CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Whole Genome Sequencing and Comparative Analysis of XDR *Acinetobacter baumannii* from Clinical Isolates

by

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I dedicated those theses to all the great people came in my life specially my beloved parents and my supervisor who encouraged me to stand out in this world with nobility and motivated me to step ahead without any fear

CERTIFICATE OF APPROVAL

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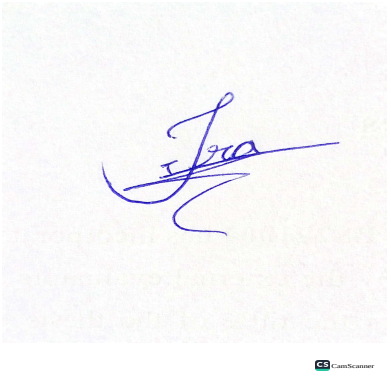

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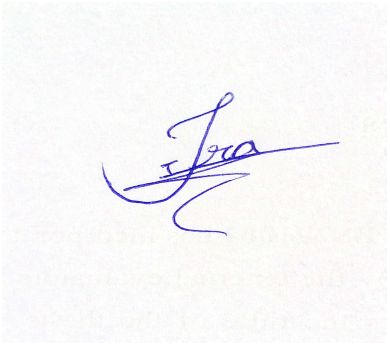

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Abstract

Acinetobacter baumannii, identified as a gram-negative coccobacillus lacking flagella, is associated with nosocomial infections, predominantly acquired within healthcare settings. It impacts various body systems, including the gastrointestinal and urinary tracts, causing infections such as ventilator-associated pneumonia, skin, wound, bloodstream, and meningitis. Its prevalence spans across all age groups, with neonates being particularly susceptible. Nevertheless, the investigation explores the genetic composition of *A. baumannii* strains by means of sequencing and analyzing four genomes obtained from local sources. The research endeavors to elucidate the genomic architecture by the utilization of comprehensive techniques such as genome sequencing, assembly, and annotation. The following comparative study offers useful insights into the genetic diversity that is inherent in the *A. baumannii* strains collected from the surrounding area. The focus of this work is to analyze the presence of virulence factors and antibiotic resistance determinants encoded within the genomes under study. The initial Results indicate the clinical significance of the strains in hospital settings is underscored by the presence of genes linked to virulence factors and antibiotic resistance mechanisms. Moreover, this research provides insight into the genetic correlation between the strains collected locally and their global counterparts. The preliminary phylogenetic analysis suggests the presence of genetically different lineages that are unique to the strains found in Pakistan. This finding offers valuable information regarding the population dynamics and evolutionary patterns of *A. baumannii* in this geographical area. In brief, this study reveals the genomic composition of *A. baumannii* strains obtained from local sources, revealing their genetic variability, possible virulence attributes, and patterns of antibiotic resistance. Through the clarification of these elements, the research enhances the overall comprehension of the clinical importance of *A. baumannii* and its consequences for healthcare initiatives at the local level.

Keyword: *Acinetobacter baumannii*, Multidrug Resistances, Virulence Factor, Multilocus Sequences Typing.

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Abbreviations

Bap	Biofilm-associated proteins
CDC	Centers for Disease Control and Prevention
CARD	Comprehensive Antibiotic Resistance Database
COGs	Clusters of Orthologous Groups
CGE	Center of Genomic Epidemiology
ESKAPE	Enterococcus faecium, Staphylococcus aureus
	Klebsiella pneumoniae, Pseudomonas aeruginosa and Enterobacter
HAP	Hospital Acquired Pneumonia
ICU	Intensive Care Units
ITOL	Interactive Tree of Life
LPS	Lipopolysaccharide
MLST	Multi-Locus Sequence Typing
OMVs	Outer Membrane Vesicles
PubMLST	Public Multilocus Sequence Typing
RAST	Rapid Annotation Utilizing Subsystem Technology
UTIs	Outer Membrane Proteins
VAP	Ventilator Associated Pneumonia
WHO	World Health Organization

Chapter 1

Introduction

Multidrug-resistant (MDR) bacteria are becoming more common, which puts human health at a high risk. These species were once limited to the medical setting, but they are now ubiquitous. Multidrug-resistant (MDR) bacteria are increasingly being found in community-acquired diseases and nosocomial infections, which is cause for concern. In fact, one of the three most significant issues affecting human health according to the World Health Organization (WHO) is antibiotic resistance (1). The abbreviation "ESKAPE," which stands for Enterococcus faecium, Staphylococcus aureus, Klebsiella pneumoniae, *Acinetobacter baumannii*, Pseudomonas aeruginosa, and Enterobacter spp., encompasses the most prevalent and dangerous MDR pathogens (2). Global public health is at risk due to the sharp increase in the prevalence of *Acinetobacter baumannii* infections resistant to multiple drugs (3). Because of its genome's flexibility, it can adapt to different resistance mechanisms more easily, making antibiotics less useful for therapy. In addition to adding *Acinetobacter baumannii*, Pseudomonas aeruginosa, Staphylococcus aureus, Enterobacter spp., and decking the ESKAPE pathogen list, the World Health Organization has declared *A. baumannii* a Group-1 priority pathogen for which there is an immediate need for novel antimicrobials. (4). Widely distributed and classified as a Gram-negative coccobacillus due to the lack of flagella, *Acinetobacter baumannii* is a type of bacterium. It is frequently observed in a variety of environmental sources. This particular bacterium, which is opportunistic in nature, is the cause of infections that are contracted in hospital and community

settings related to human medicine. The various forms of infections that can arise include bacteremia, meningitis, skin/ wound infections, pneumonia linked to the ventilator (VAP), pneumonia acquired in a hospital, urinary tract infections, and gastrointestinal tract infections (HAP) (5). *A. baumannii* is one of the 73 species of Acinetobacter that cause a broad range of diseases in people, such include skin infections, urinary tract infections, wound infections, pneumonia linked to ventilator use, and meningitis. Depending on the strain and kind of infection, mortality rates from *A. baumannii* infections have been observed to reach 50%. Colistin is the last line of treatment for *A. baumannii*, as it has effectively developed resistance to all antimicrobial medications currently on the market. Its propensity to develop biofilm on medical equipment and in hospital settings that support colonization and antibiotic-resistant chronic infections, but it also presents treatment issues (6). Concerns should be raised about *A. baumannii*'s capacity to form biofilms and exhibit resistance to desiccation and disinfectants, as these traits help to ensure its effective persistence and spread in healthcare environments. The genome of *A. baumannii* exhibits characteristics like as antibiotic resistance, environmental persistence, and the absence of recognized host-damaging toxins, suggesting that its virulence potential is likely attributed to a strategy centered around persistence and resistance. Moreover, these bacterial organisms possess the capacity to withstand complement-mediated death and oxidative stress. The existence of a malleable genome in *A. baumannii* at the genetic level results in notable diversity among isolates, hence bringing complexity to the study of *A. baumannii* as a separate organism. Therefore, the presence of *A. baumannii* is a current and worldwide challenge that requires continuous surveillance by the public health sector. The microorganism exhibits rapid growth on commonly used laboratory culture medium, such as Blood agar, MacConkey agar, and CHROM-agar. Following an incubation period lasting between 18 and 24 hours at a temperature of 37°C, the organism demonstrates the emergence of smooth, glossy, and mucoid colonies on blood agar. The observed colonies exhibit a lack of color, do not cause hemolysis, and possess a diameter that falls within the range of 1 to 2 millimeters. The bacterium demonstrates the attribute of generating colorless colonies on MacConkey agar, which have a smooth and slimy

appearance and a tomb-shaped structure. This observation indicates that the bacterium does not possess the ability to metabolize lactose. The Acinetobacter bacteria grow into pink colonies when cultivated on Leeds Acinetobacter Medium supplemented with specific nutrients. Aspects of resistance and epidemiology of Acinetobacter species as nosocomial infections (7). The molecular mechanisms underlying the pathogenicity of *A. baumannii* remain poorly comprehended, despite its significant clinical relevance. *A. baumannii* exhibits limited virulence determinants, such as Proteins associated with the membrane, including outer membrane protein A (OmpA), two-component regulatory systems, and elements involved in acquiring metals and synthesizing lipopolysaccharides (LPS) (8). WGS is a process of compiling nucleotide sequence of a complete genetic material of an organism. Whole genome sequencing (WGS) is a cutting-edge technology that could underpin the success of such goals. Microbial WGS has become a vital tool for the discovery of novel therapies because it allows for the quick and precise identification of resistance and virulence mechanisms. In this manner, the molecular resolution delivered by microbial WGS has provided important information to insight into the divergence, emergence and dissemination of antibiotic resistance [9]. In bacteria that have not received much attention, high-throughput screens are particularly helpful in revealing novel genotype-phenotype associations. Transposon insertion sequencing.Following an incubation period lasting between 18 and 24 hours at a temperature of 37°C, the organism demonstrates the emergence of smooth, glossy, and mucoid colonies on blood agar In recent years, methods like transposon-directed insertion site sequencing (TraDIS), insertion sequencing (INSeq), transposon sequencing (Tn-seq), and high-throughput insertion tracking sequencing (HITS) have become powerful tools for thoroughly screening for gene functions throughout a bacterial genome. Researchers have identified new virulence factors in Pseudomonas aeruginosa, Yersinia pseudotuberculosis, and Haemophilus influenzae by employing comparable techniques with animal infection models (9). the organism demonstrates the emergence of smooth, glossy, and mucoid colonies This study is designed to evaluate the *Acinetobacter baumannii* at genomic level to investigate the genetic diversity, emergence, epidemiological typing, and drug susceptibility, detection of virulent and resistant genes.

1.1 Problem Statement

Gram-negative *Acinetobacter baumannii*, sometimes known as *A. baumannii*, is a bacterium that causes a variety of diseases, particularly in hospital environments. The development of multidrug-resistant strains of *A. baumannii* has complicated the management of infections with this pathogen and has become a major world-wide health problem in recent years. Creating effective treatment plans requires an understanding of the genetic underpinnings of multidrug resistance in clinical isolates of *A. baumannii*.

1.2 Aims and Objectives

The research methodology is designed with following objectives:

1. Isolation characterization and susceptibility testing to Antibiotics of local *Acinetobacter baumannii* isolates
2. Analysis of Whole genome sequences of isolates with sequences assembly and annotation.
3. Identification of antibiotics resistances determinants, virulence factors, phylogenomic distribution and sequence types in genes of *Acinetobacter baumannii*..

Chapter 2

Literature Review

This study focuses on the review of the relevant literature. In 1911, the bacteria were initially isolated by the Dutch scientist Beijerinck from soil using minimum media that was enhanced with calcium acetate. Brisou and Prevot proposed the name *Acinetobacter* (derived from the Greek word "akinetos," which means non-motile) to distinguish Micrococcus calco-aceticus from the motile species found in the genus Achromobacter about 43 years after Micrococcus calco-aceticus was originally identified. In 1968, Baumann published a comprehensive examination of a range of organisms, such as Bacterium anitratum, Alcaligenes hemolysans, Mima polymorpha, Moraxella lwoffi, Herellea vaginicola, and Micrococcus calco-aceticus. Based on morphological features, the study concluded that these organisms belonged to a single genus and could not be further separated into distinct species(10). The genus *Acinetobacter* was formally recognized in 1971 by the subcommittee on the Taxonomy of Moraxella and Allied Bacteria, based on the conclusions of Baumann's 1968 paper. The currently recognized genus *Acinetobacter* contains gram-negative, strictly aerobic, non-fermenting, non-fastidious, non-motile, catalase-positive, oxidase-negative bacteria with a DNA G + C content of 39% to 47%. There are currently 26 recognized species and nine genomic species in the *Acinetobacter* genus, as a result of DNA-DNA hybridization investigations carried out by Bouvet and Grimnot in 1986 (11). The A. calcoaceticus-complex is the term used to describe a group of four *Acinetobacter* species (A. calcoaceticus, *A. baumannii*, *Acinetobacter* genomic species 3, and *Acinetobacter*

genomic species 13TU) that share so many morphological characteristics that it is difficult to distinguish between them. This nomenclature can be misleading because The A. calcoaceticus complex comprises three other species that may be the most clinically relevant because to their association with community-acquired infections as well as nosocomial infections. In contrast, the environmental species A. calcoaceticus has not been linked to clinical disease(12).

2.1 Species

*Acinetobacter*s are classified as coccobacilli that are non-motile, non-fermenting, Gram-negative, catalase-positive, oxidase-negative, and non-motile down to the genus level. However, the organisms are commonly misclassified as Gram-positive because they are often difficult to de-stain. Metabolic tests are not able to differentiate *Acinetobacter*s from other Gram-negative bacteria that do not ferment. The Juni transformation assay—which uses crude DNA from any *Acinetobacter* species to transform the mutant A. baylyi strain BD413 trpE27 into a wild-type phenotype—is a commonly used method for genus-level identification (13). The 28 phenotypic assays that are now available have shown to be 95.6% successful in detecting *Acinetobacter*s generated from human skin at the species level. Phenotypic testing by itself, however, has not been able to identify *Acinetobacter* genomic strains that have been found more recently(14). To identify *Acinetobacter*s down to the species level, more sophisticated molecular diagnostic techniques have been developed. These techniques include:

- Gene restriction analysis using amplified 16S rRNA
- Amplification of fragment length polymorphism for high-resolution fingerprint analysis (AFLP).
- Ribotyping.
- fingerprinting using tRNA spacers
- Restrictions on the intergenic spacer sequences connecting 16S and 23S rRNA are analyzed

- Sequencing analysis of the 16S–23S rRNA gene spacer region.
- RNA polymerase β-subunit (rpoB) gene sequencing, along with flanking spacer sequencing (15).

2.2 Natural Habitat

Because they can be found in nearly all samples of soil and surface water, Among the *Acinetobacter* genus' members are frequently regarded as being widely distributed in nature. This knowledge has added to the general misperception that *A. baumannii* is equally commonplace. Although not every *Acinetobacter* species finds a home in the natural world, a comprehensive and methodical investigation into the various *Acinetobacter* species' natural occurrence in the environment has not yet been studied.*A. baumannii* is a pathogen that mostly attacks mucous membranes and other wet tissues. And exposed skin from accidents or injuries. *A. baumannii* infections of the skin and soft tissues first begin as "peau d'orange," or orange skin, and then progress to a sandpaper-like look until clearing vesicles form on the skin.

Hemorrhagic bullae are apparent in areas of skin rupture, and bacteremia is observed after a visible necrotizing process (16). If treatment is not received, this infection may result in septicemia or even death. Although *A. baumannii* is probably the source of these distinct characteristics, pathogens such Enterococcus faecalis, Candida albicans, and Klebsiella pneumoniae are also considered to have a role. natural occurrence in the environment has not yet been studied.*A. baumannii* is a pathogen that mostly attacks mucous membranes and other wet tissues.These co-pathogens have the potential to induce necrotizing infection and open a channel of entry for *A. baumannii* into the bloodstream. *A. baumannii* is rarely detected as a normal skin microflora, despite its link to skin diseases. One study estimated that the bacterium colonizes only 3% (at most) of the population. Remarkably, *Acinetobacter* was found in 22% of whole body lice samples taken from homeless individuals,and indicating the possibility of another significant reservoir for the infection (16).

