument maintenance and function checks, (v) assessment of test performance through testing previously analyzed specimens and internal blind testing samples, and (vi) assessment of problem-solving skills. It is not necessary to perform every assessment, and it is up to the laboratory director to develop an appropriate plan for training. All employees should undergo competence assessment semiannually during their first year in the laboratory and then annually thereafter (71–73). The laboratory should maintain records of all employees' training.

Proficiency Testing

Any lab that is CLIA certified must be enrolled in a CMS-approved proficiency program. These proficiency programs are performed at least twice a year, and all samples that are received by the laboratory must follow the protocol of standard specimen care. Although these proficiency programs may not be directed to validate the MALDI-TOF MS system, this proficiency is important for overall laboratory validation because it is an external review of the laboratory's methods and procedures. Failure to score an 80% indicates an unsatisfactory performance, and the laboratory must undergo retraining and potential modification of planned procedures. If a laboratory fails two consecutive testing events, the CMS may impose sanctions on the laboratory (10).

Instrument Calibration

MALDI-TOF MS systems are highly complex instruments that must be checked to ensure proper working components. In accordance with the manufacturer's direction, a laboratory must have the instrument serviced at specified intervals. The laboratory should place all paperwork with the validation packet as confirmation that the system is properly calibrated. Additional maintenance may be necessary as indicated, for instance, in the case of continual failure of controls or if the system is moved to a different location within the laboratory.

Multiple Instrument Testing

In laboratories that contain multiple instruments, the laboratory must demonstrate that each instrument performs equally. CAP guidelines state that equivalency testing takes place twice a year (67). Equivalency testing is composed of testing the same specimens, either quality control organisms, archived specimens, or defined patient specimens, on all instruments. The instruments are considered equivalent if they successfully identify the isolates and the confidence scores fall within acceptable ranges. Any instrument that does not meet these specifications should not be used for clinical testing until any problems are corrected.

SUMMARY

The current CLIA guidelines on verification and validation of MALDI-TOF MS are not specific and do not address the challenges of introducing the technology to the microbiology laboratory. Our goal for this chapter was to provide guidance by defining the current guidelines and recommending study design to assist laboratories in maintaining good laboratory practices. As stated previously, the recommendations within this chapter are only guidelines and should not be considered regulatory standards. Any laboratory planning on introducing new MALDI-TOF MS

systems and methods should confirm that verification and validation standards have not changed. Furthermore, laboratories should determine if there are any additional local or state regulations that were not addressed within this chapter.

The process of assay verification may be time consuming and costly for a laboratory, but this process is essential to determine the limitations of an assay and ensure accurate and precise testing. With proper planning before testing the laboratory should be able to efficiently accomplish assay verification. The method of verification will aid in the decision to introduce the test by creating a summarized packet demonstrating the precision and accuracy of the new assay. Ultimately it is the responsibility of the clinical laboratory director to answer the question "How good is good enough?"

Just as important as verification is the ongoing validation of the test. Proper validation will ensure that the quality of the test is maintained. In some cases, laboratories may want to test their validated assays against new assays on the market. Continued validation and testing of laboratory tests will guarantee that the laboratory offers patients the best care possible.

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section XII

The Business of Diagnostics

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Improved Diagnostics in Microbiology: Developing a Business Case for Hospital Administration

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55

Health care in the United States has seen dramatic changes over the last decade, including an increasing emphasis on the cost of delivering care. Although it has been estimated that approximately two-thirds of health care decisions are based on laboratory results (Silverstein M, An approach to medical errors and patient safety in laboratory sciences, White paper presented at Quality Institute Conference, 23 January 2003), according to the Congressional Budget Office, the laboratory accounts for only 4% of health care expenditures (1, 2). Nonetheless, this amount approaches $60 billion annually and is constantly a target for reduction. Thus, in this era of Accountable Care, hospital administrators are facing significant revenue shrinkage from all payers. As revenues dry up, so does internal funding for new capital initiatives and internal competition for scarce capital dollars becomes fierce.

For laboratories to remain competitive they must be able to quantify their "value" in a business case in order to receive funding for new technologies. The ability to articulate, in the appropriate language and delivery, initiatives that are the best investments for an institution is not a skill taught to those on a technical scientific track in college. Nonetheless, a detailed and cohesive business plan is essential for success, and the first step is to know one's audience at the top echelons of hospital management.

From the C-suite perspective (i.e., the chief executive officer, chief operating officer, chief financial officer, and chief medical officer), there are multiple issues that come into play. The focus of the chief executive officer is usually on patient experience and satisfaction, clinical quality, and cost reduction through process improvement. Cost control strategies are usually the purview of the chief financial officer and generally focus on labor efficiencies and cost reductions via process improvement and supply chain efficiencies. The major challenges for hospital executives today are reduced reimbursements, physician shortages, and in some geographical areas, organized labor. Finally, they are often primarily rated (paid) on the basis of the hospital's operating margin and achievement of clinical quality and patient satisfaction targets (3). When preparing a business case for laboratory initiatives involving new technologies, the impact of these new technologies on elements of vital interest to hospital administrators must be considered.

A business case is defined as "A document that forms the basis of advice for executive decision-making for an asset investment. It is a documented proposal to meet a clearly established service requirement. It considers alternative solutions and identifies assumptions, benefits, costs and risks" (4). A business case is essentially a well-conceived story with three possible themes: (i) it enhances net revenue for the organization, such as a laboratory outreach *pro forma*; (ii) it decreases expenses, such as an insourcing justification, a laboratory consolidation, laboratory automation, or switching reference laboratories; and (iii) it contributes to the cost avoidance care continuum such as high-risk patient screening for methicillin-resistant *Staphylococcus aureus* or rapid diagnosis of *Clostridium difficile* enterocolitis. A business case is important because it provides an accounting of costs; documents/quantifies benefits and achievement of goals; articulates identified issues, risks, and solutions; communicates the resources required for implementation; and demonstrates a need for an increased operating budget (4). It should also cover those areas of greatest concern to the organization's finance department: how much will it cost, and how soon is the payback. The business case should cover several key points in addition to the business need or requirement. It should include options that best address the business need or requirement and an analysis of the benefits and costs of those options. Recommendations regarding the preferred option should be addressed as well as risk identification and mitigation strategy. Finally, an implementation plan should be included (5).

