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Isolation and Identification of Prevalent Microbial Pathogen From Domestic Kitchen Associated with Housefly

by

Samra Bibi

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Dedicated to **Allah Almighty**, **Hazrat Muhammad (S.A.W.W)** and my family.My grandmother and grand father prayers have always enlightened my way throughout my life. It's also dedicated to my mother,father and uncle who taught me that the best kind of knowledge to have is that which is learnt for its own sake. They taught me that even the largest task can be accomplished if it is done one step at a time.

CERTIFICATE OF APPROVAL

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Abstract

Houseflies *Musca domestica* are found everywhere and are vector for many diseases. *M. domestica* lives as commensals with animals and complete its life cycle within habitations of humans and domestic animals. The structure of fly is excellent adapted to carry pathogens and collect them. Pathogens varies in its characteristics depending on the area of collection. Houseflies carry different pathogens comprising of *Salmonella spp, E. coli spp* and *Staphylococcus* etc. Houseflies were collected by using Nylon net from domestic kitchen of Chakwal city. Pathogenic bacteria were isolated from the housefly and were cultured on nutrient agar and on differential media.

Biochemical characterization was done by Gram staining, Urease test, Catalase test, Citrate test and was confirmed using API kit20E. The prevalent isolated pathogens were *E. coli, Staphylococus, Klebsiellla spp.* based on biochemical characterization. 16S RNA sequencing was performed for one of the prevalent strain and suggested its close association with *Staphylococcus xylosus*. The phylogenetic analysis of the prevalent strain concluded a similarity of 99.9% with *Staphylococcus xylosus*. The strain antibiotic sensitivity was checked against nine antibiotics Cefotaxime, Chloramphenicol, Ciprofloxacin, Fusidic acid, Gentamycin, Imipenem, Nalidixic acid, Norfloxacin and Tazobactam. This strain showed 80%resistance against Cefotaxime, 86.60% against Ciprofloxacin and 93.30% resistance against Gentamycin. This strain showed least resistance against Imipenem with 93.3% sensitive with no intermediate values. Tazobactam was sensitive with 86.60%. This strain sequence was submitted to NCBI. Exposure of houseflies to animal farming and human habitats has led to greater prevalence of antibiotics resistant bacteria.

Contents

List of Figures

List of Tables

Abbreviations

AMR	Antimicrobial resistance
API	Analytical Profile Index
BLAST	Basic Local Alignment Search Tool
C. coli	*Campylobacter coli*
C. jejuni	*Campylobacter jejuni*
CoNS	Coagulase-negative staphylococci
CoPS	Coagulase-positive staphylococci
e.coli	*Escherichia coli*
EMB	Eosin Methylene Blue Agar
H. pylori	*Helicobacter pylori*
M.domestica	*Musca domestica*
Macc	MaCconkey Agar
MDR	Multidrug resistance
MEGAX	Molecular Evolutionary Genetics Analysis
MRSA	Methicillin-resistant Staphylococcus aureus
MSA	Mannitol Salt Agar
NCBI	National Center for Biotechnology Information
PM	Peritophic matrix
S. aureus	*Staphylococcus aureus*
S. enterica	*Salmonella enterica serovar enteritidis*
S. epidermidis	*Staphylococcus epidermidis*
S. saprophyticus	*Staphylococcus saprophyticus*
S. sciuri	*Staphylococcus sciuri*
S. typhimurium	*Salmonella typhimurium*

S. xylosus	*Staphylococcus xylosus*
TSB	Tryptic soy broth
T.trichiura	*Trichuris trichiura*
UPGMA	Unweighted Pairwise Groups with Arithmetic Mean

Chapter 1

Introduction

1.1 Background

Musca domestica commonly called as housefly mostly mentioned as filth flies [1]. From the start of human life houseflies also learnt how to live in association with humans [2, 3]. Housefly [*Musca domestica*] belongs to order Diptera and family Muscidae. They are cosmopolitan in nature present worldwide [4, 5].

Their habitat includes decaying organic matter like animal manure, litter and animal bedding, where they undergo reproduction and development. *M. domestica* belongs to specie that is endophilic and syanthropic in nature which means that it completes its lifecycle in human habitats [6].

The environment is contaminated by these degrading flies with antimicrobial resistant bacteria. The fly adapt in any environment with that of humans and animals as well as its maggots these all are enriched with organic matter and possess microbial flora [7]. Houseflies are abundantly present all over the places including home, public places, clinics, food areas and animal houses creating annoyance to everyone including poultry animals and other organisms. It behaves as a very strong vector causing numerous diseases[8].

Male and female houseflies have the ability to feed on any kind of fruit or food, debris, garbage, sweat, and animal dung [2]. The adult house flies are highly mobile and they carry the bacteria from septic environment by coming in contact with these surfaces like wings, feet, bodies [5]. Interactions that are actually present between the microorganism and the host is categorized as the source of nature for modulating animal physiology, fitness and social behavior of host [9–11]. Flies adapt 4 ways to transmit diseases as the surface of its body, vomitus regurgitation, the hair and alimentary canal passages [7].

The structure of fly is excellent adapted to carry pathogens and collect them. It has a profusion of hairs that have the ability to collect environment detritus. Bacteria can be isolated from external surfaces, internal surfaces, vomitus, feces of the house fly samples [12]. The maggot of fly are rich in microbial flora [7] They can carry pathogens physically as they have exoskeleton made up of cuticle and that of the double layer Type 11 peritrophic matrix [PM], that help to provide site of attachment [13].

The houseflies got a prime importance as the main agent for the transmission of certain diseases. According to U.S. Food and Drug Administration, houseflies act as a tributary factor for the dispersion of various infectious and other food borne diseases [14]. Houseflies have the ability to carry 100 agents of different etiological diseases like viral, bacterial and protozoan diseases [14,15] The diseases caused by the houseflies as vector include diphtheria, dysentery, intestinal parasites, typhoid, leprosy, and fowl cholera [2].

Molecular analysis depicted that groups of microorganisms are being dispersed by house flies [16]. On the basis of evidence it is found that *M. domestica* play an important role in the transmission of diseases, It is found that risk of diarrhea is more in the area where the number of flies increases suggesting the link between its transmission by housefly [17–20]. Life-threatening diseases in humans and animals are caused by pathogens that are carried by house fly. Association of more than 100 pathogens like bacteria, viruses, fungi and parasites [protozoans and metazoans] have been found with the house fly [21, 22].