2.3 Clinical Importance of *Acinetobacter baumannii*

Microorganisms that belong to the genus *Acinetobacter* are thought to be commonplace. Alongside the advancement of molecular and sequencing techniques, its taxonomy has changed. Some species, not *A. baumannii*, whose habitat is still unknown, are found in ambient water and have been known to infect human skin in certain cases. However, this species has the potential to invade hospital patients. As an opportunistic pathogen It is the *Acinetobacter* species that has the greatest clinical significance. and is primarily linked to infections in critically sick patients admitted to intensive care units (ICUs). Ventilator-related pneumonia and bloodstream infections are the most common hospital-acquired infections caused by *A. baumannii*, and they are linked with a significant morbidity and fatality rate. Additional infections include urinary tract infections, wound infections, infections of the skin and soft tissues (such as those in burn victims), and, less frequently, secondary meningitis (3). Consequently, particularly in the last 20 years, *A. baumannii* has grown to be one of the more problematic opportunistic infections in clinical settings. It now belongs to the category of microbes. known as ESKAPE (*Enterococcus faecium*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *A. baumannii*, *Pseudomonas aeruginosa*, *Enterobacter spp.*), which stands for Enterobacter spp. Its potential to swiftly develop antimicrobial resistance to various antibiotic classes is mostly linked to its therapeutic importance. Its remarkable capacity to persist on dry surfaces for extended periods of time makes it one of the Gram-negative bacilli that enhances its spread in the nosocomial setting (17).

2.4 Clinical Symptoms

Numerous anatomical locations, as well as patient outcomes and severity, are linked to *A. baumannii* infections. The true clinical significance of infection and

its connection to patient death are hot topics of discussion. While several research have found that *Acinetobacter* infections negatively impact patient outcomes, other comparable investigations suggested that infections had little to no impact on outcomes (18). The fact that different studies have used different methodologies some have used prospective samples, while others have used retrospective samples is most likely the cause of the lack of agreement. *Acinetobacter calcoaceticus-baumannii* complex infections have been documented in many studies. This may suggest colonization with the environmental species *Acinetobacter calcoaceticus* along with a polymicrobial infection, rather than a monomicrobial infection with a virulent *Acinetobacter* species like MDR *Acinetobacter*. But rather than identifying the organism to the species level, the results have only done so up to the genus level (19)

2.4.1 Pneumonia Acquired in Hospitals

Infection is often related with ventilator-associated pneumonia (VAP). The recognized risk factors for *Acinetobacter*-related VAP are longer hospitalizations, longer stays on mechanical ventilation, and past antibiotic use. Health care personnel who have poor personal hygiene and have colonized hands have also been linked to nosocomial outbreaks; these persons may behave as opportunistic carriers of an epidemic stain. An outbreak may potentially start inside a hospital due to intra-hospital transmission or contaminated ventilators or respiratory care equipment (19).

2.4.2 Pneumonia Acquired in the Community

Acinetobacter-caused pneumonia that was contracted outside of a hospital has been reported in Australia and Asia. Strict carriage, which affects up to 10% of community members who drink excessively, could be the cause of the infection. It has a death incidence of 40% to 60% and is distinguished by a harsh and abrupt start along with a subsequent bloodstream infection (20).

2.4.3 Bloodstream Infections

1.3% of all nosocomial monomicrobial bloodstream infections in the US were caused by *Acinetobacter*. *Acinetobacter* was found to be the primary cause of bloodstream infections in intensive care units (ICUs) more often than in other settings (1.6% vs. 0.9% of bloodstream infections, respectively).). The overall crude fatality rates resulting from bloodstream infection caused by *Acinetobacter* were 16.3% outside of the ICU and 34.0% to 43.4% inside the ICU. Only P. aeruginosa and Candida spp. infections exhibited higher crude fatality rates in the intensive care unit (ICU) than *Acinetobacter* bloodstream infection (21).

2.4.4 Trauma from Battlefields and Other Injuries

In patients with serious burns, *Acinetobacter* is a popular infection of burning percentages that can be challenging to treat. On the other hand, skin and soft tissue infections are rare outside of military settings. Eight of the 57 SSTI patients who were the subject of a retrospective review had *Acinetobacter* infections. All of the patients in this case were male, aged between 13 and 55, and of Iraqi and American descent. The average interval between the trauma and the *Acinetobacter* infection diagnosis was 15 days. The clinical presentation of SSTI was comparable in all eight patients; they all had "peau d'orange"-looking cellulitis and a severe infection that caused bullae to grow on the skin's surface. In this case, one of the eight patients died, making the mortality rate of 12.5%; however, since the patient had a gunshot wound to the groin when they were admitted, therefore an infection cannot be the only cause of death(21).

2.4.5 Meningitis

Nosocomial, following brain surgery Meningitis caused by *Acinetobacter* is on the rise, and numerous other Gram-negative bacteria are also posing issues for postoperative care. An external ventricular drain installation creates a space for opportunistic infections. Although it is impossible to identify the exact cause of death,

the mortality rate may reach 70% (22).

2.5 Potential Pathogenesis and Virulence

Little is still known about this emerging pathogen's true pathogenic potential or virulence repertory, despite a great deal of research being done on its virulence potential. A member of the Outer membrane proteins (OMPs), OmpA, has been found to considerably contribute to the disease-causing capability of *A. baumannii*, even though it is thought that other variables may contribute to the pathogen's virulence potential. *A. baumannii* OmpA attaches itself to the host's epithelia and mitochondria. Once inside the mitochondria, OmpA causes the mitochondria to enlarge and promotes malfunction. Apoptosome development results from the release of the heme protein cytochrome c after this. Each of these responses' aids in the cell's demise. OmpA, the pathogen's most prevalent surface protein, is also involved in two essential stress survival strategies, complement resistance and biofilm formation, in addition to perhaps important virulence-related variables that facilitate bacterial survival both in and outside of the host (23). Because *A. baumannii* may form biofilms, it can thrive continuously in unfavorable settings and conditions. In fact, it has been demonstrated that *A. baumannii* has the ability to build biofilms on both biotic (such as epithelial cells) and abiotic (such as glass and equipment used in healthcare units) surfaces. The most frequent elements that influence the development of biofilms are the availability of nutrients, the existence of pili and outer membrane proteins, and the secretions of macromolecules. After *A. baumannii* connect to specific surfaces, the onset of biofilm development and maturation is facilitated by both pili assembly and the creation of biofilm-associated protein (BAP). Pili begins the process of forming microcolonies on abiotic surfaces, which is followed by the complete development of biofilm structures. Bacterial cell surfaces have BAP, which stabilize the mature biofilm on biotic or abiotic surfaces, aiding in the growth and maturity of biofilms. In addition to influencing the production of biofilms, environmental cues like metal cations also help *A. baumannii* stick to specific surfaces longer. Phospholipase D and C are two more important proteins that have been demonstrated to support

the virulence of *A. baumannii*. While phospholipase D is essential for pathogenesis, resistance to human serum, and epithelial cell evasion, phospholipase C promotes toxicity to epithelial cells. Fimbria, which are also expressed on the bacterial cell surface, work in tandem with OmpA to facilitate the pathogen's adherence to host epithelia(24).

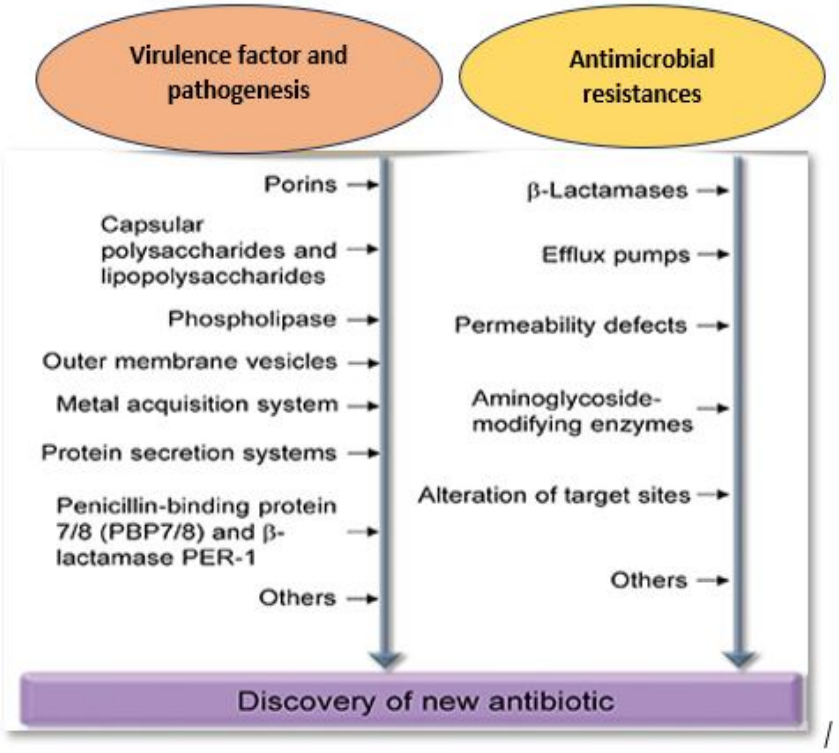

FIGURE 2.1: *A. baumannii* virulence factor and pathogenesis(24)

2.5.1 Outer Membrane Proteins (Omps)

A. baumannii's outer membrane proteins (OMPs) are essential to the pathogenicity of the organism as well as its interactions with its surroundings. Through its diverse roles, OmpA, the main and most investigated outer membrane protein in *A. baumannii*, is linked to virulence. These include the triggering of apoptosis in host cells by the production of apoptosis inducing proteins, attachment to epithelial cells through the utilization of host fibronectin, and participation in biofilm formation. The protein known as Outer membrane protein A (OmpA) has been found to be closely linked with the enhancement of adhesion, particularly to the epithelial cells located in the respiratory system. The compound localizes

within the mitochondria and nucleus, leading to the activation of the proapoptotic molecule cytochrome c and subsequent initiation of cellular apoptosis. With the help of OmpA, *A. baumannii* successfully nullifies factor H, a crucial regulator of this pathway, evading the complimentary pathways' death mechanism. The phenomenon that has been observed is usually known as *A. baumannii* serum resistance. It has been observed that OmpA promotes CD4+ cell differentiation and accelerates dendritic cell maturation and activation. Furthermore, it has been discovered to cause dendritic cells to undergo premature apoptosis (25).

2.5.2 Outer Membrane Vesicles (Omvs)

The release of extracellular vesicles originating from the outer membrane, which carry several proteins associated with virulence (such as phospholipases, catalase, superoxide dismutase, and proteases) enhances the activation of the inborn immune response in the immediate vicinity of the infection site, ultimately resulting in tissue injury. The outer membrane vesicles additionally enhance the process of biofilm formation on non-living surfaces. The polysaccharide capsule seen in Gram-negative bacteria is widely recognized as a significant virulent factor. This process plays a critical role in shielding microorganisms from phagocytosis. by the inherent immune system of the host(26)

2.5.3 Lipopolysaccharide (LPS)

Because lipopolysaccharide may influence colonization and the generation of proinflammatory responses, it is crucial for the survival of bacteria. Evidence was discovered by Chin et al. that altering lipopolysaccharide (LPS) can result in decreased sensitivity to antibiotics and the emergence of resistance mechanisms. The surface of bacteria is covered by a protective capsule that shields them from changes in the environment and certain antimicrobial substances, thereby aiding their survival, especially in serum. The lipopolysaccharides in *A. baumannii*. comprised of three primary parts a carbohydrate core, O-antigen as well as a fat.

a moiety. Lipopolysaccharides are a chemotactic and inflammatory cell-drawing agent and encouraging them to secrete their cytotoxic materials (27)

2.5.4 Phospholipase

A further significant virulence factor exhibited by *A. baumannii* is the production of phospholipase enzymes, specifically phospholipase C and phospholipase D. The lipolytic action of these entities is linked to phosphatidylcholine, a substrate for phospholipases found in eukaryotic membranes. Phospholipases exhibit the capacity to hydrolyze human erythrocytes, hence assuming a significant role in the acquisition of iron. Additionally, these enzymes are essential for the invasion of epithelial cells and the creation of serum resistance (26).

2.5.5 Biofilm-Associated Proteins (Bap)

It is commonly known that *A. baumannii*'s most potent virulence mechanism that contributes to multidrug resistance is biofilm development. The pathogen stated above possesses the capacity to employ biofilm-associated proteins (Bap) to aid in the development of biofilm architecture in response to unfavorable environmental conditions. The formation of biofilms contributes to the colonization and survival of bacteria, much like other traits that increase their capacity to cause illness. Bacteria can attach to both living and non-living surfaces through biofilms, which increases the risk of illnesses linked to medical equipment in hospital environments. Prior research has proved that QS, or quorum sensing, plays a role in the biofilm formation process, with autoinducers acting as signaling molecules. Moreover, Csu pili and type IV pili are significant. because they help to promote the development of biofilms. The capacity of *A. baumannii* to generate biofilms on both living and non-living surfaces has been extensively investigated as a recognized mechanism of resistance. In order to endure adverse conditions, it undergoes metabolic inactivity within the deeper strata of biofilms. Antibiotic-resistant bacteria are more harmful due in part to their poor penetration into bacterial cells and restricted capacity to target metabolically dormant bacteria. The strains of

A. baumannii linked to epidemics are notably resistant to desiccation and capable of growing biofilms on biotic surfaces. The capacity of *A. baumannii* to produce pellicules when polysaccharides, such as the csuA/B usher protein and poly-N-acetyl glucosamine, are present, acts as a defense against the effects of antibiotics. Many virulence factors, including biofilm-associated protein (BAP), outer membrane protein A (OmpA), BAP like protein-1 (BLP1), and BAP like protein-2 (BLP2), are additional components that have contributed to the evolution of biofilms. Furthermore, *A. baumannii*'s long-term survival depends on its ability to obtain nutrients from the host, which enables it to elude the immune system and encourage the infection's spread. To achieve this, *A. baumannii* employs a variety of strategies to obtain iron, zinc, and manganese. For each individual element, these procedures involve the synthesis of siderophores, the use of a zinc-scavenging mechanism, and the use of transporters that are individuals belonging to the NRAMP (natural resistance-associated macrophage protein) family (28).

2.5.6 Type II Secretion System (T2SS)

It is remarkable that *A. baumannii* has protein secretion systems because they enable interaction between the microbe and its host organism as well as with its surroundings. The Type II secretion system (T2SS) is essential, as it uses a biphasic mechanism to facilitate the extracellular transportation of virulence-associated effector proteins, such as CpaA, LipA, and LipH. These proteins play a crucial role as enzymatic factors. Another important system is Type VI secretion system. (T6SS), used by *A. baumannii.* to selectively target various bacteria by delivering toxins, including nucleases, peptidoglycan hydrolases, and toxins that specifically target the cell membrane. This mechanism is particularly significant in the development and progression of polymicrobial infections (29)the capacity to employ biofilm-associated proteins (Bap) to aid in the development of biofilm architecture.which are small hormone-like molecules, are synthesized by bacteria. These chemicals aid the bacteria.In order to endure adverse conditions, it undergoes metabolic inactivity within the deeper strata of biofilms textitA. baumannii employs a variety of strategies to obtain iron, zinc, and manganese.