An important initial exercise when preparing a business case is to understand the factors driving the need for any new technology. A successful business case should include realistic strategic planning to achieve its goals and such planning should include a SWOT analysis (SWOT: strengths, weaknesses, opportunities, threats). A SWOT analysis allows the discovery of internal and external issues that are key to moving forward and continuing or improving outcomes. A listing of strengths should include resources, abilities, and competitive advantages within the laboratory. Weaknesses should address where and how the laboratory can improve its reputation and services for the organization. Opportunities identify those areas where

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TABLE 1 ROI of mass spectrometry identification of bacteria and yeast

Assay	Annual reagent costs	Annual labor cost	Equipment costs	Annual reagent savings	Annual labor savings	Total annual laboratory savings[a]	ROI
Current biochemical identification	$66,712	$52,381	—	—	—	—	—
Mass spectrometry identification	$3,942	$22,131	$200,000	$62,770	$30,250	$93,020	2.15 years

[a]••••••••.

growth, advancement, and change exist and how they can be exploited. Finally, no organization is immune to threats, and laboratories/hospitals too often miss these threats, thinking that their practices are all inclusive and that there are enough patients to go around. When performing a SWOT analysis as part of the business case for administration, the laboratory should consider the following: (i) avoid gray areas; be specific when analyzing current practice and future needs; (ii) ask for input; third party opinions can be supportive and are often necessary; (iii) be realistic; stay grounded when judging strengths and weaknesses; (iv) apply context; ask where the laboratory has been and where it's going and identify steps for further growth; (v) avoid complexity; keep it short and simple; (vi) analyze realistically; compare the business case to key competitors and decide if it's better; and (vii) achieve change; update the business plan and goals as soon as problems are identified and identify steps toward directional change (5, 6).

In developing a business case for any new laboratory service, it is important to keep in mind key stakeholders, especially those outside the laboratory such as physicians, nurses, pharmacists, and infection control practitioners. Gaining their support for an initiative is often necessary for the success of a project. The laboratory should also gain the trust and respect of finance personnel by working with and through them throughout the planning process. The finance department may be the first hurdle in any *pro forma* development and often times a project is ready for presentation to the C-suite when finance says it is ready. In the final analysis hospital administration will want to know what a project will cost, what it will save and how long it will take to get a return on the investment (ROI). In layman's terms, advancing new laboratory technology, involves knowing the cost difference between the old and new approach.

Using mass spectrometry (MS) for bacterial identification (ID) as an example, the costs of both the reagents and labor needed to perform testing drop significantly. Additionally, there is a dramatic improvement in turnaround time for isolate identification. These gains will translate into additional gains in quality, efficacy, and improved patient outcomes. The reagent costs are determined by calculating the annual volume of IDs performed and multiplying this volume by the cost of MS reagents per ID to determine the annual reagent costs. The labor costs are determined by calculating the total amount of time to perform an ID and the annual volume of IDs performed. Labor time per ID and annual volume multiplied together determine the annual hours of staff time required to perform a procedure, and multiplying these annual hours by the cost of labor (wage plus benefits) will determine annual labor costs. The current annual conventional biochemical reagents and current labor to perform those tests can then be calculated. Next, it is important to calculate the cost of acquisition, which includes the capital cost of the equipment, service contracts, cost of remodeling (if applicable), cost of interfacing equipment, and the cost of project planning/implementation/validating the equipment (7). The ROI is calculated by determining the annual reagent and staff cost savings of the MS ID system ([MS reagent + MS labor] − [Biochemical reagents + Biochemical labor costs]) and dividing the result by the cost of acquisition. This calculation will determine how long it will take to recuperate the cost of converting to MS ID (Table 1).

This quantitative total has also been referred to as the economic justification index (EJI). An EJI of 1 is equal to 1 year, 0.5 equals 2 years, and 2 equals 6 months of a payback period for the reduction in labor costs. Factors that are much more difficult to quantify would need to be monitored so gains to the organization due to reduced waste, quality, and turnaround time could be captured (5, 7, 8).

Qualitative changes can be evaluated by examining their strategic justification index (SJI). The SJI is determined by looking at six change factors and assigning them a value of 0 to 2 (0 = not important, 1 = moderately important, 2 = very important). These factors are quality, safety, procedure enhancement, audit trail, more timely decisions, and flexibility. The SJI is determined by dividing the sum of the six change factors by 12 (highest possible score). An SJI of 1 is equal to a very high value, indicating that all six change factors are very important to the organization (8).

The EJI and SJI for a project can be plotted on an *x*-*y* graph with a minimum of 0 and a maximum of 1 to determine the overall project justification (Fig. 1). This ap-

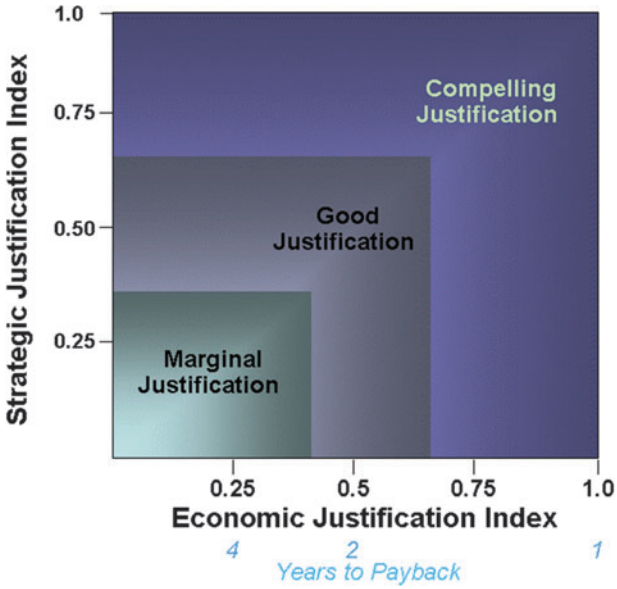

FIGURE 1 Use of SJI and EJI for overall project justification. From Hamilton, SD. Justifying laboratory automation. LA invited article. www.labautopedia.org/mw/index.php/justifying_laboratory_automation, 30 November 2011. (This content is available under GNU free documentation License 1.2).

TABLE 2 Executive summary of outcomes: integration of mass spectrometry identification with antibiotic stewardship for patients with sepsis

Study	Sepsis target	Reduction in time to identification (hours)	Impact on earlier appropriate therapy (hours)	Decrease in hospital length of stay (days)	Decrease in ICU stay (days)	30-day readmission rate with same bloodstream infection (%)	Hospital cost avoidance per patient
Methodist Hospital Houston, TX [Perez et al. (11)]	Gram-negative bacteremia (n = 201)	22.7	75 vs 29 (P = 0.004)	11.9 vs 9.3 (P = 0.01)	7.3 vs 6.3 (P = 0.05)	Not reported	$19,547
University of Michigan [Huang et al. (10)]	Aerobic Gram-positive organism, Gram-negative organism, or yeast (n = 501)	28.1 (P < 0.001)	90.3 vs 47.3 (P < 0.001)	14.2 vs 11.4 (P = 0.06)	14.9 vs 8.3 (P = 0.014)	3.5 vs 1.6 (P = 0.262)	$19,253

proach was originally proposed by Zymark Corporation (Zymark Corp, Laboratory Robotics Handbook, 1988) and later modified as a simple go/no-go approach to use prior to investing time in a more detailed justification later (8, 9). An EJI of >1 (meaning less than 1 year for ROI) will most likely occur in very large-volume laboratories where the business case is less problematic. As a rule of thumb, companies like to see an ROI with a payback period of less than 4 years, but this may vary given what a company uses for expected lifetime of technology. Administrators may have several good ROI proposals presented to them, so undoubtedly, the shorter the ROI, the better. For the most part, a stronger EJI is more compelling than a stronger SJI for the simple reason of being able to quantify the tangible benefits of a business case. If the combined EJI and SJI of a project is low (e.g., 0.25), this would equate to only marginal justification, while an SJI and EJI score of 0.75 to 1 would equate to a much more compelling justification (Fig. 1). In the end, these calculations provide a simple tool for determining if a project is worth the more detailed organization-specific analysis, while providing technical staff a means to quantify to nontechnical staff the justification of the laboratory needs.