Allergy cases that occurred due to the houseflies are rare but respiratory allergy due to the occupational exposure have been listed [23]. The allergens collected from house fly are recombinant allergens [24]. The trachoma that transmitted by the housefly causes childhood blindness for which 6 million cases reported annually. The vital role as decomposers of animal waste is performed by housefly larvae in ecosystem [25]. Flies fly to long distances and sits/visits food and human associated goods causing exposure of humans to different pathogens [26].

As the antibiotic resistance develop many bacterial isolates which play a significant role in clinical terms as it is estimated that flies not only carry specific pathogenic bacteria but also carry different nonpathogenic bacteria that are actually carriers of different antibiotic resistant genes [26]. Pathogens varies in its characteristics depending on the area of collection.

The samples of houseflies that are collected from the hospitals have high number of bacteria as compare to other locations and are highly resistant to cephalothin and gentamycin [27]. Antimicrobial resistant of bacteria and fungi are present in houseflies especially sample of those been taken from hospital environment or animal farms [21, 27–32].

Transmission of nosocomial infections found to be associated with hospital environment [21, 33]. It has affirmed relationship with the foodborne pathogens *Escherichia coli*, *Salmonella* and *Shigella spp.* [26]. Antimicrobial resistance [AMR] is considered a global risk to human health. The reason behind is the presence of multidrug-resistant (MDR) bacteria that take a long time to be treated with different combination therapy.

This probable causes of the global resistome include excessive use of antibiotics with irregular manner and periods in animals and humans. The other major causes behind resistance in antibiotics that, they are sold over-the-counter, poor sanitation / hygiene. The resistance spread more rapidly in families where the people are unaware of their risk factors. These factors may result in genetic diversity for the emergence of multi drug resistant infections in the different geographical environments [34].

1.2 Problem Statement

The habitual movement of housefly from unhygenic substrate such as garbage, human and animal feaces and carcasses makes them significant candidate for transmission of disease such as food poisoning, diarhea,cholera when settling on food. Association of synanthropic flies with pathogens varies from environment to environment. There is a need to explore the microbial fauna found in association with the houseflies under different environments.

1.3 Objectives

This study entails the following objective:

1. To isolate pathogenic bacteria associated with housefly from domestic kitchens.
2. To examine the prevalence or status of antibiotic resistance in isolated bacteria.

Chapter 2

Literature Review

2.1 Morphology

Housefly (*Musca domestica*) is a worldwide human health affecting and agricultural pest [35]. At the start of 20^{th} century, the house fly also referred as Typhoid fly because it was responsible for the spreading of typhoid fever [36].

It actually belongs to the taxonomic group Cyclorrhapha as it is one of the most broadly spread fly everywhere throughout the world since it represents 91% of all flies that are present in human residence [37].

According to FDA, housefly transmit various diseases and pathogens [29]. Different parts like internal organs, wings, and vomitus, debris and external organs being used for isolation of bacterial sample. Structure of fly make this happen because it is better suited for the carriage of different pathogens.

Fly secret sticky material on hairy legs and pads on them. The sticky material pulvillus makes fly susceptible to transfer pathogens [4, 12]. Human entozoan and feces are present in the flies in different situations. Female flies tend to be more contaminated than the male [38].

2.2 Habitat

Adaptation of insects life cycles are being mediated seasonally [39]. Below freezing temperature it is the exception for flies to be Omnipresent especially in southern region but in terrestrial environment flies are Omnipresent. For maturity and reproduction flies need sugar and protein [40]. House flies require protein for egg production because of this property they are termed as anautogenous [41]. Sex pheromones i.e. synthesis of cuticular hydrocarbons in female flies need specific diet [40, 42].

A pheromone is present in abdominal integument being synthesized by female flies to attract male flies [43]. Factors like ambient temperature, diet of flies, age, photoperiod, quality and quantity of diet of larvae and the accessibility of substrates for oviposition effect the fertility rate of house flies [5,44]. Sugar and proteins needed for the production of eggs [44]. There will be no egg, very low egg production and larval development in unfavorable condition [44–46].

Temperature promotes the hatching of house fly within 24 hours. Organic substrates like livestock waste of cattle and poultry are the places where larvae feed [44, 47, 48]. Various bacteria help in larval development they are present on the place where the eggs are deposited by the flies and on the membrane of flies [49–52].

Temperature mostly relates with light period, and both these factor are responsible for optimizing different environmental conditions including overwintering strategies that may vary geographically, e.g., across latitude vs altitude [39].

House flies as adults hibernate in the buildings. They can do this in other stages of life if they are exposed to the temperature greater than -50℃ [41,53]. Flies like human, animal manure, open privies, litter, waste around food, animal bedding, House fly develops on decaying organic matter as larvae [4, 38, 41], 8mm long pupa get developed after maggot crawl towards the dry and cool place, its color is reddish brown [5, 41]. Availability of breeding places, temperature, sun shine and humidity changes number of fly in any locality [54].

Temperature is directly related with the growth rate and reproduction rate of houseflies [55]. Scientist suggest that April month favors the reproduction of flies [56]. As the colder climates are concerned they live with association to that of humans. They have the competence to carry 100 different pathogens which are responsible to cause salmonellosis, cholera, typhoid, parasitic worms and tuberculosis [20, 57, 58].

Female houseflies produces eggs but change in environmental condition can lead to houseflies to stop its egg production, where after they may remain in the environment until spring [39].

On liquid and semi -liquid substances the housefly feed, on the other hand, solid materials are soften by the saliva or vomiting [59]. Water is vital requirement for housefly, and housefly cannot survive without it more than 48 hours. Housefly diet also include milk, liquids , juices, fruits, chicken and all the food materials present in human habitat [2]. As these flies intake a large amount of food they have the capacity to deposit a large amount of pathogens. As they are domestic flies their habitat is close to humans and can fly far away from their breeding places. The ability of houseflies to develop and feed on the decaying organic matter has a prime importance for the recycling of nutrients in soil. The researchers have concluded that this may help to fight against the increasing waste in the environment [59]. The larvae of the houseflies can be used in animal manure in control manner so that the waste can be minimized and reduction of environmental risks [29].

Houseflies are always found in association with humans and this is actually the reason for their spread all over the planet. Their larvae plays a significant role as decomposer for the animal wastes. The other insects whose genomes were sequenced, houseflies bear a unique niche as compared to them [25]. They lived very close to animals as well as develop on their manure, are not restricted to a single habitat [60]. The houseflies and blowflies are the first species which actually started to develop on the crumbling organic matter and it is important for their feed, reproduction, and development [11]. They are important in nuisance creation for the animals [26].