2.5.7 Quorum Sensing

Bacteria have the ability to communicate with each other through a process called quorum sensing. This allows them to respond collectively to changes in their environment. Autoinducers, which are small hormone-like molecules, are synthesized by bacteria. These chemicals aid the bacteria in keeping an eye on the density of their population and adjusting their surroundings as needed. *Acinetobacter* generates specific signaling molecules, such as acyl homoserine lactones, for intra- and inter-species communication, just like other Gram-negative rod-shaped bacteria. Additionally, it generates signaling chemicals that are less researched, like Retention factor 1 for 2-hydroxy-4-quinolone, as well as diketopiperazines (30).

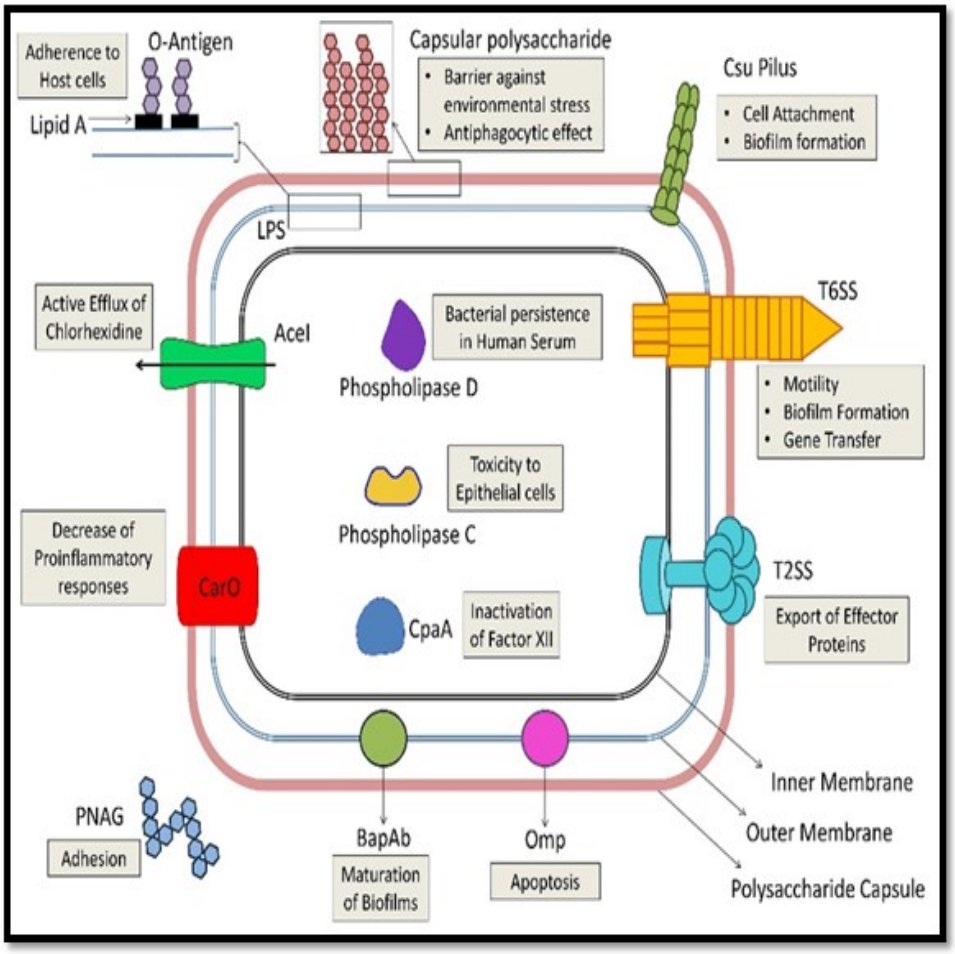

FIGURE 2.2: Illustrates the virulence Factors possessed by *A. baumannii* (30)

TABLE 2.1: Pathogenesis-Virulence Potential (23).

Sr#	**Virulence Factors (Genes)**	**Potential Contribution to the Pathogenesis**
1	Outer membrane protein A (OmpA)	The phenomenon involves different biological processes, such as surface motility, biofilm development, apoptosis induction in host cells, adhesion and invasion of epithelial cells, and resistance to serum.
2	Biofilm-associated protein (Bap)	The creation of the biofilm and the subsequent cell-to-cell binding that occurs inside the finished biofilm.
3	Lipopolysaccharide (LPS)	The evasion of the host immunological response resistance to cationic antimicrobial peptides, attenuation of TLR4 signaling, promotion of desiccation survival, and start of the host inflammatory response.
4	Penicillin-binding protein 7/8 (pbpG)	The process of cellular proliferative capacity, stability, and peptidoglycan production.
5	Outer membrane vesicles (OMV)	The transportation of virulence genes genetic material between bacterial cells and into the cytoplasm of host cells.
6	Phospholipase	The in vivo survival of bacteria, their ability to withstand serum, and their dispersion.
7	*Acinetobacter* or method of iron acquisition mediated by siderophores	In order to maintain its survival, the bacteria take iron from the host, which also causes cell death.
8	Capsule	The protein under consideration has a function in mediating cationic antimicrobial peptide resistance, as well as resistance to serum and survival inside a living organism.
9	Phospholipase C	Demonstrating hemolytic activity towards erythrocytes and facilitating iron absorption.
10	Poly-β-1-6-N-Acetylglucosamine (PNAG)	Cell adhesion to one another and the creation of defense mechanisms against the host's natural defenses are two processes involved in the production of biofilms.
11	Two-component regulatory system, BfmRS	The chaperone-usher assembly system's expression in Csu and how it affects cellular shape and biofilm formation.
12	Synthase for autoinducer AbaI	Typical biofilm development.
13	Chaperone usher pili assembly system CsuA/BABCDE	The process of pilus assembly and the production of biofilms on nonliving surfaces.

2.6 The Development of Large Spectrum Antibiotic Resistance

It is challenging to compare different regions and conduct an objective examination of the microorganism's evolution of resistance due to the lack of extensive surveillance studies conducted between the 1970s and 1990s. Furthermore, the spread of particular clonal lineages may be linked to the resistance issue. For instance, the first *Acinetobacter* spp. nosocomial isolates. that were obtained within Portugal throughout only able to survive first generation and occasionally the 2nd generation aminopenicillins and cephalosporins during the 1970s and 1980s. Furthermore, Certain isolates were unable to been due to poor identification, *A. baumannii* is a prevalent species in clinical settings these days. Following 1999, there was a significant shift in the situation, with most isolates exhibiting sole vulnerability to colistin, amikacin, and tobramycin. This was connected to the European Clone II's extensive national distribution (31). Numerous European countries have reported comparable rises in the isolation of *A. baumannii* that is resistant to drugs. Although *A. baumannii* is thought to be a low-virulence pathogen, it is undeniably that some lineages of the organism have a remarkable ability to acquire resistance genes. It frequently demonstrates resistance to most antibiotic classes, such as carbapenems and occasionally colistin, which are thought to be the final effective treatments for infections brought caused by antibiotic-resistant Gram-negative bacteria(32).

2.7 Antibiotic Resistance

After the discovery of antibiotics, a significant breakthrough has been observed in the annals of contemporary medicine. When penicillin was discovered, which was accompanied by aminoglycosides and sulfonamides, encouraged scientists to hypothesize the discovery of a potent remedy capable of eradicating infectious infections had been achieved. However, regrettably, the circumstance does not align with previous assumptions. In his Nobel talk, Fleming expressed his belief that

antibiotics will become readily accessible to the general population in the future. However, he also cautioned about the potential negative consequences of unqualified individuals, referred to as "quacks," who may inadvertently contribute to the development of antibiotic resistance by subjecting microorganisms to prolonged and suboptimal treatment regimens. The initial administration of penicillin occurred in 1941, with the subsequent identification of penicillin-resistant isolates in 1942. In a similar vein, the introduction of methicillin occurred in 1960, followed by the reporting of methicillin-resistant organisms in 1961 and subsequent years. Presently, there have been documented instances of *A. baumannii* isolates exhibiting resistance to all antimicrobial agents currently in existence. The diligent efforts of the scientific community have led to the discovery of numerous antibiotics; nevertheless, their improper utilization has resulted in a significant level of resistance. One could argue that we are entering a period reminiscent of the pre-antibiotic age, characterized by the resurgence of bacteria possessing heightened lethality. *Acinetobacter* possesses the genetic capacity for expedited acquisition of antibiotic resistance, thereby earning the designation of a natural transformant. The scientific literature contains numerous findings characterizing it as one of the most resilient bacteria. At the start of 1970s, *Acinetobacter*. infections were well managed with the administration of carbenicillin, ampicillin, nalidixic acid, and gentamicin either through standalone treatments or using combination therapy. However, a significant increase in resistance rates was observed subsequent to 1975. Currently, a number of important pharmaceuticals, including extended pectrum cephalosporin, narrow spectrum and aminopenicillins. ureidopenicillin, chloramphenicol, tetracycline, cephamycin's like cefoxitin, and the majority of. aminoglycosides, have experienced a decline in their effectiveness against *Acinetobacter* (33)

2.7.1 Resistance to Beta-Lactams

Penicillin, beta-lactamase inhibitors, carbapenems, monobactams, and cephalosporins are all members of the beta-lactam antibiotic class. Consequently, these antibiotics impede the transpeptidation process, which represents the final

stage in the manufacture of peptidoglycan. *A. baumannii* currently has inherent resistance to cephalosporins and penicillin. Resistance against beta lactam antibiotics can be acquired via the processes already discussed, namely hydrolytic inactivation, enhanced and reduced influx, and safeguarding of the target for antibiotic (34).

2.7.2 Beta-Lactamases

Beta-lactamase. are a class of enzymes which facilitate the hydrolysis of beta lactam antibiotics. These enzymes are categorized into four different classes, which are determined by their sequence motifs and variations in their hydrolytic mechanisms

2.7.2.1 Class A

Resistance to many antibiotics is attributed to class A beta-lactamases, potential to exhibit a limited range of action, or they can obtain an expanded range of antibiotic effectiveness through point mutations. Besides this Narrow range lactamases exhibit activity primarily against penicillin which can be effectively suppressed by the acid clavulanic. Beta-lactamases with an extended spectrum. (ESBLs) are enzymes that may break down certain antibiotics, including ceftazidime, ceftriaxone, cefotaxime, and aztreonam. These enzymes are found in many types of Gram-negative bacteria and can spread easily through plasmids and other genetic materials. It's important to regularly monitor for ESBL-producing bacteria and identify specific genetic markers (like. blaGES-11, blaTEM-92, blaVEB-1, blaPER-1, blaPER7, and blaGES-14,) to help with clinical decisions. Some other important members of this group include CTX-M and KPC (35).

2.7.2.2 Class B

Metallo beta-lactamases (MBLs) also known as class B enzymes are enzymes that are produced by bacteria through the expression of elements of mobile genetics like

integrons and plasmids. These enzymes facilitate the breakdown of a wide range of beta-lactam antibiotics, including carbapenems, hence leading to the development of multidrug resistance. It is important to note that MBLs do not possess the ability to hydrolyze monobactams. These enzymes necessitate the presence of any heavy metal like zinc etc. to facilitate the catalytic process. Additionally, they fall into three further subclasses for further division. based on differences in the arrangement and sequencing of each active site, such as (B1, B2, and B3). Furthermore, 4 distinct classes of metallo-beta-lactamase have also been identified in *A. baumannii.* specifically, SIM, NDM, IMP and VIM. However, the application of molecular methods, particularly next generation sequencing, is expected to provide valuable insights into the detection of these organisms. Phenotypic including carbapenems, cephalosporins, penicillin, and monobactams. Lactamases possess the techniques exhibit limited sensitivity, hence failing to identify all bacteria that produce Metallo-β-lactamases (MBLs). About 14.3% of *A. baumannii* isolates that were determined to be metallo-β-lactamase negative by the E-test were able to have their blaVIM-1 identified by the use of polymerase chain reaction (PCR). This finding underscores the significance of incorporating molecular techniques into routine clinical procedures to effectively identify concealed MBLs. In a recent study, López et al. provided evidence which indicates the presence of NDM lactamases do not have role in hindering the development of bacteria. Furthermore, they found that NDM lactamases are more advantageous compared to other metallo-beta lactamases (MBLs) due to their lack of fitness cost. This favorable characteristic has contributed to the global spread of NDM lactamases between microorganisms that are Gram-negative (33)

2.7.2.3 Class C

Beta-lactamases of class C, namely *Acinetobacter*-derived cephalosporinase (ADC), are cephalosporinases that are encoded in the chromosome and are naturally present in all strains of *A. baumannii.* The induction of lactamase overexpression can be achieved through the incorporation of ISAba125 and ISAba1 sequences preceding the gene that encodes. blaADC (previously called blaAmpC). These inserted sequences are observed to possess greater promoter activity compared to

the intrinsic promoter. Insertion sequences (IS) are a class of small transposable elements that exhibit many repetitions throughout the genome, rendering their detection challenging. A recently established open-source bioinformatics pipeline, known aspanISa, has been designed to address this issue which utilizes the whole genome sequencing (WGS) data. as its primary input. Numerous variations of ADC have been documented, a significant proportion of which demonstrate an expanded range of antibiotic resistance. As an illustration, an analog to digital converter 30 exhibits resistance to carbapenems, sulbactam, and cephalosporins in addition to those two antibiotics. The findings from the Phosphoproteomic research suggests that clinical isolates may develop imipenem resistance as a consequence of ADC dephosphorylation (33).