The total cost of ownership is another way to quantify the financial impact of deploying a new technology over the expected lifetime of the system. Of course, the gains quantified in the annual payback can rapidly disappear as equipment ages and becomes outdated. There is a price tag associated with maintaining and upgrading equipment annually, and while this is difficult to estimate, it is beneficial to include it in the planning process.

The strategies highlighted in this chapter can be leveraged by laboratorians to gain administrative approval. The key to a successful business case is to know how to present future value to the organization and quantify the benefits. With any new technology, a lack of published clinical impact studies will result in limitations to assessing costs beyond the laboratory.

OUTCOME INDICATORS: MEASURING THE PROMISE OF TECHNOLOGY

Laboratory personnel are typically excellent at ensuring that the analytical portion of laboratory testing is done correctly. However, because of the additional demands and priorities placed on staff, often once the efforts to get technology in place have been achieved, attention is focused elsewhere. When it comes to the laboratory, new cutting-edge technology is desired, but justification for the incremental expense may be difficult, unless the value is demonstrated over the continuum of patient care.

Outcomes should be measured on both the laboratory and clinical side of the balance sheet. Historically, only the laboratory outcomes have been calculated and typically more readily available than the clinical outcome data. Metrics for the laboratory consist of (i) reagent costs, (ii) labor costs, (iii) turnaround time, and (iv) impact to operations (i.e., "leaning" of operations). Clinical metrics, which may vary depending on setting (inpatient versus outpatient), can include (i) length of stay, (ii) number of diagnostic tests, (iii) antibiotic/antiviral utilization, (iv) rate of relapse, (v) mortality, and (vi) return visits (5). An example of an executive summary of clinical outcomes from published clinical impact studies for MS ID is presented in Table 2 (10, 11). By combining the impact of the laboratory outcomes with the clinical outcomes, a more persuasive ROI can be presented. Table 3 illustrates how published outcome data can be used in a business case to strengthen the ROI.

As a result of the enactment of the Affordable Care Act, laboratories are being held accountable for ensuring that the technology that is presumably advancing medicine is, in fact, improving patient care and the overall cost of care. To

TABLE 3 ROI of mass spectrometry identification of bacteria and yeast with outcome analysis

Assay	Estimated hospital saving/patient (25% of published savings)[a,b]	No. of patient interventions to break-even point	Total estimated hospital savings (n = 245)[a]	Total estimated laboratory and hospital savings	ROI
Mass spectrometry identification	$5,000	40	$1,225,000	$1,318,020	<6 months

[a]Huang et al. (10).
[b]Perez et al. (11).

do so, the laboratory must now look at clinical outcomes. These changes require the analysis of standardized metrics to guarantee that everyone is using the same ruler to measure outcomes, especially if performance is compared across institutions (or peer groups). Despite advances with electronic medical records, the mining of data from them is still in its infancy and remains a challenge for many institutions. Outcome data need to be readily available to the laboratory to track the implementation of new technologies, rather than having to do labor-intensive chart reviews. The role of an analyst in laboratory medicine, who can mine data to look at outcomes and utilization, is increasingly more important.

When presenting a business case to senior-level administration, it is useful to prepare a one-page document that includes the following elements: (i) the initiative name and business purpose; (ii) the value proposition, opportunity, and problem to be solved; (iii) any savings along with vetted estimates; (iv) income statements with net revenues, expenses, and profits for years 1 to 5, especially if a contribution margin is described; (v) projected savings by year, if the initiative focuses on cost savings; (vi) the required dollar investments for years 0 through 5 and the total investment; (vii) the ROI; and (viii) the timeline for launching the new initiative. On the day of presentation, it is very likely that senior executives will have a full agenda of speakers so the time allotted for each speaker will be limited. Speakers should practice their delivery, stay within the given time table, and avoid rambling. It is important to stay on message. Senior leaders will have questions about the business case, so be prepared with answers and know that pushback does not mean "No." Administrators will articulate what they need, thus as analysis becomes more comprehensive, be prepared to demonstrate support from other hospital services. No longer is the business case a one-stop decision process. Finally, speakers should express passion and confidence for the proposed project because this engenders confidence from the C-suite that the project will be successful.

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Molecular Diagnostics and the Changing Legal Landscape

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56

ROLE OF INTELLECTUAL PROPERTY

Molecular diagnostics is poised to transform health care in the coming decade. The molecular diagnostic industry is quickly evolving because of many factors including success with the companion diagnostic model, the rapid advance of gene sequencing and other detection technologies, the Affordable Care Act, and the Supreme Court's reworking of the boundaries of patentable subject matter. As a result, companies in this space must learn to navigate a quickly evolving industry and drastically changed legal framework if they are to be successful in protecting their innovations and investments.

For example, successes with the companion diagnostic model, particularly in oncology, place diagnostics as a valuable piece to increasing the success rate of clinical trials and managing drug life cycles within the pharmaceutical industry. A companion diagnostic test is one that is codeveloped with a therapeutic candidate, and when successful, companion diagnostics can improve therapeutic outcomes by providing tailored patient profiles for therapy, including identifying patients at risk of serious adverse reaction, and monitoring response to treatment (1–3). These diagnostic tests may not only identify patients that require treatment, but also identify those that do not, thereby reducing the number of patients treated with ineffective or potentially toxic therapies, while controlling health care costs. For example, biomarker-guided therapies for non-small cell lung cancer (NSCLC) were estimated to have a 62% success rate in phase III clinical trials as compared to about a 28% overall success rate from 1998 to 2012 (1). Given this success, it is essential that both the diagnostic services industry and the pharmaceutical industry capture the value of companion diagnostic tests.

Other factors that drive the value of the diagnostic industry include rapid advances in gene sequencing technologies and the changing U.S. health care system. For example, with the rapidly decreasing cost of nucleic acid sequencing, there is enormous potential for advanced genetic testing to diagnose illness and provide personalized treatment. This is particularly true for diseases with substantial genetic heterogeneity (3). However, cost, throughput, and accuracy still remain as hurdles, and thus innovative sequencing platforms and improvements can encourage further adoption of such tests in the industry. While as of this writing there are no FDA-approved tests based on next-generation sequencing, there are several gene-sequencing technologies under development that promise to place vast amounts of personalized genomic data at our fingertips. Intellectual property will have an important role in protecting successful innovations on this front. A widely adopted sequencing platform that serves as the basis for clinical genetic tests would likely be a lucrative technology if a successful intellectual property strategy were implemented with it.