The houseflies are important widely distributed insect and synanthropic species present in restaurants, homes, and animal production sites [26]. The houseflies migrated from the infected environment to that of close proximity to humans and start feeding on animal and human food [13, 61]. The houseflies are highly attracted to the places where food is present either prepared or processed, stored, consumed or disposed and play a significant role in their contamination. The range of dispersal of houseflies is from 0.5 upto 2 miles approximately but distances as the 10-20 miles are also reported [56].

2.3 Ecological Importance

Houseflies are synanthropic and are present everywhere in large number because they reproduce throughout the year with high reproduction rate and are adaptive in almost all kinds of environment [62]. Houseflies buzzing disturb people and are present on food which make people annoy .Because of the development and living requirement of housefly, these are responsible to annoy many people by buzzing, landing on food, and making people unfordable. Egg production has been reduced in hens and milk production also been reduced in cows more than 400 million$ since 2013 which has raised different types of economic issues due to diseases caused by houseflies [25, 63, 64].

Houseflies has developed resistance [65]. In recent times waste management for the poultry has been a serious issue which could be solved with the help of houseflies by converting wastes into protein [66]. Livestock and humans get effected by the increase production of the arthropods because of increase in animal production [67]. Adult flies feed on all substrates across all the wall of buildings, ceilings and feaces. The production properties of livestock and poultry alter vastly and there are some practices that actually encourage the development of house flies [68].

M. domestica referred as pests because they cause annoyance and are vectors for carrying different pathogens. Moreover, they are able to convert animal wastes

into valuable biomass that may be suitable for inclusion in animal diets, which has been explored for industrialization outside of the United States [69].

Muscoid flies are the universal pests of livestock and human and even for all inhabitants as well. Although houseflies do not feed blood but still they act as carriers for germs. As compared to other groups of flies i.e. stable flies, houseflies, horn fly and face fly, it is most important for livestock and poultry and it is not ecto-parasite [67]. Wounds, cow's eyes, and teats attracts flies. After the attack of flies it results into the lower milk production by the cows and less weight gain [70].

2.4 Health Importance

Worldwide flies are most important vector of human diseases and also got great medical and veterinary significance. Their random movements and ability to fly long distances, sitting on food ,visiting dirty places and on waste materials make them a best vector and increases the risk of human to get infected with the pathogens they carry. *M. domestica* act as main transporter in carrying the bacteria from places where human exposure is less to the places where human exposure is more (food) and present a hazard to human [26].

According to U.S. Food and Drug Administration, houseflies act as a vector and play important role in dissemination of the food borne diseases and other infectious [71]. Different etiological diseases like viral, bacterial and protozoan diseases can be carried by the houseflies [15, 72]. Vectors include diphtheria, dysentery, intestinal parasites, typhoid, leprosy, and fowl cholera as well as helminth eggs can be carried by houseflies and have ability to cause disease [2, 73]. All over the world, a lot of deaths in the rural area especially in children and cause of dysentery has been also related with housefly. Spread of different intestinal infections is the basic property of houseflies this property is because of its feeding habit [74]. Their physical appearance is well structured to pick pathogens from excrement or garbage [75]. When the people eat food with laid eggs by female houseflies, it leads to severe

diseases [75]. Because of liquefaction fly maggots cause the chronic respiratory diseases as they cause negative impact on the poultry manure [76].

Amongst major disease transmission of flies one is eye diseases and other enteric diseases. Mastitis in cattle being cause by houseflies [77]. Some of the major pathogen in humans include *Helicobacter pylori*, *Cryptosporidium paryum*, foot-and-mouth disease. Bovine rhinotracheitis and Aujesky's disease are caused in cattle, ungulates, pigs and sheep and cattle respectively by housefly [4, 78, 78–80]. The importance of flies as vectors is well documented and well-studied against different diseases [68].

Milk quality get effected with increase of fly number [80]. Production of milk increases with the control of flies [81]. For removing flyspecks and cleaning eggs quality of egg got effected. Quality of egg and its production rate get developed after treatment of flies [82].

2.5 Houseflies and Associated Pathogens

The pathogens association with houseflies have been found to be associated with bacterial association external and internal surface but the microbes present in the gut of house flies are still not well characterized and how they transferred via food chain [2, 83, 84]. There are different species of flies that actually feed on waste materials like animal manure [85]. Flies have developed various modes of transmission of pathogens and these all have been examined by modern research techniques by medical and veterinary entomologists. A study conducted in Japan isolated the verotoxin producing *E. coli* from the samples of houseflies in the area where the humans infected by that bacteria [24]. Houseflies have also been isolated from dairy including *Campylobacter spp., E. coli*, and *Cochlosoma anatisn*. The deposition of bacteria in these production is done by regurgitation and defecation [86]. The bacterial specie of *Campylobacter jejuni* was isolated from the sample of housefly from the poultry and that pose a health threat to humans via the consumption of contaminated meat [87]. It has been found that when *E.coli*

carried by houseflies started to divide on its surface, the bacterial replication on the regurgitation spots also observed that deposited by the fly when it landed [24]. Larva that exposes to certain pathogens contributes in biochemical transmission [88].

Different pathogens have been separated from houseflies that may include *Shigella, Yersinia enterocolitica, Actinibacter spp., Klebsiella spp., Proteus spp., Enterococcus spp., Sarcocystis sp., Enterobacter sp., Chlamydia sp, Pseudomonas sp, Toxoplasma gondii, Entamoeba coli, Giardia, Isospora, Entamoeba histolytica, Endolimax nana, Hammondia, Trichomonas and Cryptosporidium parvum Polio* [1] . A variety of studies carried out on swine and it was found that it carry strain of *Enterococcus spp* [89]. The samples of houseflies collected from fast food restaurant in Kansas carry the pathogens of *Enterococcus spp* [90]. The pathogens that are present within the gut of houseflies are different as the diet changes. *Proteus* and *Providencia* are always present within the gut of housefly [91]. The strains of *Providencia stuartii* and *Providencia rettgeri* have been isolated from intestinal tracts of houseflies larvae from turkey bedding and corn silage [5]. In Malaysia, a study reported the mechanical transmission of rotavirus by the wings and legs of the house fly and approximately 0.1mg food was contaminated [14].