2.7.2.4 Class. D

The beta lactamases in Class D, known as Oxacillinases. (OXA) or hydrolyzing class D β-lactamases with carbapenem. The induction of lactamase overexpression can be achieved through the incorporation of ISAba125 and ISAba1 sequences preceding the gene that encodes. blaADC (previously called blaAmpC). These inserted sequences are observed to possess greater promoter activity compared to the intrinsic promoter (CHDLs), has the ability to render all beta-lactams (mostly those belonging to the OXA-10 family) ineffective, therefore serving as the primary mechanism for carbapenem resistance. The enzymes in question exhibit serine dependency, similar to Class A. and C. beta lactamases. Furthermore, it is worth noting that class D beta-lactamases typically exhibit resistance to inhibition by tazobactam, sulbactam and clavulanic acid. Multiple blaOXA genes have been identified, such as blaOXA-143, blaOXA-23, blaOXA-57, blaOXA-235, blaOXA-24, blaOXA-23, and blaOXA-51. Enzyme coding genetic material is found on Different additional mechanisms could be in charge of chromosomes and plasmids. *A. baumannii*'s resistance to carbapenem is caused by upregulation of OXA-23 orOXA-51. Molecular tools like PCR and sequencing have identified carbapenems-producing *A. baumannii* in companion animals and pigeons, emphasizing the need for global surveillance (33)

2.7.3 *Acinetobacter baumannii*'s Resistance to Carbapenems

Referred to as "last line agents," Carbapenems are the -lactam antibiotics that have the widest range of action., which include imipenem, meropenem, biapenem, ertapenem, and doripenem., demonstrating excellent effectiveness with bacteria that are Gram-positive and Gram-negative. Furthermore, they exhibit a high degree of stability against most -lactamases, which is a commonly described mechanism antimicrobial -lactam resistance. However, within recent years, Increased resistance to carbapenems has been shown in gram-negative bacteria. (36). Different additional mechanisms could be in charge of *A. baumannii*'s resistance to carbapenem. One possible explanation for carbapenem resistance (CarO) is the deletion or modification of particular outer-membrane proteins. Reports sometimes link changes in penicillin binding proteins (PBPs) to carbapenem resistance. It was discovered that *A. baumannii* possesses the AdeABC efflux pump, which is a member of the resistance nodulation division family antimicrobial efflux in a range of configurations by the use of RND efflux systems. Eventually, it was shown that this pump contributed in some way to carbapenem resistance. Nevertheless, it seems that the aforementioned resistance mechanism confers clinical resistance to carbapenems just in conjunction with other factors, including the synthesis of oxacillinases that hydrolyze carbapenems (37).

2.7.4 *Acinetobacter baumannii*'s Mechanisms of Acquiring Carbapenemase Determinants

The processes by which bacteria acquire exogenous DNA have been extensively documented, but our understanding of the factors underlying the swift genetic evolution of bacteria is by no means comprehensive. It appears that some species are more likely than others to acquire foreign DNA. Without a doubt, *A. baumannii* has a great deal of genetic flexibility in order to live. Four distinct HGT pathways are available for use by members of the *Acinetobacter* genus in order to exchange genetic material. It is said that conjugation has the biggest influence

on the spread of antibiotic resistance. Although transformation and transduction are seen as less significant, new research indicates that their function may be more expressive than previously believed. Particular observations of all the HGT processes have been made in *A. baumannii*. It is discovered that a large number of blaOXA genes and certain MBL determinants, including blaNDM genes, are plasmidic, indicating that conjugation plays a significant part in the spread among these factors of carbapenemases. However, some investigations were unable to show that plasmids in this species are conjugated [44], and frequently the distribution of these plasmids is deduced from where the plasmid is located, particularly with epidemiology research where conjugation assays are not shown. Furthermore, it has been demonstrated that isolating and analyzing *Acinetobacter* plasmids is a challenging task, which could potentially constrain experimental tests. *A. baumannii* transduction has been observed experimentally in a few investigations. The genus *Acinetobacter* is known to undergo natural metamorphosis. Nevertheless, it has only been recently shown that this mechanism operates in clinical isolates of *A. baumannii*. The species *A. baumannii* has been shown to be naturally competent in two investigations and our own current research; under specific circumstances, it is capable of absorbing both chromosomal and plasmid DNA. This suggests that the dynamics of resistance genes in *A. baumannii* may be influenced by natural transformation. Additionally, it was demonstrated that OMVs can mediate plasmid DNA transfer in *A. baumannii*. This process is likely underappreciated and is not commonly mentioned to explain how resistance genes proliferate (38). The processes underlying *A. baumannii*'s horizontal transfer of carbapenemase genes. None of the blaOXA genes linked to For *A. baumannii*, carbapenem resistance has been found in integrons, as opposed to most oxacillinases in other species. Nevertheless, integrons, which are usually put into mobile elements but do not move on their own, are where certain MBLs are inserted as gene cassettes (39).

TABLE 2.2: The genetic location, horizontal gene transfer pathways, genetic context, and mobile genetic mobility of the carbapenem-containing genes of Acinetobacter baumannii [47].

Type	bla Gene	Resistance Origin	Location	Genetic Context
CHDLs	OXA-51	innate	mostly chromosomal plasmid	Sometimes ISAba1-blaoxa-51 similar Tn6080
	OXA-23	Acquired	Chromosome and plasmid	insertion sequences, transposons, and genomic islands
	OXA-40/24	Acquired	Chromosome and plasmid	Self-transmissible plasmid found in plasmids carrying mob .
	OXA-58	Acquired	Mostly plasmidic	Plasmid is self-conjugative member of replicon group GR6.
	OXA-143	Acquired	Plasmid	Flanked by two rep genes. XerC/XerD recognition site.
	OXA-235	Acquired	Chromosome and plasmid	Flanked by two ISAba1
MBLs	IMP	Acquired	Chromosome and plasmid	Several integrons in class 1 Gene not contained within an integron. Chromosomal class 1 integron.
	VIM	Acquired	Chromosome	Class 1 and 2 integrons.
	NDM	Acquired	Mostly plasmidic; chromosome	Associated to composite transposon Tn125.
. Class A -lactamases	KPC	Acquired	Chromosome	Integrated Tn4401b in a KQ-like component. Tn4401e.
	GES	Acquired	Chromosome and plasmid	Class 1 integrons.

2.7.5 The Mechanisms of CHDL Movement and Dissemination

2.7.5.1 OXA-23

The blaOXA-23 gene, formerly known as blaARI-1, was first discovered in a medical isolate of *A. baumannii* that was collected in 1985. It can be inserted into plasmids or chromosomes and is surrounded by a variety of genetic contexts, including insertion sequences (ISs), transposons (Tn2006, Tn2007, Tn2008, Tn2009, and Tn6206), and genomic islands (AbaR4, AbaR25, and AbaR26). The blaOXA-23 gene was first naturally occurring in *Acinetobacter* radioresistens strains, but it was not expressed. A series of distinct horizontal transfer events and transposition most likely led to *A. baumannii* receiving this gene transfer(40), where it is now widely distributed. *Acinetobacter* nosocomial is, *Acinetobacter* pittii, and *Acinetobacter* genomic species 15TU. Some of these species have the same genomic background as *A. baumannii*'s blaOXA-23 gene, indicating the possibility of HGT. Additionally, Three reports have been released on the identity of the gene blaOXA-23 in Escherichia coli and Proteus mirabilis, two species belonging to the Enterobacteriaceae family, whereas the gene in E. coli is situated using a plasmid that isn't self-conjugative, flanked by two IS1 elements. The gene in E. coli failed to conjugate to recipient strains of *A. baumannii* and E. coli (15). It has been demonstrated that this gene can be transferred horizontally between *A. baumannii* strains using plasmid conjugation. Additionally, the plasmid's conjugation t has been shown to contain blaOXA-23 from *A. baumannii* to A. baylyi. The concept that transposons carrying the blaOXA-23 gene migrate intracellularly by transposition is supported by target site duplications around the transposons, as discovered by nucleotide sequencing of the gene's genetic background in wildtype *A. baumannii* strains (41). This also holds true for genomic islands. The different blaOXA-23 chromosomal insertion locations among isolates from the same clonal cluster have also been used to suggest evidence of horizontal gene transfer (HGT). The discovery that multiple genetically unrelated isolates have the same genomic background provides additional evidence of the mobile element-associated blaOXA-23 gene's horizontal migration (41).

2.7.5.2 OXA-40/24

The first *A. baumannii* strain was identified in 1997 and was a clinical strain identification of the blaOXA-40 gene, which was previously known as blaOXA-24. The chromosome and plasmids contain the blaOXA-40 genes that have been identified in *A. baumannii*. *A. baumannii* and E. coli have not been conjugated by an *A. baumannii* plasmid containing blaOXA-40 genes, however, been successfully demonstrated experimentally. Nevertheless, the presence of this gene on self-transmissible plasmids, equipped with mob genes that can be mobilized by the plasmids themselves, suggests a potential for horizontal spread through conjugation. These plasmids are classified under the replicon group GR6. Upon examining the GC content of the blaOXA-40 gene in comparison to the overall genome of *A. baumannii*, it is evident that this gene has its origins in a separate species (42). he blaOXA-40 gene is not integrated inside the typical structures associated in DNA mobilization, such as ISs, but rather is flanked by conserved inverted repeats that are similar to XerC/XerD binding sites. This gene can be found on chromosomal *A. baumannii* or in many plasmids. These sites are the targets of the site-specific recombination procedures carried out by the XerC and XerD recombinases. It is hypothesized that this process can mobilize the blaOXA-40 gene inside the isolates of *A. baumannii*, which can subsequently be further dispersed by HGT. The identical structures can be found the same binding sites flank other DNA modules in numerous plasmids and chromosomes, as well as in other locations, lend credence to this theory. The broad distribution and durability of plasmids carrying the blaOXA-40 gene may be explained by the discovery of these plasmids' genes linked to the toxin/antitoxin system (43). There have been sporadic reports of this gene in other *Acinetobacter* species, including A. pittii, A. calcoaceticus, A. haemolyticus, and A. baylyi. The identical plasmid, flanked by the XerC/XerD binding sites and containing the blaOXA-40 gene, has been discovered to be carried by *A. baumannii*, A. calcoaceticus, *A. baumannii*, and A. haemolyticus. This finding suggests that the genetic element was transferred from *A. baumannii* to the other species through an as-yet-unidentified HGT mechanism. P. aeruginosa has also been shown to have the *A. baumannii* blaOXA-40 gene, within a plasmid resembling that of *A. baumannii* (43). Due to their extensive research on the

transfer of proteins and toxins to target cells during infection, OMVs of Gram-negative bacteria are typically associated with virulence factors. However, OMVs encoding the plasmid-encoded blaOXA-24 have been experimentally demonstrated by Rumbo and colleagues to be released from clinical strains of *A. baumannii* resistant to carbapenem, and these OMVs have the ability to transform a strain of *A. baumannii* susceptible to carbapenem. The transformants continued to be able to release OMVs with the blaOXA-24 plasmid encoded in them. The role of OMVs-mediated transfer in the transmission of antibiotic resistance is still mainly unknown, and our study provides the first experimental proof that OMVs containing carbapenemase determinants may be released by clinical isolates of *A. baumannii* as a method of HGT (44).

2.7.5.3 OXA-58

In 2003, the first recorded finding of the blaOXA-58 gene was in a clinical isolate of *A. baumannii*. This gene can be found in several plasmids and is typically plasmid-encoded. Numerous attempts to show conversion of this genetic transportable element from *Acinetobacter* spp. to strains of *A. baumannii* or between *A. baumannii*. have failed, despite the plasmid placement. Therefore, the widespread distribution of the blaOXA-58 gene expressed by plasmids may finally be explained by the contribution of transduction, spontaneous transformation, and/or OMVs-mediated transfer (45)(46) ISAba3 encircles the blaOXA-58 gene in *A. baumannii*, but numerous ISs, including ISAba1, ISAba2, ISOur1, IS1008, IS15, and ISAba825, disrupt the upstream ISAba3-like element often, increasing the expression of the blaOXA-58 gene. Although different *Acinetobacter* species and strains have similar blaOXA-58 genetic settings, and these mobile components surround the blaOXA-58 gene, there is little evidence that this structure has traveled by transposition. The hypothesis states that the blaOXA-58 gene and its associated genetic environment were acquired through homologous recombination events. The finding that two repeated sequences called Re27 flank different genetic constructs carrying the gene lends credence to this [57]. In A. nosocomialis and A. pittii, the blaOXA-58 gene and its surrounding gene have been found to have the same recombination sites. According to a single study, the blaOXA-58 gene was

transferred into a plasmid in A. pittii, bordered by intact ISAba3-like elements and an ISAba3. Multiple copies of the blaOXA-58 gene were connected with increased Minimum Inhibitory Concentrations of carbapenems. Two IS26 elements around the original unit have been implicated in the duplication of an ISAba2/ISAba3-blaOXA-58-ISAba3 unit in clinical *A. baumannii* isolates. (47). *A. baumannii* strains exhibit a wide distribution of the blaOXA-58 gene, has been proposed to have originated in a distinct species. This theory is supported by the gene's distinct GC content when compared to the core genome of the species. The source hasn't been found yet, though. The OXA-58 CHDL's distribution mechanism was been clarified. The production of extracellular OXA-58 CHDL was also facilitated by the development of OMVs by *A. baumannii*, however this is not precisely a route of gene transfer. OXA-58 is released selectively by OMVs via the Sec-dependent periplasmic translocation transport mechanism; in response to a carbapenem challenge, this release was amplified. Bacteria that are susceptible to carbapenem are shielded by these OMVs, which enables the cells to unexpectedly survive in situations like polimicrobial infections. *A. baumannii*'s overexpression of OXA-58 shields other bacteria while preventing genetic material exchange. Nonetheless, the genetic interchange cannot be discounted, much like in the instance of the transmission of blaOXA-40 OMVs (48).

2.7.6 Mechanism of Resistance to Aminoglycosides

According to the findings of MicroBIGG-E, it has been discovered that *A. baumannii* can develop resistance to aminoglycosides (AG) through three different mechanisms. These consist of 16S rRNA altering the active site and the existence of aminoglycoside-modifying enzymes (AMEs) that decrease AG's binding capacity. lower AG absorption as a result of decreased permeability, and methyltransferases. or elevated efflux pump activity. Acyl modifying enzymes (AMEs). can be classified into three main categories based on the specific location of modification on the AG molecule. Recent studies have shown that there are several

categories of transferases that modify AG, including acetyl-transferases, adenyl-transferases, and phospho-transferases. N-acetylation, O-phosphorylation, and O-nucleotidylation are three possible processes for these alterations. Unfortunately, *A. baumannii.* has developed resistance to aminoglycosides in a significant number of cases, ranging from 19% to 31%. This inherent resistance adds to the challenge of treating infections caused by *A. baumannii* (33). Aminoglycosides (AGs) are a group of substances that inhibit protein synthesis. They do this by penetrating the bacterial cell wall and interfering with the extension of peptides at the .30S ribosomal subunit. Resistance genes may be transmitted through various methods, such as gene cassettes, integrons, conjugative elements and transposons. At the cellular level, the transfer of AG resistance genes can occur through many mechanisms, including mobilizable or conjugative plasmids, natural transformation, or transduction. These processes enable the transmission of AG resistance genes beyond the molecular level (49).

2.7.7 Mechanisms of MBL Gene Movement and Dissemination.

A better explanation for their dispersion may be found in the fact that certain MBLs are chromosomal, whereas others are present in plasmids. Mobile genetic elements such as transposons may integrate integrons in which certain MBLs have been introduced. Integrons are genetic elements that have the ability to recombinately grab gene cassettes at specified sites. Although this has not been empirically as demonstrated by MBL-encoding genes, any gene cassette inserted into an integron that enters a cell by HGT processes may be drawn in by an integron that already exists. The MBL-encoding genes found in *A. baumannii* are present in a variety of cassette arrays. (50).