As the Affordable Care Act is implemented, the market for molecular diagnostics is likely to expand substantially as well as evolve due to the increase in the insured population and increasing focus on disease prevention. Pricing and reimbursement for diagnostic tests will remain as significant issues for the industry, but ideally, pricing and reimbursement will be tied to the value of any diagnostic test to its actual value to patient care. A well-executed intellectual property strategy will help capture pricing advantage in an otherwise very cost-sensitive environment (Park R, Trends in the U.S. IVD Market: Medicare Reimbursement and the Affordable Care Act, http://www.mdtmag.com/blogs/2014/05/trends-us-ivd-market-medicare-reimbursement-and-affordable-care-act, 2014).

As in other industries, intellectual property plays a key role in realizing the value of diagnostic technologies. Understanding the value of patent assets to the overall health care industry—for example, understanding the value to the diagnostic industry as well as the pharmaceutical industry—is the key to maximizing intellectual property value. This chapter provides a brief review of the patent process and how it applies to molecular diagnostics, provides a review of the new framework for determining patentable subject matter in the United States, and provides guidance on navigating this framework to maximize the value of diagnostic intellectual property.

OVERVIEW OF INTELLECTUAL PROPERTY CONCEPTS

There are four main categories of intellectual property: patents, trade secrets, trademarks, and copyrights, with patents and trade secrets being the most crucial to the diagnostic industry.

A trade secret includes any confidential information, material, or instrument that a business may use to gain

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commercial advantage over competitors. Trade secrets provide significant advantages to molecular diagnostics companies to the extent that the confidential technology is not easily reverse-engineered from available information and/or is unlikely to be independently discovered. Examples of trade secrets in the molecular diagnostic context can include biomarker algorithms, biomarker-associated clinical data, proprietary materials such as antibodies that are not easily accessible or re-created, and clinical tissue collections. A trade secret may be enforced indefinitely, provided that the information does not become publicly known and the requisite safeguards are in place to maintain the confidential or proprietary nature. These safeguards include the use of nondisclosure agreements, noncompete agreements, and restricting access to the confidential material or data. Significantly, while patents, trademarks, and copyrights are based on federal laws, trade secrets are enforced at the state level.

Patents are the foundational intellectual property protecting advances in the molecular diagnostic industry. Patents in this industry provide exclusive rights for molecular diagnostics companies to market diagnostics instruments, kits, and services, and as we will discuss in the last segment, can also provide exclusive rights to market a drug for a biomarker-defined patient population. In the United States, there are three types of patents: utility patents, design patents, and plant patents. While utility patents protect the functional attributes of an invention, design patents serve to protect the ornamental aspects of an invention. Utility patents are the most common type of patent application filed with the United States Patent and Trademark Office (USPTO) and are the type of patent most relevant to diagnostics. The following paragraphs present an overview of patent concepts with a focus on the U.S. system and how these concepts pertain to diagnostic inventions.

By granting exclusive rights to the patent owner, the patent system intends to encourage innovation and investment into new and useful technologies. In exchange for this exclusive right, the patent owner is required to publicly disclose the invention by filing a patent application with the USPTO that both describes the invention and enables others to practice it. A patent awarded in the United States allows the patent owner (or in some instances an exclusive licensee) to exclude others from making, using, offering to sell, selling, or importing a patented invention in the United States during the term of the patent. As such, a patent does not give the patent owner the right to practice the invention *per se*, but rather gives the right to prevent others from practicing the claimed invention. The term of a patent is 20 years from its earliest filing date. For some U.S. patents, the term of the patent may be adjusted or extended. For example, patent term adjustments are available if the USPTO fails to examine a patent application in a timely manner and/or fails to timely issue the resulting patent, although these adjustments are offset by the applicant's own delays during the examination process. Other term extensions are available to the pharmaceutical industry for drug patents based on time lost in clinical trials.

Patent applications are allowed and then granted by the USPTO through a process known as "patent prosecution." In the United States, this process often starts with the filing of a provisional patent application that provides a one-year period of pendency before the drafting of a formal nonprovisional utility application. The one-year period does not count toward the resulting patent's 20-year term. If there is a series of applications that claim priority to a provisional or a nonprovisional application, the 20-year clock begins with the date of filing of the earliest file nonprovisional application. The basic elements of a utility patent application include the specification, which includes a description of the invention and ideally experimental data to demonstrate it, as well as one or more claims that define the legal boundaries of the exclusive rights to which an applicant or applicants believe they are entitled. The patent application is examined by a technically trained patent examiner who reviews the claims, including their scope and precision of language, for compliance with all requirements for patentability. The final scope and content of the claims is determined through one or more rounds of negotiation between the patent examiner, the applicant, and the applicant's patent counsel. In some instances, the USPTO may not allow some or any of the claims. Should that happen, the applicant may choose to abandon the application or appeal the rejection of one or more of the claims.

To be successful in obtaining a patent, the patent applicant must present a set of claims that overcome four substantive hurdles for patentability: (i) patentable subject matter, (ii) utility, (iii) novelty, and (iv) nonobviousness. These are in addition to the requirement to describe and enable practice of the invention to such an extent that another person working in the same field would understand the invention and be able to practice it without undue effort.

The patentable subject matter requirement defines the types of inventions that will be considered for patent protection. Under 35 United States Code (U.S.C.) §101, patentable subject matter includes "any new and useful process, machine, manufacture, or composition of matter, or any new and useful improvement thereof." Historically, the Supreme Court has constructed these categories to be quite broad to include "anything under the sun that is made by man." However, the Court has always recognized that 35 U.S.C. §101 has limits and does not embrace every discovery or process. Specifically, the Court has long held that laws of nature, physical phenomena, and abstract ideas are not patentable subject matters. These are often referred to as the "judicial exceptions" from patentable subject matter. In practice, there is an indistinct line between abstract ideas or laws of nature on one hand and patentable matter or methods on the other. This distinction is at the heart of molecular diagnostic patent practice, and will be discussed in "Molecular Diagnostics and the Boundaries of U.S. Patentable Subject Matter," below.

The second requirement for patentability is that the invention be useful. See 35 U.S.C. §101. This is generally a low threshold and merely requires that the application disclose at least one use that is "specific, substantial, and credible" [United States Patent and Trademark Office "Manual of Patent Examining Procedure" (MPEP) 2107 (II)(a) and (II)(c)]. The utility requirement does not usually impact cases in the molecular diagnostic arena. But, for example, identification of a molecule of unknown or unclear function (such as a newly discovered microRNA), without any correlation of the biomarker to patient condition or disease state, would likely lack utility under current law, such that the molecule or methods of its detection would not be patentable in the United States.