The study in Nigeria reported the presence of hookworms and *T. trichiura* isolated from house fly [29]. The most surprising results about the housefly is that it carried approximately 6 x 106 bacteria on the external surface and more than 100 from the digestive tract, pathogenic bacteria stay alive in the body of housefly and on the body of houseflies [14]. The gene sequencing of 16S rRNA genes analysis predicted a wide variety of new and unreported bacterial species that are associated with the gut of houseflies and hence put the light on the role of housefly as reservoirs against different human pathogenic bacteria [5]. Different types of diseases like bacteremia, upper respiratory diseases, urethritis, complications of surgery and instrumentation, infants pulmonary diseases, and burning complications that all lead towards the new record about the flies [92].

TABLE 2.1: Bacterial Species Associated with House flies

S#	Bacterial Genera	Species	Medical	Ref
1	*Helicobacter*	*H. pylori*	Medically good	[78, 93, 94]
2	*Staphylococcus*	*S. aureus*	Medical	[26, 95-98]
		S. sciuri	Medical/ veterinary	[92]
		S. xylosus	Medical	[92]
		S. saprophyticus	Medical	[92]
		S. epidermidis	Medical	[95]
		Others	Medical/ veterinary	[5, 14, 16, 27, 99, 101]
3	Salmonella	*S. enterica serovar Enteritidis*	Medical	[27,102,103]
		S. typhimurium	Medical	[104]
		Others	Medical	[27, 78, 93, 94, 101, 105, 106]
4	*Campylobacter*	*C. coli*	Medical/ veterinary	[107]
		C. jejuni	Medical/ veterinary	[107,110]
		Others	Medical/ veterinary	[111,112]
5	*Enterobacter*	*Enterobacter spp.*	Medical	[14,95]
6	Alternaria	Alternaria specie.	Medical	[28, 96, 119]
7	Pseudomonas species	aeruginosa (species)	Medically	[28, 96, 113]
		Others	Medical	[114]

Table 2.1 continued from previous page

8	Choronobacter	*C. turicensis*,	Medical	[102, 103]
9	Streptococcus	*S. faecalis*	Medical	[95, 96]
		S. pyogenes	Medical	[96]
		Others	Medical	[14, 101, 113]
10	*Bacillus*	*B. sphaericus*	Medical field	[11]
		B. pumilus	Medical	[92]
		B. megatarium	None	[8]
		B. alvei	Medical field	[8]
		B. thuringiensis	None	[92]
		B. anthrax	Medical/ veterinary	[12]
		B. cereus	Medically	[92, 96]
		Others	Medical	[113, 114]
13	Aeromonas	*A. hydrophila*	Medical	[115, 116]
		A. caviae	Medical	
		Others	Medical	[5]
14	Klebsiella	*K. oxytoca*	Medical	[117]
		K. pneumonia	Medical	[7, 28]
		Others	Medical	[59, 99]
15	Enterococcus	*E. faecium*	Medical	[89,118]
		E. hirae	Medical	[89]
		E. faecalis	Medical	[33, 89, 118]
		E. casseliflavus	Medical	[89, 118]
16	Shigella specie	*S. dysenteriae*	Medical	[92]
		S. sonnei	Medically	[99]
		Others	Medical	[106, 111]
17	Proteus	*P. vulgaris*	Medically	[92]
		Proteus sp.	Medical	[98, 113]
		P. mirabilis	Medical	[28]
18	Escherichia	*E.coli*	Medically	[32, 95]

Table 2.1 continued from previous page

18	Listeria	*L. monocytogenes*	Medical	[58, 102, 103]
		Others	Medical/ veterinary	[100]
19	Serratia spp	*Serratia spp.*	Medically	[95]
20	Yersinia spp	*Y. enterocolitica*	Medically	[99]
21	Acinetobacter spp	*A. baumanni*	Medically	[14, 92]
22	Providencia spp	Providencia spp.	Medically	[5, 120]
23	Edwardshiella	*Edwardsiella spp.*	Medical	[120]
24	Morganella spp	*M. morgana*	Medically	[5, 8]
25	Micrococccus	Micrococcus spp	Medically	[14]
26	Methylbacteriam	M. persicinum	Medically	[92]
27	Lactobacillus	*Lactobacillus spp.*	Medically	[16]
28	Corynebacterium	*Corynebacterium*	Medically	[16]
27	Vibrio	*Vibrio cholera*	Medically	[106]
		Others	Medical/ veterinary	[100]
29	Burkholderia	*B. pseudomallei*	Medically	[117]

TABLE 2.2: Fungal Species Associated with Housefly

S#	Fungal Genera	Species	Medically and Veterinary	Ref.
1	*Penicillium*	*P. corylophilum*	Medically	[127]
		P. fellutanum	Medically	[127]
		P. aurantiogriseum	Medically	[128]
		P. verrucosum	Medically	[128]

Table 2.2 continued from previous page

		P. axalicum	Medically	[96]
		Others	Medically	[21, 119] [129, 130]
2	*Candida*	*C. krusei*	Medically	[89]
		C. parapsilisis	Medically	[89]
		C. albicans	Medically	[58, 112]
		C. dubliniensis	Medically	[89]
		C. glabrata	Medically	[89]
		C. tropicalis	Medical	[58, 89]
		Others	Veterinary	[21]
3	*Aspergillus*	*A niger*	Medical	[8, 127]
		A tamari	Medical	[96]
		A fumigatus	Medical	[8]
		A. flavus	Medical	[127, 128]
		A parasiticus	Medical	[128]
		Others	Medically	
4	*Alternaria*	*A. alternata spp*	Medically	
5	*Beauveria*	*B. bassiana spp*	Medically	[21]
6	*Curvularia*	*C. brachyspora spp*	Medically	[127]
7	*Chrysosporium*	*Chrysosporium species*	Medically	[119]
8	*Epicoccum*	*Epicoccum species*	Medically	[119]
9	*Moniliella*	*Moniliella species*	Medically	[119]
10	*Rhizopus*	*Rhizopus spp.*	Veterinary	[119]
11	*Mucorales*	*Mucorales species*	Medically	[21]
12	*Moniliella*	*M. suaveolans*	Medically	[128]
13	*Microsporum*	*M. canis species*	Veterinary	[13]
		M. gypseum species	Medically	[130]
14	*Mycelia*	*M. sterilia*	Medically	[127]
15	*Curvalaria*	*Curvalaria pecies*	Agricultural	[119]
16	*Eupenicillium*	*Eupenicillium species*	Medically	[119]