2.7.7.1 IMP-Type

A. baumannii has been shown to harbor at least 12 of the 52blaIMP gene variations that have been reported (GenBank accession number KT935306). On plasmids or

chromosomes are the blaIMP genes carried by the *A. baumannii* class 1 integrons. *A. baumannii* does not appear to have class 1 integrons harboring blaIMP genes, which are typically seen in transferable plasmids in Enterobacteriaceae. Additionally, there has been no success in conjugating plasmids containing the blaIMP-5 gene from A. bereziniae to *A. baumannii*. Takahashi et al. observed conjugation of a blaIMP gene across strains of *A. baumannii*. Though plasmids are sometimes challenging to isolate in this species, the scientists' failure to find any in their donor isolates raises the prospect that the blaIMP gene may have been transferred via a different HGT method. However, it was reported that a plasmid encoding the blaIMP-1 gene that was not connected to the class 1 integron was conjugated from *A. baumannii* to E. coli, indicating that blaIMP genes are not invariably embedded in integrons. The presence of the same class 1 integron in numerous bacterial species and genera is assumed to constitute evidence of HGT (51).

2.7.7.2 NDM-Type

Since the 2008 discovery of New Delhi metallo--lactamase (NDM) in India, reports of this MBL have been made worldwide. Numerous species and genera have been shown to harbor the blaNDM genes in a number of replicon type plasmids, most notably IncA/C2 and IncFIIY plasmids. These plasmids most likely account for this improved dispersion. Additional examples are provided. The blaNDM gene is now known to exist in 16 distinct variants. Among these variations, three were found in *A. baumannii* (GenBank accession number KU220611): blaNDM-1, blaNDM-2, and blaNDM-3.(52). Furthermore, in a clinical instance in China, blaNDM-1 was found in E. Coli, Citrobacter freundii, and *A. baumannii* that were obtained from the same patient. Nevertheless, blaNDM-1 was shown to be present in a very large plasmid (¿400-Kb) in *A. baumannii*, in contrast to the Enterobacteria, where it was placed in an identical plasmid. There was no analysis of the genetic environment. A 100-Kb plasmid containing the blaNDM-1 determinant was also found in a Brazilian isolate. In Switzerland, the blaNDM-1 gene was found in Enterobacteria on conjugative IncA/C- or IncF-type plasmids.All strains included a section of ISAba125 upstream of the blaNDM-1 gene, which has been

found in blaNDM-1-negative *A. baumannii*. This finding suggests that *A. baumannii* was the original spreader. This carbapenems is also present in certain mammals. A pig suffering from pneumonia and sepsis had a lung sample from which an isolate of *A. baumannii* positive for blaNDM-1 was obtained. There was a 47-Kb plasmid containing the blaNDM-1 gene. This plasmid could be conjugated into E. coli J53 with a high frequency of 1.15 × 102 per donor cell. Thus, it appears that a variety of conjugative plasmids are involved in the dissemination of carbapenem resistance in the blaNDM-type, and conjugation plays a significant role in this process (53). Nevertheless, chromosomal location of the blaNDM-1 is also possible. In fact, it seems that blaNDM-1 is typically flanked by two ISAba125 insertion sequences, which are part of the composite transposon Tn125. Tn125 acts as the main mechanism for blaNDM gene propagation in *A. baumannii*, as evidenced by the fact that this transposon was also connected to blaNDM-2 (54). According to the most current study, a carbapenem-resistant *A. baumannii* clinical strain (R2090) was revealed to have horizontally transferred the Tn125 transposon harboring the blaNDM-1 gene to a carbapenem-susceptible strain through experimentation. The other three HGT mechanisms are not considered to be involved by the authors. The translocation of the blaNMD-1 gene into the strain may have been caused by the two distinct insertion sites (ISAb125 and ISCR21) that flanked the gene on the chromosome. It was discovered through complete sequencing that this strain possessed three intact prophages within its chromosome, and it was proposed that the transduction process was facilitated by the activation of one of these prophages. However, further testing is need to confirm whether the phages found are active. This is the first study to date that shows that mobile elements may have a role in the spread of carbapenem resistant genes. Since there are roughly 20 known *A. baumannii* phages, bacteriophages may in fact have aided in the evolution of *A. baumannii*'s resistance to antibiotic selection(55)

2.7.7.3 VIM-Type

Only six of the 48 blaVIM gene variants that have been identified thus far have been linked to *A. baumannii*. P. aeruginosa has a greater number and diversity

of reports on the blaVIM genes incorporated into class 1 integrons, but *A. baumannii* has only a limited variety of gene cassette arrays where they have been found. Additionally, a class 2 integron in *A. baumannii* containing the blaVIM-2 gene has been found (GenBank accession number LC107606).It is unclear, therefore, whether the chromosomal position of the gene or the plasmid's incapacity to conjugate is to blame for this failure (56).

2.8 Emerging Therapeutic Strategies

2.8.1 Phage Therapy

Although phages are naturally occurring invaders of bacterial cells, research paradigms on phage therapy have shifted due to the rise of antibiotic resistance. The lysing activity against *A. baumannii*, the pathogenic bacteriophages AB1 and AB2.They also demonstrated their potential as disinfectants to control *A. baumannii* infections. Later research has demonstrated the effectiveness of phages in curing *A. baumannii* infections in numerous in vitro and in vivo trials (57). Phage therapy for *A. baumannii* infection in people was used with good outcomes using a combination of four phages. This inspired and encouraged the use of phage therapy as a treatment modality against a number of other infections. Capsular polysaccharide (CPS) depolymerase has been shown in a recent study to have antibiofilm activity against *A. baumannii* in vitro. The AB6 phage's tail spike protein (TSP), which contains the enzyme, was extracted. *A. baumannii* biofilms have been demonstrated to be susceptible to the antibacterial and antibiofilm properties of bacteriophage AB3 and its endolysin LysAB3. This is demonstrated by the reduction of viable cells in the biofilm. In a mouse model, a different investigation demonstrated the effectiveness of km18 phage therapy in reducing bacterial burdens; however, the therapeutic effects diminished with delayed delivery. A combination of two phages, one with lytic activity and the other with depolymerase activity, produced a phage cocktail that exhibited significant antibacterial and antibiofilm action against *A. baumannii* (58). Antibiotics such imipenem, gentamycin, tobramycin, and meropenem, along with environmental phage cocktail

combination therapy, significantly reduced the biomass of *A. baumannii* biofilms. The CRISPR system, which confers adaptive immunity and raises concerns when considering phages as a therapeutic alternative, makes it easier for bacterial cells to adopt phage resistance (59).

2.8.2 Photodynamic Therapy

The non-invasive, efficient, and quickly developing treatment method of photodynamic therapy (PDT) is used to remove biofilms. Due to the resistance era, PDT, an outdated technology, is currently being resurrected. When visible light and oxygen are present, biomolecules form a variety of reactive oxygen species (ROS) and a harmless chemical compound known as photosensitizer (PS). As a result, excessive ROS generation damages the matrix and the membrane by producing ROS in the membrane lipids, changing the outer membrane's permeability, or causing intracellular harm including organelle and DNA breakdown, which ultimately leads to cell death. The various PS for PDT methods that are available for treating bacterial infections(60). It has been shown that photodynamic therapy alone is not sufficient to eradicate the pathogen completely; however, when combined with antimicrobials like gentamycin, imipenem, and colistin, synergistic effects enhanced the number of PDR and XDR *A. baumannii* that were killed. Sublethal antimicrobial photodynamic treatment (aPDT) was found to cause a large increase in ompA expression in *A. baumannii*, perhaps as a means of making up for the damage caused and promoting bacterial survival. In addition to antimicrobials, studies have looked at using PDT in conjunction with natural substances like chitosan to successfully destroy *A. baumannii* cells linked with biofilms (61). Methylene blue demonstrated a reasonably large reduction in colony forming units, while protoporphyrin IX shown efficacy as an antibacterial photodynamic treatment photosensitizer against *A. baumannii* biofilms. This was demonstrated in another investigation. Recently, Ran et al. used Nile blue photosensitizer, which has strong antibacterial characteristics against *A. baumannii*, to strategically combine bacteriophage and PDT and successfully eliminate produced biofilms. While PDT is thought to be a dependable and safe therapy, more in vivo research is

needed to confirm this and boost PDT's effectiveness in treating biofilm infections (62).

2.8.3 Nanoparticle Therapy

The antibacterial and antibiofilm qualities of nanoparticles (NPs), such as cyclodextrins, NPs polymer, liposomes, metal NPs metal, and microemulsions have gained focus. The antibiofilm property of nanoparticles (NPs) stems from their small size, which enables them to penetrate deeper biofilm layers and engage in interactions with microorganisms to cause membrane disruption, inhibit metabolic pathways, inactivate enzymes, and alter gene expression, ultimately resulting in cell death (63). NPs can be engineered to target not just the microbe but also compounds that degrade EPS, such as chitosan, or to target EPS through an intrinsic feature that disrupts the matrix. Subinhibitory concentrations of nanoparticles of silver reduced *A. baumannii* growth and the expression of a number of virulence and biofilm-associated genes, such as kpsMII, afa/draBC, bap, ompA, and csuA/B. They also inhibited these genes' ability to build biofilms.This was validated by another research. By serving as a means of delivering drugs mechanisms or catalysts to improve medication uptake by biofilms, NPs can amplify the antibacterial effects. Before NPs are used to treat infections in humans, more in vivo research is necessary to address their cytotoxicity and safety concerns (64).

Chapter 3

Material and Methodology

The following section summarizes the methodological steps exploited to carried out the research.

3.1 Ethical Approval

The project summary was presented to departmental Ethical review committee and project was approved after discussions by Department of life and health sciences, capital university of sciences and technology, Islamabad

3.2 Samples Collection

Bacterial Samples were obtained from Nishtar Hospital Multan, Pakistan. The specimens were obtained from the sputum and blood of patients. The morphological and biochemical characterization of *Acinetobacter baumannii* isolates is an essential step in understanding and to identifying bacterial species.The isolates were performed to the Lab at the corresponding hospital for initial identification, which involved assessing their colony/cell morphology, doing biochemical and molecular characterization, as well as performing an antibiotic susceptibility assay.

3.3 Culturing the Isolates

The Isolate was inoculated onto MacConkey agar which were used as nutrient agar to enhance bacterial growth uniformly then colonies were picked and inoculated on CHROM-agar plates, which serve as a selective medium for *A. baumannii* species. After that, the plates were incubated at 37C. Following a 24-hour incubation period, observations were made on the form, colony size, texture, and margin. In order to achieve optimal growth, colonies of *A. baumannii* were subjected to re-culturing on nutrient agar plates. The colonies were chosen from the growth plates based on their size in order to harvest DNA and carry out subsequent processing. Colonies were picked on coal swabs which were sealed properly and stored at 4C.

3.4 The Isolates' Antibiotic Susceptibility Testing (AST)

Antibiotic susceptibility testing will be performed by disc diffusion method. A bacterial overnight broth culture having turbidity of 0.5 McFarland (turbidity standard) will be inoculated on MHA medium with the help of sterile cotton swab. The antibiotic discs such as piperacillin, imipenem, meropenem, Doripenem, minocycline, levofloxacin, ciprofloxacin, Amikacine, Gentamicin, Tobramycin, Tetracycline, Tazobactam will be put to MHA medium plates and given a 24-hour incubation period at 37C

3.5 DNA Extraction, Quantification, and Sequencing

The Solarbio DNA purification kit was employed for the purpose of DNA extraction. The quantity of DNA extracted from the isolate was determined through Qubit 2.0. fluorometer. The purity and quality were assessed via the utilization of Agarose Gel Electrophoresis. The DNA was diluted in TE buffer. Samples were

then sent for sequencing and the DNA was sequenced on Illumina Next generation sequencing 500 platform

3.6 Sequence Submission

The sequence information was then uploaded to the National Center for Biotechnology Information Archive (NCBI) with project Accession

3.7 Sequence Retrieval

TThe genomes of the isolated strains were downloaded from global complete genomes were retrieved from PATRIC database based on the host (human)

3.8 Assembly and QC Check

The initial step in the genome assembly process involved the assessment of the raw sequencing reads' quality using the FastQC software . The genome assembler SPAdes version 3.13.0 (https://cab.spbu.ru/software/spades/) was utilized to assemble the raw reads. SPAdes are a bioinformatic tool that has garnered considerable attention and widespread usage owing to its notable efficacy in the assembly of DNA sequences, particularly in the context of intricate genomes. SPAdes offer a precise approach for building short readings by employing a combination of de Bruijn graph and graph-based algorithms. This facilitates the ability of researchers to reconstruct genomic sequences and interpret genetic information with increased precision and enhanced continuity. The acquired data encompasses the aggregate number of contigs, genome magnitude, GC.content, and comprehensive coverage. The following parameters were determined: the total count of contigs, the size of genome, GC content, and the overall coverage. Assembled. genomes. Were then subjected for quality check through the quality evaluation tools QUAST, developed by the Center for Algorithmic Biotechnology (CAB).

QUAST (Quality Assessment) (https://cab.spbu.ru/software/quast/) utility for Genome Assemblies a bioinformatics is widely utilized for the purpose of evaluating the quality of genome assemblies. The tool offers comprehensive metrics and visualizations for evaluating the precision, comprehensiveness, and structural soundness of assembled genomes. The QUAST tool is particularly valuable for the purpose of comparing different assembly approaches and refining pipelines for the process of genome assembly. The minimum contig length option was set to ¿500, while the default values were utilized for the remaining parameters.

3.9 Specie Identification

After assembly and quality check genomes were run by FIDBC (http://fbac.dmicrobe) to confirm the species.

3.10 Genome Annotation

The compiled genomes underwent structural annotation using the Prokka pipeline with default settings. Prokka, a bioinformatics tool designed for precise and efficient annotation of bacterial genomes, streamlines the process through gene prediction, coding region identification, and functional annotation assignment to various genomic components. Widely recognized as an indispensable tool for researchers studying microbial genomes, Prokka's automated capabilities contribute to its notable efficiency. This annotation process yielded multiple output files, including a Protein FASTA file, a Nucleotide FASTA file, and an Annotation file in GFF3 format. Functional annotation was performed using the Rapid Annotation utilizing Subsystem Technology (RAST) server. The bioinformatics tool RAST (Rapid Annotation utilizing Subsystem Technology) serves as a comprehensive and user-friendly platform for genome annotation and analysis. This tool plays a crucial role in annotating microbial genomes, assisting researchers in uncovering genes, functional elements, and metabolic pathways embedded within DNA sequences.

RAST's automated and curated methods significantly accelerate the comprehension of microbial genetics and biology. The resulting output files are available in various formats, such as GenBank and EMBL.