In order to receive a patent, the invention must also be new. Specifically, under the Leahy-Smith America Invents Act (AIA) of 2011, novelty requires that the invention

was not in public use, on sale, described in a printed publication, or otherwise available to the public anywhere in the world prior to the effective filing date of the patent application. See 35 U.S.C. §102(a)(1). The invention must also not have been described in an earlier-filed patent application naming a different inventor. See 35 U.S.C. §102(a)(2). The AIA significantly amended the patent laws of the United States particularly with respect to the novelty requirement. For example, under the prior law, an inventor could file a U.S. patent application up to one year after the date of various types of disclosures of the invention, including in a printed publication made by the inventor or even a sale of a product embodying the invention. Although a 1-year "grace period" still exists for disclosures made by the inventor, the question of whether a grace period continues to exist for all disclosures, such as sales, is debatable. Regardless of the type of disclosure made by the inventor, the sensible course of action is to file a patent application describing the invention prior to engaging in these acts, thus preventing these acts from serving as a bar to patentability should a U.S. court one day take the opposite view. This course of action will also preserve patentability in countries that do not provide for any grace period after a public disclosure.

Another significant change the AIA brings to U.S. patent law is a switch from a "first-to-invent" system to a "first-inventor-to-file" system. Before AIA, an inventor in the United States could spend the time needed to perfect an invention before filing a patent application and during examination of the application he or she would be given credit for an earlier invention date based upon evidence of the invention date submitted to the patent office. Under the new system, an inventor who waits to file an application risks losing his or her patent rights to an inventor who invented later but filed first. This aspect of AIA does away with the previous Patent Interference system, which had been a unique aspect of U.S. patent practice. Therefore, under AIA, diligent and timely patent filings are a critical component to any patent strategy to avoid losing rights to others that win the race to the patent office.

The fourth hurdle to patentability is the nonobviousness requirement. The test for nonobviousness is whether the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious to a person having ordinary skill in the art at the time the invention was made. See 35 U.S.C. §103. Accordingly, a patent may be denied if the invention was of such minimal creativity that it would have been obvious to someone of ordinary skill in the relevant art. For example, as explained by the Supreme Court in *KSR International Co. v. Teleflex Inc.*, 550 U.S. 398, 82 USPQ2d 1385 (2007) ("*KSR*").

> [w]hen a work is available in one field of endeavor, design incentives and other market forces can prompt variations of it, either in the same field or a different one. If a person of ordinary skill can implement a predictable variation, §103 likely bars its patentability. For the same reason, if a technique has been used to improve one device, and a person of ordinary skill in the art would recognize that it would improve similar devices in the same way, using the technique is obvious unless its actual application is beyond his or her skill. *Id.* at 417, 82 USPQ2d at 1396.

As mentioned above, a patent is a property right, and the essence of this right is exclusive use. As such, a patent owner may rightfully seek remedies against another party for unauthorized use of the invention. Specifically, a patent may be enforced against another party for direct infringement, inducement to infringe, or contributory infringement. A party directly infringes a patent by making, using, selling, offering to sell, or importing an article or process covered by at least one claim of the patent. For example, performing a diagnostic test in the United States that is covered by another's U.S. patent, without authorization from the patent owner, is direct infringement. A party "induces" infringement by promoting direct infringement. For example, selling a reagent that is useful in a particular diagnostic test, while promoting this use of the reagent in the market, might be found to induce infringement where the particular diagnostic test is the subject matter of an issued U.S. patent. In addition, a party "contributes" to infringement by selling, offering to sell, or importing a material component of a patented product knowing it to be especially made or adapted for infringement and that it has no substantial noninfringing use. There are additional bases for establishing patent infringement based on importation of products, including where the product is made outside the United States by a process patented in the U.S. 35 U.S.C. §271(g). Infringement is ultimately decided by a court and potential remedies include an injunction to compel an infringer to cease its activities and/or pay monetary damages to the patent owner.

On the other hand, the validity of a patent may be challenged in court by a defendant in a patent litigation suit or by a party that has been threatened by such a suit. Alternatively, the patentability of any claims of an issued patent may be challenged at the USPTO through various procedures including reexamination, *inter partes* review, and post-grant review. These procedures differ in the types of unpatentability positions that may be asserted by a third party, and they vary in the level of the third party's participation. Particularly, it is common in patent infringement suits for the accused infringer to assert that the patent in suit is invalid and thus its infringement should not incur liability to the patent owner. Invalidity of the patented claims is established for each claim independently by showing that any one of the statutory requirements for patentability as described previously is not satisfied.

MOLECULAR DIAGNOSTICS AND THE BOUNDARIES OF U.S. PATENTABLE SUBJECT MATTER

The landscape of what may be claimed in a patent continues to evolve, particularly in the United States. Certain types of patent claims that were once commonplace for issuance are no longer considered to be "patentable subject matter" or enforceable in view of a handful of U.S. Supreme Court decisions. In this section, we will dissect these decisions that narrow the scope of patentable subject matter, and we will review the basics of a viable patent strategy under the new legal framework.

The Supreme Court reworked the scope of patentable subject matter in the cases of *Mayo Collaborative Services v. Prometheus Labs., Inc.*, 132 S. Ct. 1289 (2012) ("*Prometheus*"), *Ass'n for Molecular Pathology v. Myriad Genetics, Inc.*, 132 S. Ct. 1794 (2012) ("*Myriad*"), and *Alice Corporation Pty. Ltd. v. CLS Bank International*, Case No. 13-298 (Supr. Ct., June 19, 2014) ("*Alice*"). The first two decisions directly impact molecular diagnostics patents, while the third relates to the patenting of software and impacts computer-implemented diagnostic tests, such as those that

are common for gene expression tests, among others. The basis for patents in the diagnostic field, as well as a few other industries, has been substantially limited by the Supreme Court through these decisions in an apparent effort to "untangle" the patent landscape (*Myriad*).

Patent claims to biomarkers or reagents as merely isolated components, even claims that have already been blessed by the USPTO and granted in an issued patent, can now run afoul of the patentable subject matter boundaries as recently interpreted by the Supreme Court. The Supreme Court invalidated claims in patents owned by U.S.-based Myriad Genetics, Inc., which had developed assays that identify mutations in human *BRCA1* or *BRCA2* genes. A small but significant percentage of human breast and ovarian cancer cases are associated with those mutations. A mutation in either of these genes results in a syndrome called hereditary breast and ovarian cancer syndrome and is associated with a significantly increased risk of developing cancer in those tissues (http://www.myriad.com) (4–6).

After researchers at Myriad developed a molecular diagnostics assay to determine whether a patient carries either *BRCA1* or *BRCA2* or both gene mutations, Myriad filed numerous patent applications with a combined total of over 500 patent claims.