Table 2.2 continued from previous page

17	*Nigrospora*	*Nigrospora species*	Agricultural	[119]
18	*Scopulariopsis*	*Scopulariopsis species*	Veterinary	[119]
19	*Rhodotorula*	*Rhodotorula species*	Medically and Veterinary	[21]
20	*Mucor*	*M. cirinelloides*	Medically	[8]
21	*Fusarium*	*F. verticilliodes*	Medically	[128]
		F. oxysporum	Medically	[96]
		F. proliferatum	Medical	[128]
		Others	Medical	

Table 2.3: Viral Species Associated with House flies

S #	Viral family	Species	Medically and Veterinary	Ref
1	Filoviridae spp	Ebola virus	Medically	[121]
2	Hytrosaviridae	hypertrophy virus (MdSGHV) Musca domestica Salivary gland	Importance in Veterinary	[122]
3	Orthomyxovirida e	Avian (birds) Influenza virus (H5N1)	Veterinary	[123]
4	Paramyxoviridae	virus of Newcastle disease	Medically and Veterinary	[124]
5	Picornavirus	Seneca virus A	Medically and Veterinary	[125]
6	Arteriviridae	respiratory syndrome virus and Porcine reproductive	Importance in Veterinary	[126]

TABLE 2.4: Parasites Species Associated with House flies

S#	Parasite Genera	Species	Medical or Veterinary Importance	Ref
1	Entamoeba	*E. histolytica*	Medical	113, 131
2	Trichuris	*T. suis*	Medical/ veterinary	58
		T. trichiura	Medical	113, 131, 132
3	Metastrongylus	*M. spp*	Veterinary	58
4	Crytosporidium	*C. parvum*	Medical/ Veterinary	125
5	Enterobius	*E. vermicularis*	Medical	1
6	Hymenolepis	*H. nana*	Medical	131
7	Hookworm	Ancylostoma duodenale/ Necator americanus	Medical	113, 132
8	Strongyloides	*S. ransomi*	Veterinary	58
		S. stercoralis	Medical	113
9	Haematopinus	*H. suis*	Veterinary	58
10	Giardia	*G. lamblia*	Medical	1, 131
11	Taenia	*T. spp.*	Medical/veterinary	131, 132

2.6 Antibiotic Resistance

Antibiotics are those kinds of compounds that are produced by certain microorganisms that could destroy or inhibit other microorganisms growth. Nowadays many

synthetic compounds with similar functions including βlactams, cephalosporins, and carbapenems are also called antibiotics.

Following the initial discovery, the antibiotics were commonly used as a veterinary medicine in humans, and were important for protecting human and animal health from pathogens. Number of times bacterial infections have been successfully treated with antibiotics, the likelihood of survival and quality of life of humans and other animals worldwide is greatly enhanced [133].

There are lot of of uses of antibiotics, seen just after the first applications of antibiotics in the 1930s when sulfonamide-resistant Streptococcus pyogenes, methicillin-resistant *Staphylococcus aureus* (MRSA) and streptomycin-resistant *Mycobacterium tuberculosis* were first isolated in hospitals [134]. The rapid development of hospital-acquired infections by antibiotic-resistant (ART) pathogens and opportunistic pathogens such as MRSA,*Clostridium difficile*, vancomycin-resistant *Enterococci* (VRE) and Resistant to Fluoroquinolone *Pesudomonas aeruginosa* (FQRP) has become a major public health concern in recent years [37].

2.6.1 Examples of Antibiotics Used in Treatment of Infections Caused by Bacteria

Imipenem is a broad-spectrum antibiotic shown in figure 2.1. It is recommended in initial therapies and is used for the treatment of severe bacterial infections including nosocomial infections, febrile neutropenia, ventilator associated pneumonia (VAP), hospital acquired pneumonia(HAP) [135]. Imipenem the IUPAC name (N-formimodoyl-thienamycin) is not used individually because it is rapidly degraded by enzyme dehydropeptidase which produced by the human kidney and has toxic effects on kidney. Therefore it is used with cilastatin in the ratio of 1:1. That act as an inhibitor of the dehydropeptidase enzyme and neutralize the toxic effect of the antibiotic. Transpeptidases enzymes also called penicillin binding proteins (PBPs) cross link the peptidoglycan and provides the rigidity to bacterial cell wall. These PBPs are the main targets for imipenem. Imipenem inactivates

the transpeptidases moreover it inhibits the Dalanine carboxypeptidase in *E.coli* [136].

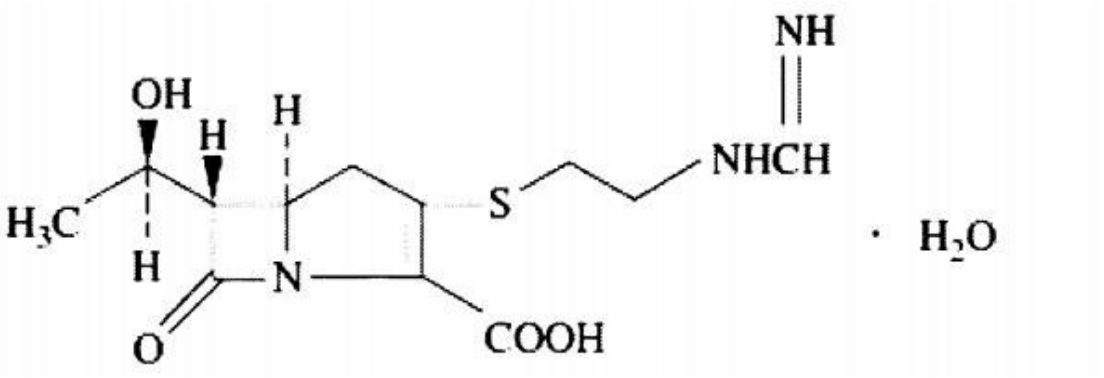


FIGURE 2.1: N-formimodoyl-thienamycin (Rodloff et al., 2006)

Tazobactam is a therapeutic drug that is involved in inhibition of bacterial activity mainly for SHV-1 and TEM group. It is used along with other antibiotics to make them less vulnerable to organisms,commonly available in salt form for its use. It is also used against infections caused by *Pseudomonas aeruginosa* [137].

Gentamicin, is a drug used against common respiratory infections, urinary tract infections, common cold and other inflammatory diseases. It is given by injection or as ointment [138].