3.11 Genome Analysis

3.11.1 MLST (Multilocus Sequence Typing)

The MLST Software version 2.0.9 was utilized to analyze the contig sequences of one isolate. Renowned as a cutting-edge bioinformatics tool, MLST Software 2.0.9 is widely employed for Multi-Locus Sequence Typing (MLST) analysis. This updated version enhances precision and effectiveness in characterizing genetic diversity among microorganisms, making it an indispensable instrument for researchers and physicians investigating microbial evolution and epidemiology. Seven housekeeping genes (cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB) were used in the investigation, which made use of the Institute Pasteur scheme (MLST-IP). to determine the Allelic profiles or Sequence Types (STs) of each isolate. Profile validation was conducted through PubMLST (Public Multilocus Sequence Typing), a robust bioinformatics tool for microbial strain typing and epidemiological studies. PubMLST facilitates the evaluation of genetic differences in multiple genes, enabling the monitoring and understanding of infectious disease transmission. Moreover, PubMLST fosters collaboration and data exchange within the scientific community through standardized and publicly accessible databases.

3.11.2 Phylogenatic Analysis

To enhance the understanding of genetic variants and evolutionary connections, the CSI Phylogeny 1.4 program from the Center of Genomic Epidemiology was employed. This bioinformatics tool is widely embraced in evolutionary research, showcasing expertise in constructing phylogenetic trees and exploring genetic relationships among organisms. The phylogenetic relationships among the four genomes,

along with fifteen other global strains and one reference genome, were established using this software. Subsequently, the Interactive Tree of Life (iTOL) web program (version 6.8) was utilized to visualize the output Newick file. iTOL version 6.8 (https://itol.embl.de/tree/395823044382491703954690) stands out as a state-of-the-art bioinformatics application commonly employed to visualize and annotate phylogenetic trees and hierarchical data. Its online accessibility, extensive customization options, and interactive features make it indispensable for researchers and scientists exploring and communicating complex evolutionary relationships and taxonomy. The finalized tree was rendered and saved in PNG.

3.11.3 Identification of Virulence Genes

The identification of virulence factors involved analyzing the genomes of one isolated strain using VFDB , a comprehensive database dedicated to cataloging virulence factors. VFDB, or the Virulence Factor Database, is a computational tool developed for a thorough analysis of bacterial virulence factors. It serves as a repository of well-organized data on pathogenicity-related genes, proteins, and functional annotations. Researchers can leverage VFDB to deepen their understanding of the molecular processes underlying bacterial infections and to formulate preventive and therapeutic strategies. The analysis utilized an input file in Nucleotide FASTA format, and a reference genome was chosen for comparison purposes. The outcomes were obtained and documented in an Excel spreadsheet as the final output.

3.11.4 Identification of Resistance Genes

The antibiotic resistance determinants within the sequenced genomes of isolates were identified using the CARD database. The Comprehensive Antibiotic Resistance Database (CARD) (https://card.mcmaster.ca/rgi/results/brf3f0-JifBo KcMiXbOfc8HC5yR1qZnnEXjz9EIVw) is a specialized bioinformatics tool designed to advance the investigation and analysis of antibiotic resistance. This platform provides a comprehensive compilation of carefully curated data related to

antibiotic resistance genes, their associated proteins, and the molecular pathways contributing to antibiotic resistance. Scientists can leverage the Comprehensive Antibiotic Resistance Database (CARD) as a valuable resource for examining and interpreting genetic information related to antibiotic resistance. This tool plays a pivotal role in improving understanding and facilitating the mitigation of this urgent global health issue. The Resistance Gene Identifier version 2 was employed to access the CARD database.

3.12 Overview of Methodology

The overview of methodology acquired is represented in figure 3.1

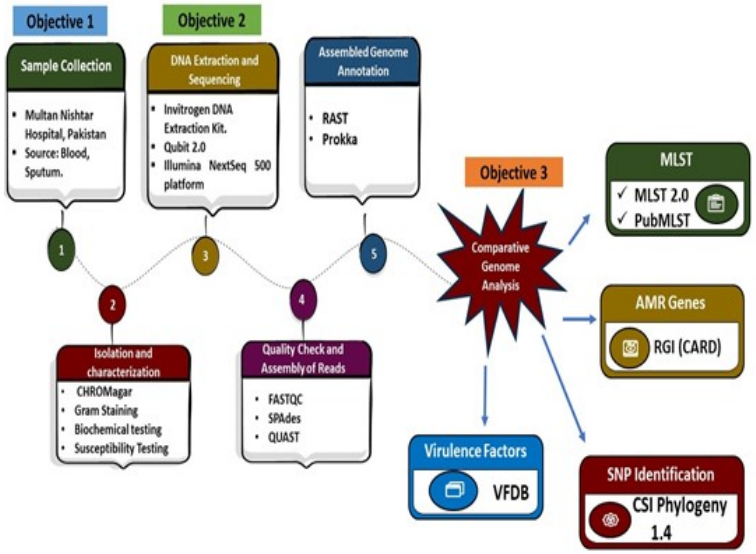

FIGURE 3.1: Methodology used for Comparative Genome Analysis.

Chapter 4

Results

4.1 Morphological and Biochemical Characterization of *A. baumannii* Isolates

The morphological and biochemical characterization of *Acinetobacter baumannii* isolates is an essential step in understanding and identifying this bacterial species. Here are some general characteristics and methods commonly used for the morphological and biochemical characterization of *A. baumannii*. Morphological characterization of colony is Red on CHROMagar and Pale yellow on Nutrient agar. Its form is smooth and texture is Mucoid. Microscopy and Biochemical Tests in *A. baumannii* is a Gram-negative bacterium. Its oxidase test is negative and catalases test is positive producing bubbles when hydrogen peroxide is added.

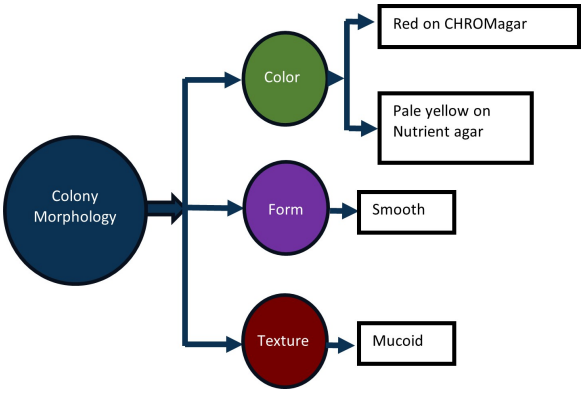

FIGURE 4.1: Flowchart of Morphological colony.

TABLE 4.1: Biochemical Characterization of *A. baumannii*..

Biochemical	Characterization
Gram Staining	Gram Negative
Catalase Test	Catalase positive
Oxidase Test	0Oxidase negative

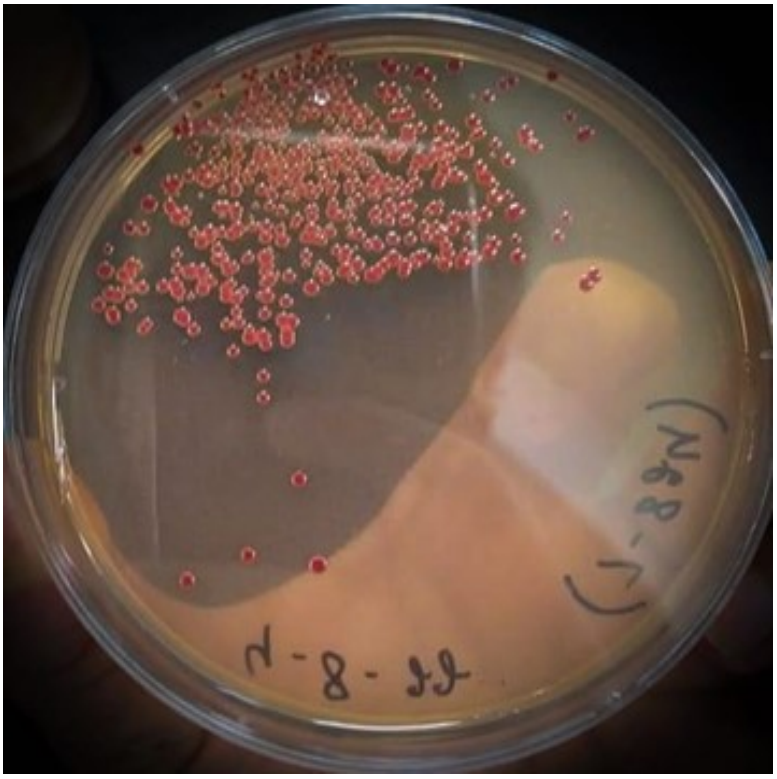

FIGURE 4.2: Morphological characterization of colony

4.2 Phenotypic antibiotic resistance of the isolates

TThe strain BAC8924 exhibited resistance to a variety of antibiotics. Specifically, it displayed resistance to the following antibiotics: Piperacillin, Imipenem, Meropenem, Doripenem, Minocycline, Levofloxacin, Ciprofloxacin, Amikacin, Gentamicin, Tobramycin, Tetracycline, Tazobactam. This antibiotic resistance profile indicates that BAC8924 is resistant to a broad spectrum of antibiotics, including beta-lactams, carbapenems, fluoroquinolones, aminoglycosides, and tetracyclines. Monitoring and understanding the antibiotic resistance patterns of bacterial strains like BAC8924 are crucial for effective clinical management and public health efforts.

TABLE 4.2: Phenotypic and genotypic antimicrobial resistance profile of local *Acinetobacter baumannii* strains BAC8924

Antibiotic	**Disk Contents**	**BAC8924 PR**	**BAC8924 GR**
Piperacillin	10μ g	R	ND
Tazobactam	100/10 μ g	R	ND
Imipenem	10 μ g	R	adeN, OXA-66, adeI, adeJ
Meropenem	10 μ g	R	adeN, OXA-66, adeI, adeJ
Doripenem	10 μ g	R	adeN, OXA-66, adeI, adeJ
Minocycline	30 μ g	R	adeN, adeA, adeB, adeC
Levofloxacin	5 μ g	R	rsmA, adeN, adeI, adeJ
Ciprofloxacin	5 μ g	R	rsmA, AbaQ
Amikacin	30 μ g	R	APH(3")-Ib, ANT(3")-IIc
Tobramycin	10 μ g	R	APH(3")-Ib, ANT(3")-IIc

4.3 Genomic Characteristics

The de novo assembly of BAC8924 Illumina reads resulted in 116 contigs, each exceeding 500 base pairs in length. The overall GC content of the assembly is 38.87%. The assembly statistics include an N50 value of 88,343, N75 value of 59,298, L50 value of 11, and L75 value of 25. The longest contig in the assembly is 459,641 base pairs. The total genome size of BAC8924 is determined to be 4,017,350 base pairs. Furthermore, the annotation of the BAC8924 genome identified a total of 3,817 predicted coding sequences (CDS). Additionally, BAC8924 is found to harbor 49 tRNA genes and 3 rRNA genes within its genomic content.

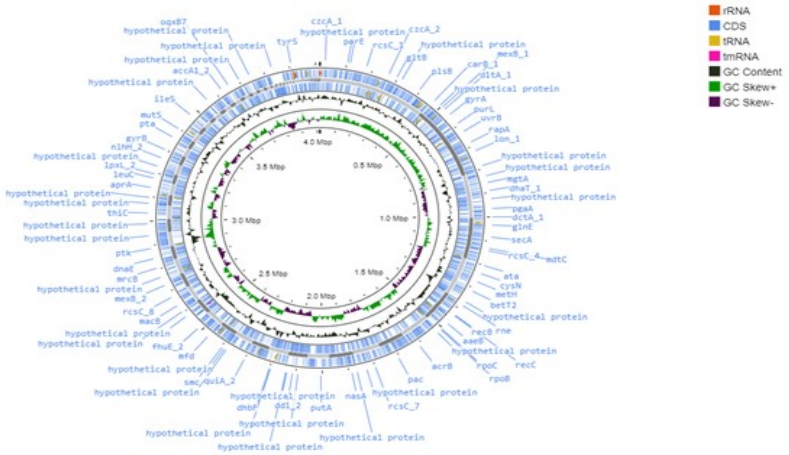

FIGURE 4.3: Circular visualization of whole genome of *A. baumannii*

TABLE 4.3: Characteristics of BAC8924

Characteristic	**Value**
Organism	*A. baumannii*
Genome size (bp)	4017350
Contigs	116
GC content %	38.87%
N50	88343
N75	59298
L50	11
L75	25
Longest contig (bp)	459641
CDS	3817
tRNA	49
rRNA	3
tmRNA	1
ST	2
NCBI Accession number	SAMN 3916321

4.4 Whole Genome of BAC8924

A reference genome constitutes a thoroughly investigated and annotated genome of either the same species or a closely related one. In the context of *A. baumannii*, a suitable reference genome could be derived from a well-characterized strain. The provided figure illustrates the alignment of BC8924 with reference genomes submitted to the NCBI database. In the figure, the brown color highlights the reference genomes, while the black color represents the GC content. The purple color indicates positive GC skew, and the green color signifies negative GC skew. Additionally, blue denotes coding sequences (CDS), pink highlights assembly gaps, and parrot color is used to represent tmRNA.

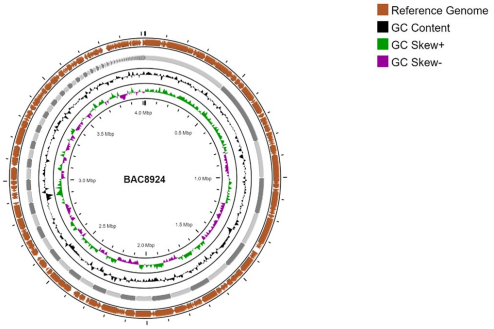

FIGURE 4.4: BAC8924 with references genome

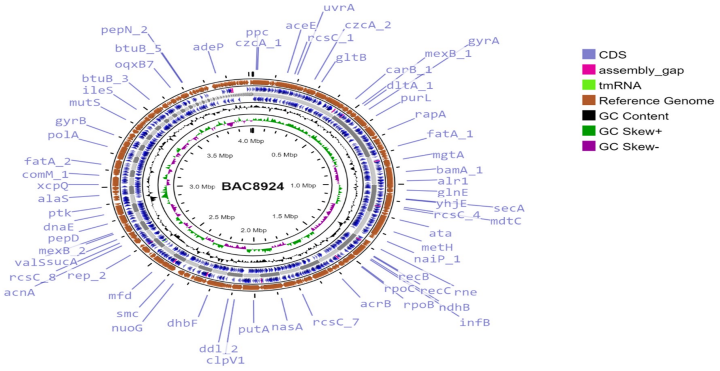

FIGURE 4.5: whole genome sequences analysis of BAC8924.

4.5 Specie Identification

After assembly and quality check genomes were run by fbac.dmicrobe.cn to confirm the species. The specie name is *Acinetobacter baumannii*. Taxonomy are Bacteria, Proteobacteria, Gammaproteobacteria, Pseudomonadales, Moraxellaceae; Acinetobacter.

TABLE 4.4: Taxonomic Hierarchy

Taxonomic Level	**Classification**
Taxonomy	Bacteria Proteobacteria Gammaproteobacteria Pseudomonadales Moraxellaceae Acinetobacter

4.6 Sequence Retrieval

A total of 216 complete genome sequences of *A. baumannii* were retrieved from PATRIC reported from around the world on 19th Dec 2023. Also 30 draft genomes of *A. baumannii* from Pakistan were retrieved from PATRIC database on the same date. The *A. baumannii* strains included in the study were all reported as MDR and causing infections in human host.