The first two claims of Myriad's U.S. patent 5,747,282 serve as useful examples for what is now considered patent-ineligible material under *Myriad*:

1. An isolated DNA coding for a BRCA1 polypeptide, said polypeptide having the amino acid sequence set forth in SEQ ID NO:2.
2. The isolated DNA of claim 1, wherein said DNA has the nucleotide sequence set forth in SEQ ID NO:1.

When these claims were filed, Myriad was following a long-standing practice to file and have granted claims to isolated biomarkers or biological reagents (such as DNA or antibodies) since it was generally held by U.S. courts that *the act of isolating naturally occurring molecules* set the composition apart from that which occurred in nature. In fact, this premise was set by decades of court decisions on the topic. Thus, historically, defining DNA, protein, peptide, or other biomarker as "isolated" or "purified" in the patent was sufficient to distinguish the composition from naturally occurring matter.

This all changed on June 13, 2013, when the Supreme Court issued a decision in *Association For Molecular Pathology v. Myriad Genetics, Inc.* ("*Myriad*") and unanimously held against certain of Myriad's issued patent claims, and in particular, claims to "a naturally occurring DNA segment," finding that such compositions are "a product of nature and not patent eligible merely because it has been isolated." The Court made their position clear with the statement: "In this case, . . . Myriad did not create anything. To be sure, it found an important and useful gene, but separating that gene from its surrounding genetic material is not an act of invention." The decision appears to impact any "isolated" molecule from nature, and so far, the USPTO has taken this position by including "nucleic acids . . . proteins and peptides; and other substances found in or derived from nature" as natural products that are subject to the same analysis set forth in *Myriad*.

According to the Supreme Court, there must be some structural distinction of a molecule from that which exists in nature (beyond just isolation) to place it within the realm of patentable subject matter. The message that can be gleaned from the Supreme Court decision is that patent claims to biomarkers or naturally occurring reagents as compositions (such as oligonucleotides, proteins, and antibodies) must now include some nonnatural feature. Exemplary modifications to a natural molecular structure that would likely bring the composition within the realm of patentable subject matter include a detectable label, conjugation or coupling to components of a detection system such as a bead or array, heterologous sequences including sequences that control expression, and nucleotide or amino acid substitutions from the natural sequence. As a result of the *Myriad* decision, a patent application intending to claim reagent components that are naturally occurring must do more than simply describe the sequence or structure of the new biomarker or reagent and its diagnostic utility; it must describe various manners in which it could be implemented in a detection assay or in a kit with a combination of assay components, with as much detail as possible. Of course this adds a layer of complexity to getting valuable patent claims that are not designed around by mere selection of a noninfringing detection platform.

On the same day of the *Myriad* decision, the USPTO issued a memorandum that contains this preliminary guidance to its patent examiners:

> As of today, naturally occurring nucleic acids are not patent eligible merely because they have been isolated. Examiners should now reject product claims drawn solely to naturally occurring nucleic acids or fragments thereof, whether isolated or not, as being ineligible subject matter under 35 U.S.C. § 101. Claims clearly limited to non-naturally-occurring nucleic acids, such as a cDNA or a nucleic acid in which the order of the naturally occurring nucleotides has been altered (e.g., a man-made variant sequence), remain eligible.

However, a patent applicant should take note that even cDNA is not patent eligible without a clear distinction from naturally occurring genetic material, as the Supreme Court indicated in *Myriad*:

> cDNA is not a "product of nature" and is patent eligible under §101, except insofar as very short series of DNA may have no intervening introns to remove when creating cDNA. In that situation, a short strand of cDNA may be indistinguishable from natural DNA.

The USPTO further clarified, and broadened, its guidance to its Patent Examining Corps in a memorandum of March 4, 2014 (A.H. Hirshfeld. "2014 Procedure for Subject Matter Eligibility Analysis of Claims Reciting or Involving Laws of Nature/Natural Principles, Natural Phenomena, And/Or Natural Products"; http://www.uspto.gov/patents/law/exam/myriad-mayo_guidance.pdf). According to the USPTO, patent claims will not be allowed for abstract ideas, laws of nature/natural principles, natural phenomena, and natural products. According to the memorandum, a natural product includes but is not limited to nucleic acids, proteins, peptides, and other substances found in nature. Thus, as a result of *Myriad* and USPTO interpretation of the decision, claims in patent applications to nucleic acids, proteins, and peptides are being rejected if they are identical in sequence to naturally occurring molecules, including short sequences that do not include any clearly synthetic component.

Thus, the mere term "isolating" or "purifying" will not sufficiently distinguish a composition from a product of nature. As a general rule, the more a composition (as

described in the patent) has clear aspects of human intervention or ingenuity, the lower the likelihood that an inventor will face a difficult rejection based on *Myriad*. For example, the specification may include a description of recombinant or synthetic construction of polynucleotides, or, if appropriate, various modifications of a sequence (e.g., mutations, chemical modifications, or fusions with heterologous sequences) that would likely define and support patent eligible subject matter. A description in the specification of the patent application should include how the recombinant polynucleotide structurally differs from a naturally occurring molecule. The recombinant polynucleotide may contain a promoter region that regulates the expression of a heterologous nucleic acid of interest, or the nucleic acid of interest may contain other heterologous components such as a reporter gene.

Myriad itself may have followed this example in its claims to an expression system that comprises isolated DNA that is operably linked to a suitable control sequence. These claims were not invalidated by the Supreme Court decision, although defining the regulatory sequence and the DNA as mutually heterologous, such that they do not naturally occur together in any organism, would have made for a stronger claim. Claims to host cells comprising the same expression system and several method claims were also not found to be invalid under 35 U.S.C. §101.

In summary, when preparing a patent application to protect an invention based on a molecule found in nature, it is important to describe all the ways that the molecule could be designed or manipulated to be distinct from the naturally occurring molecule, with a focus on how the molecule will be employed in a commercial context. These descriptions may include vectors, fusions, and labels; components of a detection system such as an array, bead, or sensor; or even a kit including a series of naturally occurring molecules that do not occur together in a natural setting.

Mayo Collaborative Services *v. Prometheus* Laboratories, Inc.

While *Myriad* raises the bar for protecting biomarkers and reagents as compositions through patents, "*Mayo v. Prometheus*" or simply "*Prometheus*," raises the bar for the diagnostic method claims. Prior to *Prometheus* it had become commonplace to obtain patent coverage for diagnostic methods that involve testing for one or more biomarkers in patient samples, and on the basis of these measurements, making correlations to patient condition. *Prometheus* established that even these claims are invalid if the "activity" recited in the claims could be viewed as conventional or routine in the art. In an era in which most genetic markers are embodied in some manner on commercially available arrays or other types of detection platforms, *Prometheus* leaves far less room for diagnostic method claims to be patented.