Fusidic acid is a drug used against gram positive bacteria. It dont kill the bacteria but behave as inhibitor of synthesis of its protein. It is also termed as "bacteriostatic" [140].

Ciprofloxacin is a drug used for treating infections caused by bacteria. Different infections of joint, respiratory, skin, urinary infections, fever are treated [141].

Chloramphenicol This drug is used against bacterial infections. Commonly used for treating eye infection conjunctivitis, cholera and fever [142].

Cefotaxime this drug is used againts bacterial infections including fever, respiratory tract infections, urinary tract infections, gonorrhea.

2.6.2 History of antibiotics

As for as origins of antibiotics is concern, many people would relate it to the famous discovery of penicillin, *Penicillium notatum* produce penicillin, by Nobel Prize winner Alexander Fleming in 1928, which would later be widely used in World War II. Since it is saving millions of lives however, the earliest discoveries for antibiotics can be dated back to the 19th century [143]. In 1887, Rudolf Emmerich demonstrated that developing cholera in animals with artificially infected with *Streptococci* were protected; and the soil mold Penicillium first discovered in 1896, by a French medical student, Ernest Duchesne, that was able to inhibit the growth of certain bacteria [29]. Later,*Bacillus pycyaneus* was used by Emmerich and Oscar Löw for the extraction of pyocyanin (now called Pseudomonas aeruginosa) which can be used to kill bacteria i.e. with ability to cause cholera, typhoid, diphtheria and anthrax [144]. Eventual clinical introduction of natural penicillins and synthetic sulfonamides in the 1930-40s resulted by early discoveries and by search of antimicrobial agents [145]. This success of antibiotics made people immunized in the battle against bacterial infections and led to considerable excitement in medicine. The discovery of various new classes of naturally occurring and synthetic antibiotics occured (Table 2.5). In fact, most recently used classes of antibiotics were discovered between 1940 and 1962 which is also known as the Golden Age of antibiotic development. It took almost 10 years partly to develop both synthetic and natural antibiotics because of the believe of people that those bacterial infections were no longer a problem apart from that most pharmaceutical companies did not view antibiotics as a profitable market. In the 1980s, studies for new antibacterial agents were resumed. This was done because ART pathogens were increasing in clinical settings. However scientists are performing continuous research in antibiotic development since 1980 but only three new classes of antibacterial have entered the market those includes pseudomonic acid antibiotic mupirocin in 1985, the oxazolidinone linezolid in 2000 and the lipopeptide daptomycin in 2003 [146]. Only 7 new antibacterial drugs, four synthetic drugs have been launched in last decade (i.e. Gemifloxacin, Fosfluconazole, Ceftaroline fosamil and Telavancin) belonging to three structure classes

(quinolone, β-lactam, and lipoglycopeptide) and three naturally derived drugs (i.e. Daptomycin, Doripenem and Tigecycline) belonging to three classes (daptomycin, quinolone, and tetracycline) [146,147].

TABLE 2.5: History of Antibiotic Development

S#	Classes of Antibiotics	Intro.	Derivation	Example	Mechanism
1	Sulphonamide	1935	Synthetic	Sulfapyridine	Antifolate
2	β-lactam	1941	NP- derived	Penicillin	Bacterial cell wall
3	Bacterial peptide	1942	NP- derived	Bacitracin, Polymixin	Bacterial cell wall, Bacterial cell membrane
4	Aminoglycoside	1944	NP- derived	Streptomycin	Protein synthesis
5	Cephalosporin	1945	NP- derived	Cephalosporin	Bacterial cell wall
6	Nitrofuran	1947	Synthetic	Nitrofurantoin	Various
7	Hexamine	1947	Synthetic	Methenamine mandelate	Release of formaldehyde
8	Chloramphenicol	1949	NP- derived	Chloramp-henicol	Protein synthesis
9	Tetracycline	1950	NP- derived	Chloro-tetracycline	Protein synthesis
10	Isoniazid	1951	Synthetic	Isoniazid	Fatty acid biosynthesis
11	Viomycin	1951	NP- derived	Viomycin	Protein synthesis

12	Macrolide	1952	NP- derived	Erythromycin	Protein synthesis
13	Lincosamide	1952	NP- derived	Lincomycin	Protein synthesis
14	Streptogramin	1952	NP- derived	Virginiamycin	Protein synthesis
15	Cycloserine	1955	NP- derived	Cycloserine	Bacterial cell wall
16	Glycopeptide	1956	NP- derived	Vancomycin	Bacterial cell wall
17	Novobiocin	1956	NP- derived	Novobiocin	DNA synthesis
18	Ansamycin	1957	NP- derived	Rifamycin	RNA synthesis
19	Nitroimidazole	1959	Synthetic	Tinidazole	DNA synthesis
20	Ethambutol	1962	Synthetic	Ethambutol	Bacterial cell wall
21	Quinolone	1962	Synthetic	Nalidixic acid	DNA synthesis
22	Fusidane	1963	NP- derived	Fusidic acid	Protein synthesis
23	Diamino pyrimidine	1968	Synthetic	Trimethoprim	Antifolate
24	Phosphonate	1969	NP- derived	Fosfomycin	Bacterial cell wall
25	Pseudomonic acid	1985	NP- derived	Mupirocin	Protein synthesis
26	Oxazolidinone	2000	Synthetic	Linezolid	Protein synthesis

27	Lipopeptides	2003	NP- derived	Daptomycin	Bacterial cell membrane

2.6.3 Resistance of Antibiotic

Antibiotic resistance was detected in 1930s when sulfonamide-resistant *Streptococcus pyogenes* detected in military hospitals [134]. Subsequently, Streptomycin resistant *Mycobacterium tuberculosis* and methicillin resistant*Staphylococcus aureus* (MRSA) had also been detected [134]. The US Surgeon General, Dr. William H. Stewart in 1969 made a testimony to Congress we have to close the book on infectious diseases as infectious disease has been controlled largely and the fight against pestilence is now over. however, sooner antibiotic resistance emerged [145]. After a few years nosocomial vancomycin-resistant *Staphylococcus aureus* become common while *Enterococcus faecalis* strains resistant to vancomycin also emerged and very common in a short period of time [148]. With the passage of time antibiotic resistant bacteria become a serious problem in the mid-1980s where 1% to 5% of *S. aureus* were detected as methicillin-resistant. Further, 60% to 70% were multidrug-resistant MRSA occurring in hospitals today. In 2007, CDC published a report that MRSA strain are close to 100,000 per year [149]. Studies on *Salmonella Typhi* isolates were showing that 54.0% were nalidixic acid conducted in 2006 (introduced in 1967) and 19.6% of *Campylobacter* isolates were ciprofloxacin resistant (introduced in 1987) as compared in 1999 (19.2% and 12.9%) [29, 151, 152]. The situation become more worse with the emergence of multi-drug resistant bacteria due to the emergence of diverse resistant traits begun to gather within single resistant strain. The application of combination therapy also played a part to treat difficult infection. For instance, enteric and Gram-negative bacteria such as*Escherichia coli, Shigella* and *Salmonella* in the 1950s were the initially source of multi-drug resistance detection, and was blamed for the re-occurrence of extensively drug-resistant (XDR),multidrug-resistant (MDR) and tuberculosis