4.7 Multi Locus Sequence Typing of *A. baumannii* Isolates.

The strain BAC8924 belongs to ST2, according to the genomic analysis. All 216 *A. baumannii* samples were subjected to MLST analysis using the Pasteur scheme, which includes seven housekeeping genes: cpn60, fusA, gltA, pyrG, recA, rplB, and rpoB. Allelic profiles were obtained for each strain, and strains with known STs were assigned new STs. MLST analysis revealed that 360 strains that show high genetic diversity between *A. baumannii* genomes. The most frequent sequence type was ST2 (n=269) but none of the isolated strains belong to sequence type 2. Among other STs shared by *A. baumannii* isolates, the most frequently encountered were ST1 (84 isolates), ST437(13 isolates), ST10 (9isolates), ST57 (10isolates), ST570 (5 isolates) ST85 (8 isolates), ST78(11 isolates), ST79(6 isolates), ST622(12 isolates) ST636(13 isolates), ST40(8 isolates), ST16(8 isolates), ST422(11 isolates), ST49(14 isolates) and ST72(7 isolates).

TABLE 4.5: Multi Locus Sequence Type of selected *A. baumannii* strains

Seven Housekeeping Genes	**Sequence Types**	**Number of Isolates**
cpn60, fusA, gltA,	ST2	269
	ST1	84
pyrG, recA, rplB	ST437	13
	ST10	9
and rpoB	ST57	10
	ST636	5
	ST85	8
	ST78	11
	ST79	6
	ST622	12
	ST636	13
	ST40	8
	ST16	8
	ST422	11
	ST49	14
	ST72	7

4.8 Identification and Comparative Analysis of Antibiotic Resistance Genes

Several antibiotic Resistance Genes were identified through RGI of Comprehensive Antimicrobial Resistance Database (CARD) in isolated strain of *A. baumannii*. Which cause resistance to several class of drugs. 30 resistance genes were predicted in BAC8924 respectively.

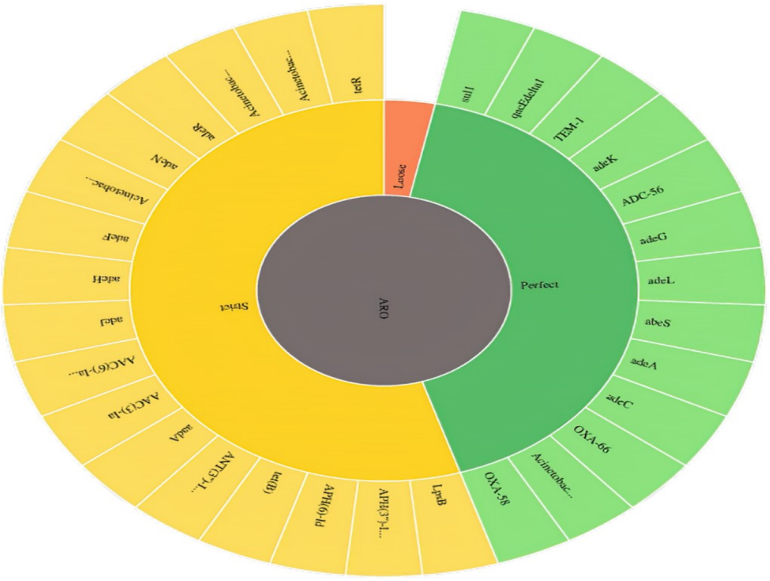

FIGURE 4.6: Indicates the resistance genes found in isolates of *A. baumannii*..

Which show resistance towards multiple class of antibiotics like sulfonamide antibiotic (sul1 , sul1) disinfecting agents and antiseptics (qacEdelta1, qacEdelta1, *Acinetobacter baumannii* AmvA) monobactam, cephalosporin, penam, penem (TEM-1) macrolide antibiotic, fluoroquinolone antibiotic, lincosamide antibiotic, carbapenem, cephalosporin, tetracycline antibiotic, rifamycin antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic, penem (adeI, adeK, adeG, adeF, adeJ, adeK) cephalosporin (ADC-56) macrolide antibiotic, aminocoumarin antibiotic (abeS) carbapenem, cephalosporin, penam (OXA-66, OXA-58) peptide antibiotic (LpsB) aminoglycoside antibiotic (APH(3")-Ib, AAC(3)-Ia, ANT(3")-IIc, aadA, APH(6)-Id, AAC(6')-Iaf) tetracycline antibiotic(tet(B), *Acinetobacter baumannii* AmvA, tetR) fluoroquinolone antibiotic, tetracycline antibiotic(adeH, adeE, adeN, adeR, adeJ) fluoroquinolone antibiotic(parC, gyrA).Several antimicrobial

gene families encoding for MFS, RND and SMR antibiotic efflux pump are also identified providing resistance against macrolide, fluoroquinolone, cephalosporin, cephamycin, penam and tetracycline antibiotics. Resistance mechanism adopted by *A. baumannii* against antibiotics includes antibiotic efflux, antibiotic target replacement, antibiotic inactivation and reduced permeability to antibiotics. The resistance profile of the above BAC8924 strains have been compared with global publicly available strains which was selected on the base of sequence typing ST profile.

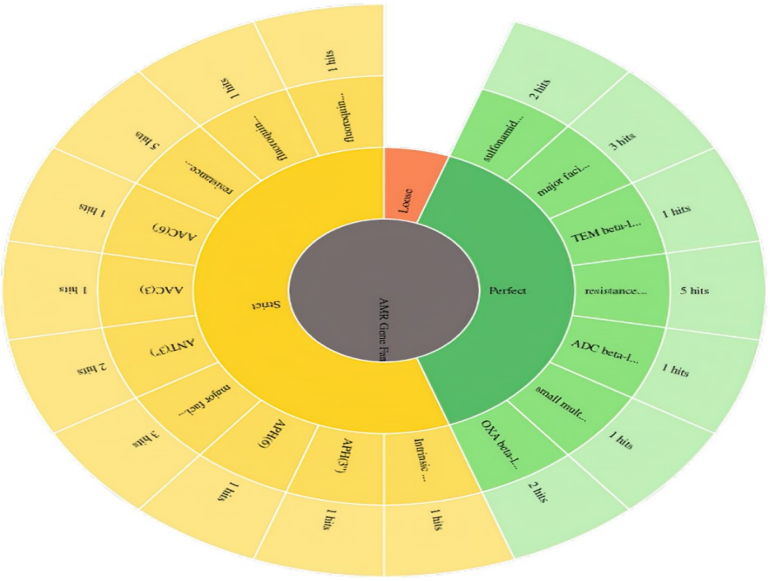

FIGURE 4.7: Indicates the family of resistance genes found in isolates of *A. baumannii*.

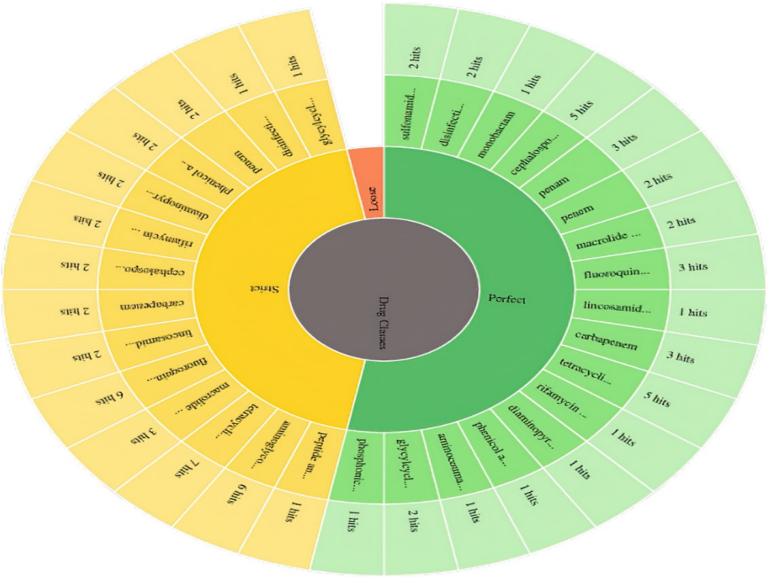

FIGURE 4.8: Drugs class against which *A. baumannii* isolates are resistant.

Table 4.6: Resistance genes, drug class, and mechanism of resistance in *A. baumannii* isolates

ARO. Term	AMR. Gene Family	Drug Class	Resistance Mechanism
sul1	sulfonamide resistant sul	sulfonamide antibiotic	antibiotic target replacement
qacEdelta1	MFS antibiotic efflux pump	disinfecting agents and antiseptics	antibiotic efflux
TEM-1	TEM beta-lactamase	monobactam, cephalosporin, penam, penem	antibiotic inactivation
adeK, adeJ, adeN	RND antibiotic efflux pump	multiple antibiotics	antibiotic efflux
ADC-56	ADC beta-lactamase	cephalosporin	antibiotic inactivation
adeG, adel, adeH	RND antibiotic efflux pump	fluoroquinolone, tetracycline	antibiotic efflux
abeS, AmvA	SMR antibiotic efflux pump	macrolide, aminocoumarin	antibiotic efflux
adeA, adeC, adeR	RND antibiotic efflux pump	glycylcycline, tetracycline	antibiotic efflux
OXA-66, OXA-58	OXA beta-lactamase	carbapenem, cephalosporin, penam	antibiotic inactivation
AbaF	MFS antibiotic efflux pump	phosphonic acid antibiotic	antibiotic efflux
LpsB	Intrinsic peptide resistant Lps	peptide antibiotic	reduced permeability
parC, gyrA	fluoroquinolone resistant	fluoroquinolone antibiotic	target alteration
ANT, aadA, AAC			
APH, APH, APH	aminoglycoside antibiotic	antibiotic inactivation	
tetR, tet(B)	MFS antibiotic efflux pump	tetracycline antibiotic	target alteration, antibiotic efflux

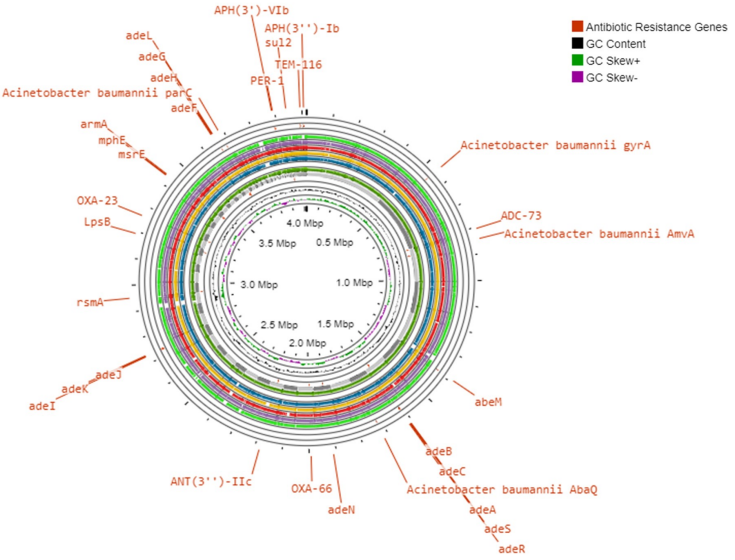

FIGURE 4.9: Circular visualization of BAC8924 comparative analysis of antibiotic resistance determinants via GC Viewer. Black color shows the GC contents, green color show positive GC skew and purple color show negative GC skew and red color show antibiotic resistance determinants

*SMR; Small multidrug resistance antibiotic efflux pump

*MFS; Major facilitator superfamily antibiotic efflux pump

*RND; Resistance-nodulation-cell division antibiotic efflux pump This heatmap show the comparison of all available common genes with global strain like sulfonamide antibiotic (sul1 , sul1) disinfecting agents and antiseptics (qacEdelta1, qacEdelta1, *Acinetobacter baumannii* AmvA) monobactam, cephalosporin, penam, penem (TEM-1) macrolide antibiotic, fluoroquinolone antibiotic, lincosamide antibiotic, carbapenem, cephalosporin, tetracycline antibiotic, rifamycin antibiotic, diaminopyrimidine antibiotic, phenicol antibiotic, penem (adeI, adeK, adeG, adeF, adeJ, adeK) cephalosporin (ADC-56) macrolide antibiotic, aminocoumarin antibiotic (abeS) carbapenem, cephalosporin, penam (OXA-66, OXA-58) peptide antibiotic (LpsB) aminoglycoside antibiotic (APH(3")-Ib, AAC(3)-Ia, ANT(3")-IIc, aadA, APH(6)-Id, AAC(6')-Iaf) tetracycline antibiotic(tet(B), *Acinetobacter baumannii* AmvA, tetR) fluoroquinolone antibiotic, tetracycline antibiotic(adeH, adeE, adeN, adeR, adeJ) fluoroquinolone antibiotic(parC, gyrA).This purple color shows the 0% prevelances, yellow color show 100% prevelance and sky blue color shows 60% prevelances.

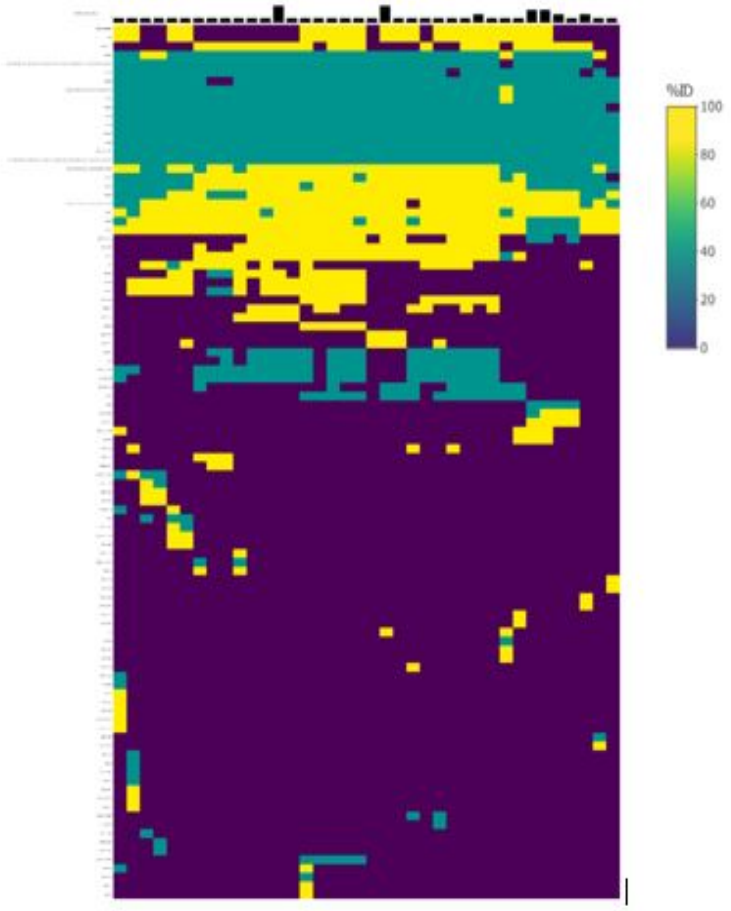

FIGURE 4.10: Heatmap of BAC8924 comparative analysis of antibiotic resistance determinants

4.9 Identification and Comparative Analysis of Virulence genes

Genomes of isolated strains were checked for both structural and secreted virulence factors and it was determined that all of the strains carry different number of virulence genes i.e. strains BAC8924 carry 48 genes respectively. This includes genes clusters encoding for Adherence, Biofilm formation, Enzyme, Immune evasion, Iron uptake, Regulation Serum resistance and Stress adaptation etc. the adherences virulence factors are Outer membrane protein and LPS O-antigen (P. aeruginosa) (Pseudomonas) Biofilm formation virulence factors are Biofilm-associated protein, Csu pili and PNAG (Polysaccharide poly-N-acetylglucosamine). Enzyme virulence factors are Phospholipase C, Phospholipase D. Immune evasion virulence factors are Capsule. Iron uptake virulence factors are Acinetobactin and Heme

utilization. Regulation virulence factors are Quorum sensing and Two-component system. Serum resistance virulence factors are PbpG. Stress adaptation virulence factors are Catalase (Neisseria). These virulence factors were further compared with global isolates. The predicted virulence factors in *A. baumannii* isolates were common between global strains and local isolates. A comparison of virulence factors of several *A. baumannii* given in figure.