The facts behind the dispute in *Prometheus* concern the use of thiopurine drugs as "an effective treatment for inflammatory bowel diseases such as Crohn's disease and ulcerative colitis" (U.S. patent 6,680,302) (7) The administered thiopurine drugs at issue include azathioprine, which is nonenzymatically converted to 6-mercaptopurine *in vivo*, and 6-mercaptopurine. However, a small percentage of patients are deficient in the enzyme thiopurine methyltransferase and they accumulate 6-thioguanine nucleotide, a cytotoxic metabolite of 6-mercaptopurine. Monitoring the level of 6-thioguanine in patients undergoing treatment with a thiopurine drug is essential to avoid serious side effects that can occur in the minority that are deficient in thiopurine methyltransferase (U.S. patent 6,680,302) (7).

Prometheus was the exclusive licensee of U.S. patents 6,355,623 and 6,680,302, which contain only method claims. An exemplary claim of patent 6,680,302 is the following:

> A method of optimizing therapeutic efficacy for treatment of an immune-mediated gastrointestinal disorder, comprising:
> (a) administering a drug providing 6-thioguanine to a subject having said immune-mediated gastrointestinal disorder; and
> (b) determining the level of 6-thioguanine in said subject having said immune-mediated gastrointestinal disorder. . ."
> wherein the level of 6-thioguanine indicates a need to increase or decrease the amount of the drug subsequently administered to the patient.

Mayo Collaborative Services provided a testing service for patients treated with thiopurine drugs, and at one point Mayo began to produce and use its own diagnostic assays for that purpose. Prometheus sued Mayo for infringement in the Southern District Court of California, which ruled in favor of Mayo. The court's reasoning was that the patent claims were invalid because they amounted to nothing more than "data-gathering" followed by an unpatentable mental step (an abstract idea).

Ultimately, the case found its way to the Supreme Court, which, on March 20, 2012, unanimously concluded that the Prometheus claims were unpatentable. According to the Supreme Court in *Prometheus*:

> [b]ecause methods for making such determinations were well known in the art, this step simply tells doctors to engage in well-understood, routine, conventional activity previously engaged in by scientists in the field. Such activity is normally not sufficient to transform an unpatentable law of nature into a patent-eligible application of such a law.

In other words, the Supreme Court affirmed that the methods claimed in the two patents were merely routine or conventional biomarker detection steps together with a statement of a natural correlation and were thus insufficiently transformative to be considered patentable subject matter. In its decision, the Supreme Court made clear that something "more" than data-gathering and a mental step tailoring the treatment based on what the data reveal is required to render the claims patentable. The *Prometheus* decision has left the USPTO and the patent community trying to distinguish the Supreme Court's new test for patentable subject matter from a novelty/nonobviousness analysis, since the Supreme Court has now incorporated elements of what is "well understood, routine, conventional activity previously engaged in by scientists in the field" into the test for patentable subject matter.

After *Prometheus*, the USPTO provided its "interim guidance" in a memorandum to its patent examiners (http://www.uspto.gov/patents/law/exam/2012_interim_guidance.pdf) in an attempt to place a consistent framework behind the *Prometheus* analysis. In this memo, the USPTO takes the official position that a process claim should be examined by asking three questions:

(1) Is the claimed invention a process?
If the answer is no, this analysis is not applicable. If yes, proceed to question 2.

(2) Does the claim focus on the use of a "natural principal", that is, a law of nature, a natural phenomenon, or naturally-occurring relation or correlation?
If the answer is no, this analysis is complete and the claim should be analyzed to determine if an abstract idea is claimed. If yes, proceed to question 3.
(3) Does the claim include the "additional steps" or element combinations that move the invention beyond the natural principal, as demanded by the *Prometheus* decision?
If the answer is no, the claim is not patent-eligible and should be rejected. If yes, the claim is patent-eligible, and the analysis is complete.

Of course, what is meant by "additional steps" or element combinations that move the invention beyond the natural principal is subjective. The memorandum attempts to provide guidance with several examples. One example is a claim to the disinfecting property of sunlight. A process claim focusing primarily on such a natural principal would be rejected if it does not introduce an element of human intervention or ingenuity such as additional steps that "could involve constructing a sanitizing device that uses ultraviolet light for disinfection with steps that integrate the ultraviolet light into the device." A second example is based on the relationship between blood glucose levels and diabetes as a natural principle. According to the memorandum, a claim that sets forth the relationship between blood glucose levels and the incidence of diabetes would require additional steps that do significantly more to apply this principle than conventional blood sample testing or diagnostic activity based on recognizing a threshold blood glucose level. Such additional steps could involve a testing technique or treatment steps that would not be conventional or routine.

While the USPTO guidelines certainly clarify the relatively simple scenario in which the diagnostic method involves a novel or unconventional device or reagent (these claims are still patentable subject matter), the USPTO guidelines provide very little to clarify the majority of situations that involve detection of known genes or known biomarkers using commercially available tools and analyzing this information in new and nonobvious ways. In these cases, it will most likely be necessary to rely on a combination of features, even if these features are routine and conventional in isolation. For example, patentable subject matter might be found where the claim recites some combination of the following features:

1. a patient subpopulation in which the prior art does not specifically teach detection of the biomarker(s) of interest.
2. a particular detection platform or reagent that is adapted for use with the diagnostic test, or which is not conventionally used with the biomarker(s) of interest, such as a custom microarray containing a set of probes for detecting the particular genes of interest, or an antibody that is defined by a particular epitope that is important to the method.
3. a computer-implemented algorithm trained on biomarker measurements, for example, gene expression measurements tied to clinical patient information; and
4. a subsequent method step for treating a patient (or withholding treatment) based on the information from the test such as an administration step that maintains the drug's level, or more specifically maintains the level within a specified range.

While this is just an exemplary list, the point is to find some combination of features that, even if commonly used or routinely used in isolation, cannot be shown to have been used together. While tests that rely on computational aspects, such as a computer-implemented algorithm, may have an advantage where the algorithm is sufficiently defined, it is critical not to rely on generic language merely implicating a computer in the process, since the Supreme Court has weakened this approach in its decision in *Alice Corp. v. CLS Bank International*. In *Alice*, the Supreme Court found that certain claims to a computer-implemented, electronic escrow service for facilitating financial transactions were invalid as being directed to an abstract idea. Merely reciting that the process was performed on a computer was not sufficient to transform the claims to patent eligible subject matter.

Because disclosures cannot be added to patent applications after filing, it is important that the applicant consider describing all of these factors that potentially relate to unconventional or nonroutine activity, either in isolation or in combination with other features of the claim. By describing all aspects of the method in as much detail as possible, there will be many more opportunities to distinguish the method from a mere "natural law" according to the *Prometheus* decision. Because the USPTO's current guidelines do not provide much guidance past methods that incorporate clearly novel and unique reagents or devices, it is important to prepare for this unpredictability with an extremely detailed patent application that does not rely on generic statements of patient population, biomarkers sets, reagent sets, computational aspects, and diagnostic decision-making, but instead sets forth detailed descriptions for each, including various alternative embodiments.