in the 1980s [134, 153, 154]. Many previously affordable and effective antimicrobial treatments became unsuitable for their original usage due to increasing 17 resistance in bacteria.

Many resolutions have been proposed, and a number of recommendations has been made but no appropriate progress seen. Unfortunately, antibiotic resistance is a global issue, effecting the treatment period as well. Several physiological, genetical and biochemical procedures and mechanisms have been proposed to overcome this issue which may steer this resistance. Furthermore, the lack of data on these specific issues is a vital, causing a lack of significant progress in resistance. The discovery of antibiotics was a defining moment in the history of mankind that revolutionized medicine and saved countless lives. Unfortunately, these "magic bullets" have been accompanied by these emerging resistant strains of pathogens. Currently, experts in medical sciences are struggling hard for a return to the pre-antibiotic age. the available bacterial genomes paved a way to analyze it in different ways, but it has been concluded that resistance genes are present that may be target or influx-efflux,; however, the resistance determinants in several microbes have been found less in number than other. Among enteric bacteria including *Shigella, Salmonella* and *Escherichia coli* , the resistant strains causing economic losses, in developing countries where the lack of resources may cause a huge burden each year to be treated in future and ultimately more time for transmission of resistance strains among the community. The increasing antimicrobials therapy results in the increase incidence of resistance, mainly in the developing countries where the drugs were freely taken from any medical store without any prescription or legal requirement.

Most of the antibiotics are generated by microbes or environmental fungi where saprophytic bacteria are predominantly applied for its production, however, some are modified synthetic, and some are entirely synthetic e.g fluoroquinolones and sulphonamides. Various organisms evolved defensive phenomena where inhibition of drug or distribution may degrade the antimicrobials. Although very few has been found in human pathogens, production of enzymes (β-lactamases) resistance mechanism and has an impact on human health [155, 156].

It is well known that numerous antimicrobial compounds released by bacteria stop the growth of other organisms, a mutual benefit. Earlier it has been suggested that antimicrobial substances concentration in the soil is much lower, and unable to stop the growth of other neighboring bacteria. Further, the data available so far suggest that antimicrobial molecules with concentrations in sublethal have a significant effect on physiology, evolution, and may have signaling molecules required for gene or proteins expression. Resistance developed in natural antimicrobials and synthetic antimicrobials. A few saprophytic bacteria prepare broad spectrum antibiotic. These results need some more queries about the impacts of these antibiotics and their economical concern.

Etiology of resistance to antibiotics has been investigated widely and found that it has many factors behind. The major are inadequate regulations, deficiency in awareness which steers inept the use of such antibiotics, antibiotics usage in poultry as growth promoter instead of treatment.

Similarly, antibiotics resistance in tuberculosis is also a serious problem. Many drugs are being used for the treatment and cure of tuberculosis infections. Along with drugs, different control programs are developed for prevention from this disease. But unfortunately TB infections are increasing because the TB strains are becoming resistant to the drugs. The causative agents are resistant and increasing the risk of severe forms of infection in society. Due to this resistance of Strains.

Many antibiotics are in use for its control along with many programs but still resistance is also developed making this diseases to evolve in more severe form now. The overuse of antibiotics is a major cause behind resistance evolution. According to Sir Alexander Fleming, the antibiotics kill sensitive bacteria but resistant strains survive which reproduce through natural selection. This overuse is strongly discouraged, but still a large number of population are not aware about this phenomena and there remains over-prescription causing more panics. Majority of investigation revealed that 30%–50% of the cases are misusing antibiotics. In livestock, majority, of antibiotics are being used as a growth promoter. About 80%

of the antibiotics are used in the US as growth promotor and infection control in animals [34].

2.6.3.1 Resistance Antibiotics Mechanisms

The most common survival strategy of ART bacteria survival strategy is by reducing the concentration of the inner cellular antibiotic to the sub-lethal level in the presence of environmental antibiotic. ART bacteria use three major mechanisms of this strategy i.e. permeability reduction of the cell wall to antibiotics, antibiotics expulsion, and antibiotics destruction by upgrading an antibiotic-inactivating enzymatic pathway [145, 157]. Target-mediated AR is another less frequent strategy, variant target molecule of certain antibiotics with lower binding affinity with the antibiotics were produced by using targeted mediated AR strategy for the normal or near normal metabolic function [158, 159].

Bacteria follow three routes to develop AR: specific natural cellular property makes it intrinsically resistant (insensitive), target gene under strong selective pressure and transmit the gene vertically to the offspring are the conditions when it accumulate mutations, and acquire resistance through horizontal transfer [157, 160]. Compared to the limited cases of intrinsic resistance and the low frequency of mutation (around 10^8-10^9), horizontal transmission of antibiotic resistant determinants play an important role in the rapid dissemination of AR.