The core-genome phylogenetic analysis of a global strain set in *A. baumannii* demonstrates that the phylogenetic relationships inferred from the core-genome align with the phylogenetic tree based on globally distributed SNPs. This consistency reinforces the reliability of the phylogenetic relationships determined using core-genome data. The phylogenetic tree was examined in further detail together with a matrix that showed which *A. baumannii* genomes included core and auxiliary genes. The findings of this analysis showed that the number of unique genes increased in tandem with the number of genomes. This discovery suggests that *A. baumannii*'s genomic repertoire extends with the incorporation of other genomes, indicating an open pangenome. The fact that the total number of genes is rising in tandem with the increasing number of *A. baumannii* genomes highlights how dynamic the genomic content of the species is. The open pangenome idea emphasizes the diversity and adaptability found in the worldwide *A. baumannii* population and represents the continuous acquisition of new genetic components

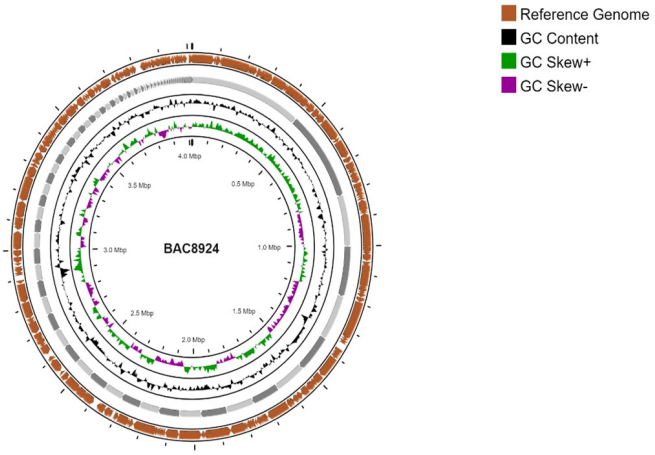

FIGURE 4.11: Circular visualization of Virulence genes with reference genom

TABLE 4.7: Virulence's Factor A. baumannii isolates.

VF class	Virulence's Factor	Related Genes
Adherence	Outer membrane protein LPSO	ompA (Pseudomonas) (Pseudomonas)
Biofilm formation	Biofilm-associated protein, Csu	adeF, adeG, adeH, bap csuA/B, csuA, csuB csuD, csuE, pgaA, pgaB
Enzyme	Phospholipase C, PhospholipaseD	plc, plcD
Immune evas	Capsule	Undetermined
Iron uptake	Acinetobactin Heme utilization	barA, barB, basA, basB, basC, basD, basF, basG, basH, basI, basJ, bauA, bauB, bauC, bauD bauF, ebtE, hemO
Regulation	Quorum sensing, Two-compont	abaL, abaR, bfmR
Serum resis	PbpG	PbpG
Stress adapt	Catalase (Neisseria)	katA

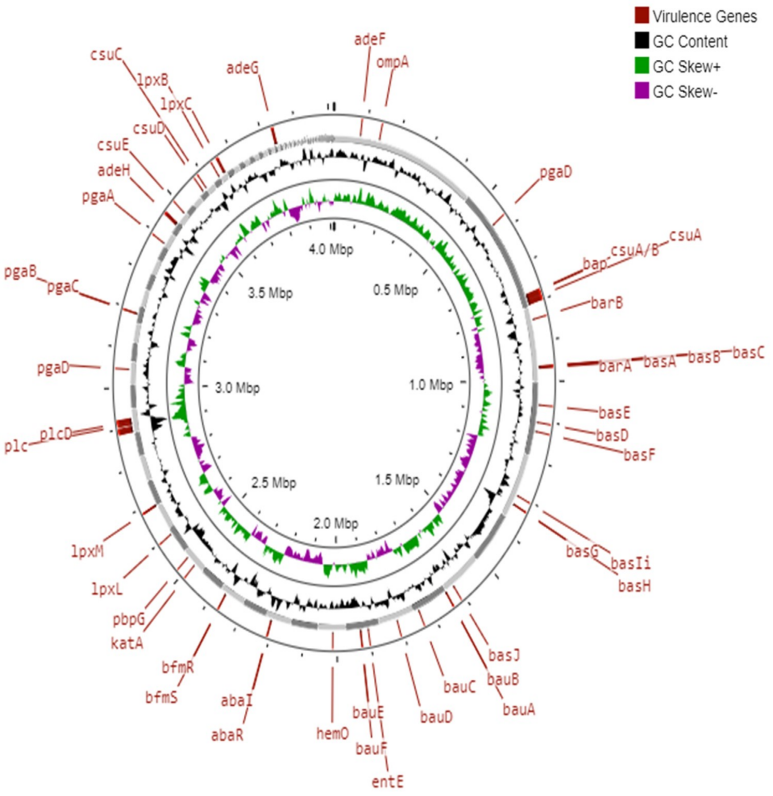

FIGURE 4.12: Circular visualization and Comparative Analysis of Virulence genes

4.10 Phylogenetic analysis

The SNP-based phylogeny revealed the presence of two main subclades. Notably, the first subclade encompassed three genomes that shared a clade, namely BAC8924, CAb65, CAM1801, and 361512. The phylogenetic analysis, which was based on the alignment of concatenated SNPs, demonstrated overall agreement with the global patterns detected in this study. The observed subclades and their composition, particularly the grouping of BAC8924, CAb65, CAM1801, and 361512 in the first subclade, were consistent with the broader phylogenetic trends identified globally.it is classified as one of the ESKAPE infections. Because of this, the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have identified it as a serious global health issue. By analyzing 216 genomes of *A. baumannii*, this study reveals important information on the genetic variations and mechanisms of resistance in this bacterium. The pangenome analysis offers a concise representation of the genomic terrain. This suggests that the genetic relationships inferred from the alignment of concatenated SNPs are in accordance with the evolutionary patterns observed on a larger scale within the *A. baumannii* population under investigation.

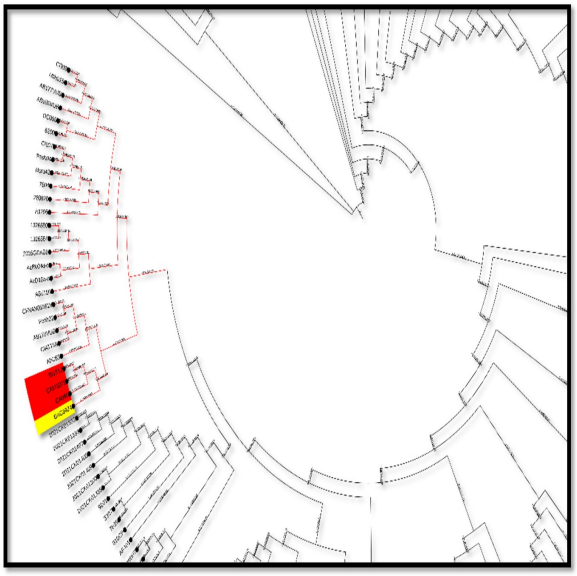

FIGURE 4.13: the grouping of BAC8924, CAb65, CAM1801, and 361512 in the first subclade, were consistent with the broader phylogenetic trends identified globally

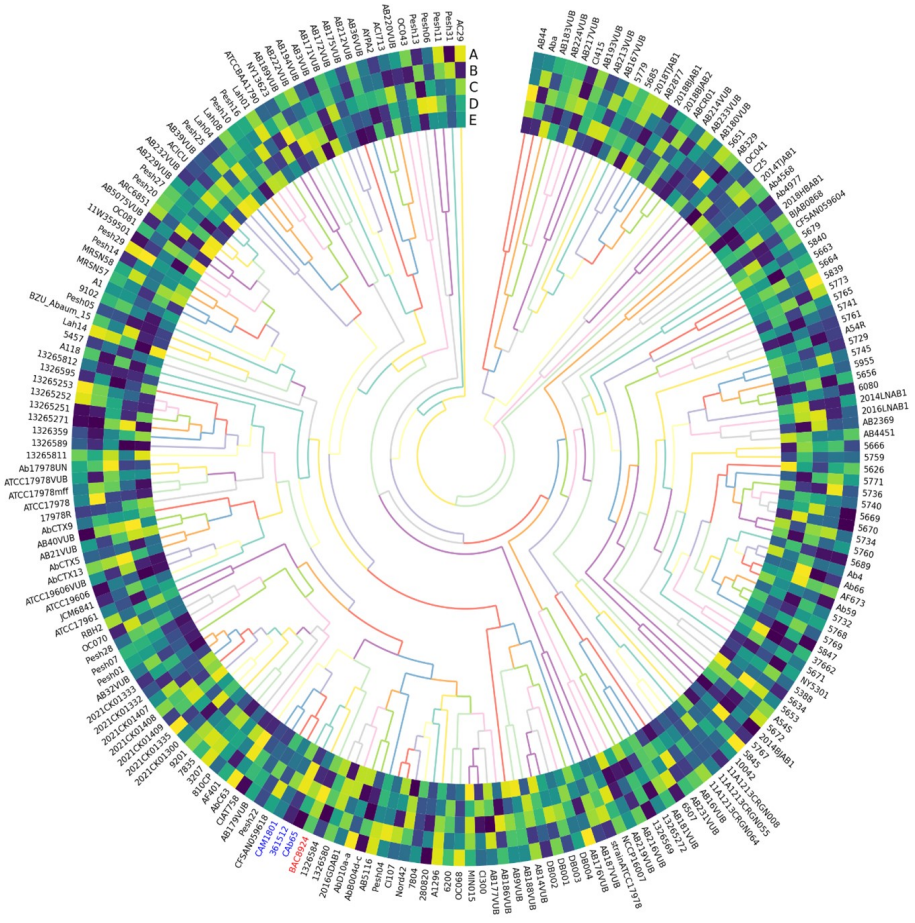

FIGURE 4.14: Circular visualization of SNP phylogenetic tree of BAC8924

Chapter 5

Conclusion and Future Prospects

This study offers a thorough analysis of the genetic composition, antibiotic resistance mechanisms, and virulence factors of Multi-Drug Resistant (MDR) *A. baumannii*. Bacteria with a coccobacillus-like morphology that react negatively to the Gram stain are known as *A. baumannii*. It is well known for its capacity to seize opportunities and spread diseases in a variety of contexts, most notably medical ones. Because *A. baumannii* is resistant to antibiotics, it is classified as one of the ESKAPE infections. Because of this, the World Health Organization (WHO) and the Centers for Disease Control and Prevention (CDC) have identified it as a serious global health issue. By analyzing 216 genomes of *A. baumannii*, this study reveals important information on the genetic variations and mechanisms of resistance in this bacterium. The pangenome analysis offers a concise representation of the genomic terrain. *A. baumannii*'s resistance profile is far more serious than previously thought, as revealed by the identification and comparative analysis of antibiotic resistance genes. The isolated strains have predicted resistance genes that span several drug classes, such as aminoglycosides, tetracyclines, cephalosporins, carbapenems, phenicols, and tetracyclines. The analysis of worldwide strains demonstrates the presence of both common and distinct resistance genes, indicating regional variations. The high occurrence of resistance-associated genes such as AbaQ, AbaF, abeS, adeL, and adeI underscores the necessity for antibiotic stewardship programs tailored to specific regions. An extensive range of variables contribute to the pathogenicity of *A. baumannii*, as demonstrated by

virulence gene analysis. The presence of genes linked to immune evasion, biofilm formation, adhesion, and iron uptake improves our comprehension of the processes by which *A. baumannii* causes infections. These factors can contribute to the processes of evolution, adaptation, or interactions with the host environment. The study's importance rests in its potential to influence infection control tactics and therapeutic procedures. The genomic variety, resistance mechanisms, and virulence variables that have been uncovered provide a foundation for precise and focused therapies. Further investigation could focus on the creation of antibiotic guidelines tailored to individual regions, taking into account the distinct resistance profiles. The study's results highlight the pressing necessity for ongoing monitoring and investigation to proactively address *A. baumannii*'s adaptive tactics. Effective infection control strategies and antibiotic stewardship programs require collaborative efforts among researchers, doctors, and policymakers. Ultimately, this study offers a thorough comprehension of MDR *A. baumannii*, by integrating genetic studies with functional observations.The SNP-based phylogeny revealed the presence of two main subclades. Notably, the first subclade encompassed three genomes that shared a clade, namely BAC8924, CAb65, CAM1801, and 361512. The phylogenetic analysis, which was based on the alignment of concatenated SNPs, demonstrated overall agreement with the global patterns detected in this study. The observed subclades and their composition, particularly the grouping of BAC8924, CAb65, CAM1801, and 361512 in the first subclade, were consistent with the broader phylogenetic trends identified globally. our local BACK8924 are closely related global strain from Malysia, Combodia, Russia. These strains also shares common virlence and resistance profile. The genetic relationships inferred from the alignment of concatenated SNPs are in accordance with the evolutionary patterns observed on a larger scale within the *A. baumannii* population under investigation.The genetic diversity, antibiotic resistance, and virulence variables that have been identified provide a basis for future research and intervention efforts. This work offers practitioners and researchers important direction in addressing the increasing issue of *A. baumannii*-caused multidrug-resistant infections in healthcare settings. As the antibiotic resistance is increasing day by day because of the excessive use of antibiotics. Therefore, alternative strategies need to be adopted to

overcome the challenge. Vaccine development and combination therapy can play their role in combating the challenge. For better understanding of *A. baumannii* more genomes would be made available to study genetic diversity and population structure. Also, appropriate surveillance of *A. baumannii* will be needed to determine its prevalence in the region, routes of infection and local antibiotics resistance trends.

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