MOLECULAR DIAGNOSTICS INTELLECTUAL PROPERTY VALUATION

It is rarely straightforward to place a discrete value on patent assets. This is certainly the case in the molecular diagnostic industry, because the role that patents play, and hence their value, varies widely. Further, while historically broader platform-type patents have more value over narrower implementation-type patents, the Supreme Court's recent interpretation of Section 101 places a risk of invalidity over broad patents in the diagnostic space. Thus, valuating patents in the diagnostic space is particularly difficult.

For the sake of convenience, we will discuss diagnostic patents in three buckets: patents that cover (i) laboratory services, (ii) patents that cover diagnostic test kits and consumables, and (iii) companion diagnostic tests (laboratory services that are tied to approval of a drug product).

Patents that cover laboratory services, which once had been quite valuable to this sector, are more difficult to valuate now under the interpretation of patentable subject matter set forth in *Prometheus*. Broad diagnostic claims that control the use of a biomarker for a particular diagnostic test will be at risk of invalidity upon third party challenge in the courts or USPTO; this is despite their apparent dominating position otherwise. On the other hand, narrower patents, while they are more easily defensible, will be at risk of design around by competitors. Thus, the

scope of patents in this space must be carefully balanced with their ease of enforcement under the new interpretations of patentable subject matter.

Diagnostic method patents can be licensed or provide exclusivity for a Clinical Laboratory Improvement Amendments (CLIA) lab test, and can be valued based on the income produced by the test, which often must be priced for reimbursement by the ultimate payer (e.g., the insurance company or Medicare). This pricing will in turn be related to one or more of the following: the cost of alternative diagnostic means, the availability and cost of therapy for the condition tested for, and the ability to identify patients that do not actually need the expensive therapy. These savings in diagnosis or in therapy will largely translate to the value of the diagnostic test.

Prior to commercialization or market penetration, the type of test will likely also impact the value of the intellectual property. For example, is the test based on a well-accepted platform that has fewer industry barriers, such as a gene expression or DNA sequencing-based test? Or, is the test in niche area that has yet to be fully accepted by industry? These factors relate to the barriers for commercialization and market penetration and should be considered where the test is not yet commercialized.

Patents that cover commercial diagnostic kits and consumables have a similar valuation issue after the *Myriad* decision, in that components of the kit or product that are essentially isolated biological molecules or fragments of natural biological molecules will be more difficult to protect through U.S. patents in the absence of a feature that is markedly different from the natural molecule. Commercially, some tests are embodied in kits or cartridges for a testing system. Patents can certainly be framed to cover kits and cartridges (without controversy!). The value of the patent assets in this situation is largely based on their ability to protect sales of the kit or test consumables. The value of the patent assets is then proportional to the price and profitability of the kit or consumables and their ability to prevent similar tests from being marketed. This is, for example, an important model in the point-of-care diagnostic market.

Patents that cover true companion diagnostics, and therefore are connected commercially with pharmaceutical products, should be viewed quite differently. Molecular diagnostic patents that protect a companion diagnostic test are critical to the exclusivity position of the underlying drug product and can have a value based on the test's contribution to supporting the revenue of the pharmaceutical product. Specifically, a companion diagnostic test can provide for life cycle management for the corresponding drug product and thereby have an important role in extending the original drug product exclusivity. More particularly, a patent claiming the companion diagnostic test, for example, in the form of a method can help prevent early generic entry and provide an advantage to a branded drug that is marketed with the companion diagnostic. It will be difficult for a generic to gain FDA approval and enter the market without infringing or otherwise invalidating or designing around the diagnostic patent. In these instances, the value of the diagnostic patent assets is based in part on the ability to extend initial market exclusivity for the drug.

Alternatively, the diagnostic test may help a second-generation product surpass the efficacy or safety of a first-generation product and thereby help the second-generation product penetrate the market. In some cases, a companion diagnostic may save a clinical candidate that is otherwise too ineffective for a more broadly defined patient population, thereby securing a very important and valuable role in the original product exclusivity position. In any of these instances, the value of the diagnostic patent is related to the value added to the underlying pharmaceutical product.

Given any of these possibilities, and given the difficult patent law framework for purely diagnostic claims, it is important to claim diagnostic innovations as methods of treatment where possible. For example, claims that recite a "method for treating" a patient population defined by a diagnostic marker not only have potential to escape significant *Prometheus*-based scrutiny but also have the ability to capture more value to the pharmaceutical industry since such claims can be listed in the Orange Book (and are thus useful for protecting against early generic entry). The purely diagnostic claims remain important for licensing an exclusive diagnostic test to a commercial partner, and the two are complementary strategies for capturing the value of companion diagnostic inventions.

Finally, while there are an enormous number of detection platforms known and continually being developed, which often renders the detection platform and its intellectual property as a mere commodity, a truly innovative detection platform that changes the pricing, accuracy, or throughput dynamics of the industry are potentially lucrative. This might be the case, for example, in some of the DNA sequencing technologies currently being developed. However, in most cases, while the implementation and detection platforms can offer significant value if patented, it is the overall biomarker and its diagnostic information that is historically the most valuable patent asset, and of course, now the most difficult to protect with patents.

By having a thorough understanding of how a diagnostic patent can be leveraged in industry to either support a laboratory service, test consumables, or pharmaceutical products, coupled with a strong understanding of how diagnostic patents can still be successfully obtained in the United States even with the Supreme Court's recent landscape of decisions will allow for value to be continually captured in the diagnostic patent arena.

MAKING SENSE OF THE CHANGING LEGAL LANDSCAPE

The Supreme Court's recent decisions concerning the reach of the judicial exceptions to patent subject matter, and particularly *Myriad* and *Prometheus* decisions, are having a pronounced effect on how the diagnostic industry is planning the protection of its intellectual property. While some of these effects might have a negative impact on the industry, some may be neutral or positive. Biotechnology companies that rely heavily on existing claims to isolated DNA or broadly claimed analytical methods may find the bedrock of their strategy to be shaky at best. Some may be forced to spend valuable time and capital shoring up their existing U.S. patents and/or filing for new patents to capture minor modifications to their technology. It is likely that biotechnology and diagnostic companies will make greater use of trade secrets rather than file for some U.S. patents, since trade secrets now provide greater protection than U.S. patents for some types of inventions. On the other, these court cases may have the beneficial effect of untangling the patent landscape by essentially invalidating a significant amount of the molecular diagnostic patent landscape already in place in the U.S., while narrowing

the window of patentability for patent applications going forward. While this may provide more space for entities to work without risk of infringement, which in theory could translate to reduced costs for patients, it remains to be seen whether the new legal framework will have a chilling effect on innovation and new enterprises in this industry. Without clear guidance regarding the availability of U.S. patents for new diagnostic technologies, company financings in the diagnostic industry, particularly in the United States, could become few and far between.

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Subject Index