2.6.4 Maintenance of Antibiotics

Imposition of additional metabolic cost on the host strain occur by the carriage of AR on plasmids or transposons, thus reducing bacterial fitness to the environment. Based on this concept, once the use of certain antibiotics is banned or restricted ART bacteria are expected to gradually disappear. The so called phenomenon easy-to-get, hard-to-lose, contrary to our expectation is observed in many cases. In order to understand this unexpected phenomenon, AR persistency being investigated by the researchers. Studies indicated compensatory mutations or

counteracted by the beneficial effects of AR determinants may be used to eliminate negative effects of AR genes [161–163]. For example, host's adaption to elevated pH in the environment would raise by mdfA and tetL [164]. Fitness advantage to the new host being conferred by Apramycin resistance plasmids [165]. In these cases, in the absence of any antibiotic selective pressure, ART isolates would not disappear but rather gradually replace antibiotic susceptible isolates. Strains with mosaic resistance genes are of little or no cost since no additional proteins need to be synthesized. Therefore In these cases resistance phenotype can be considered selectively neutral, After the removal of selection pressure it may also accounts for the persistency of ARs. In addition, Li et al. (2011) described plasmid stabilization mechanisms, such as the toxin-antitoxin (TA) 24 and TA independent mechanism and this mechanisms to persist resistance on plasmids [200]. It is also worth noting the impact of constant horizontal gene transfer between commensal and pathogenic microorganisms. The basic cellular biology, including transmission, maintenance of AR and the mechanisms of dissemination, it differs little between pathogenic bacteria and commensal. Microorganism specially commensal microorganisms on a host live harmlessly, Stable reservoir of resistance gene are commensal microorganisms. For example, in oldest antibiotics such as tetracycline, ampicillin high level of acquired resistance can be detected in commensal bacteria even in rural area with minimal antibiotic exposure or organic pig in animals that never treated with antibiotics [166, 167].

Chapter 3

Materials and Methods

3.1 List of Equipments

Autoclave , Magnetic stirrer, Measuring balance, Laminar flow, Incubator, Vortex, Microscope, Shaker, pH meter, Micro Centrifuge, Centrifuge, Microwave Oven, Refrigerator.

3.2 List of Apparatus

Beakers, Spatula, Conical flasks, Eppendorf Tube, Micropipette, Petri dishes, Spirit lamp, Insect Nylon net , Inoculation loop, Glass rod, Dissecting needle, Dropper, Parafilm and Graduated cylinders.

3.3 List of Chemicals

Nutrient Agar[g/liter], 0.7% saline, MacConkey Agar, Distilled Water, Safranin, Mannitol Salt Agar, Oxidase test, Catalase test, Urease test, Eosin Methylene Blue Agar, Crystal violet

3.4 Methodology Chart

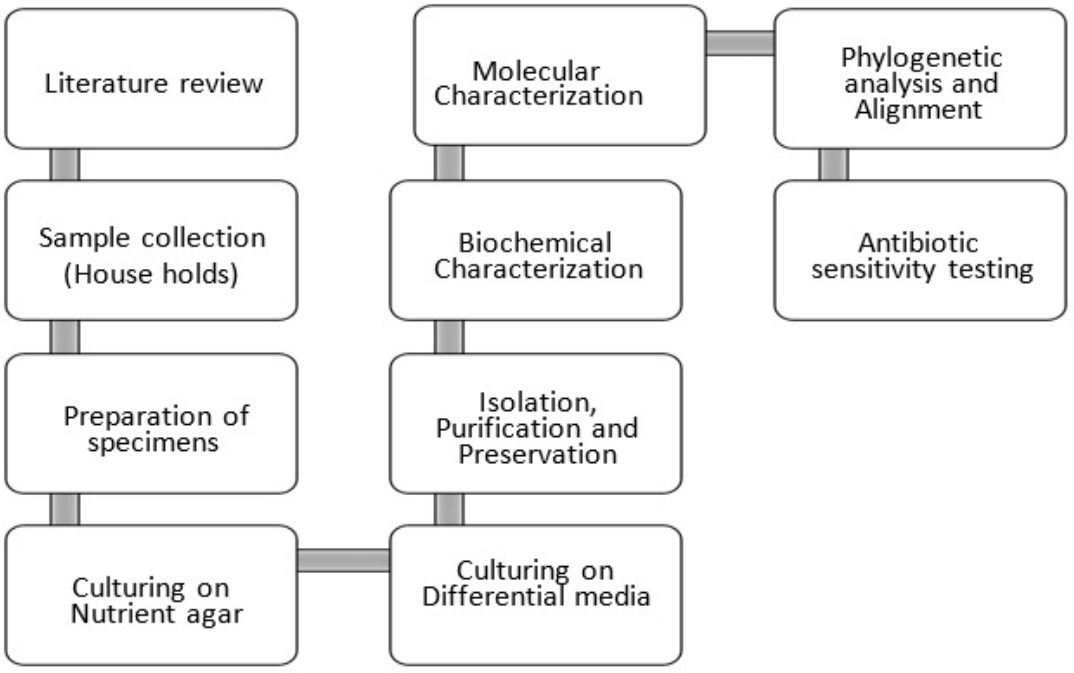


FIGURE 3.1: Methodology of Project

3.4.1 Sampling Locations

Three locations were selected from Chakwal district named as Odherwal, Chakora and Bhaun. The sampling areas were the kitchens of domestic houses. In each location 3 consective streets,in each street 5 houses were selected for sample collection. Altogether from 3 locations 300 samples were collected. Collection was done during month of July to September.

3.4.2 Collection Using Insect Net

Different locations were used for collecting adult houseflies of Households in Chakwal by the use of nylon insect net. Flies were transferred to the glass bottles and immediately transported to the laboratory. They were kept in refrigerator at -2℃. For isolation, 50 houseflies from each location were transferred to autoclaved centrifuge tubes containing 10ml Phosphate buffer Saline solution [PBS] and 100ml of

distilled water. Allcentrifuge tubes were vortex for 3-5 minutes. Centrifuge tubes were labelled according to location from where sample was collected [143].

3.4.3 Nutrient Agar Preparation

To verify the presence or association of bacterial pathogens with the houseflies, the sample was cultured on the Nutrient agar. Nutrient agar was used for the growth of bacterial pathogens. Nutrient Agar of 5.6g was weighed by measuring balance and added in the 200 ml of distilled water. The mixture was autoclaved at 121℃ for 15 to 20 minutes. Autoclaved 20ml of media was poured into sterile petri plates uniformly under laminar flow. Centrifuge tube was transferred to the petri dishes through an autoclaved tips and micropipette. The sample was spread uniformly on the Petri plate through spreader 5ml of prepared sample was poured in plates were spread on 10 plates containing Nutrient Agar. Each location was replicated 5 times. Plates incubated for 48 hours on 37℃. Plates were incubated in upside down direction to avoid the moisture.

3.4.4 Growth on Differential Media

Differential media were used for identification of bacteria including MaCconkey Agar [Macc], Mannitol Salt Agar [MSA], and Eosin Methylene Blue Agar [EMB].

3.4.4.1 MaCconkey Agar [Macc]

250 ml distilled water was added to 13.75g of dry powder of MaCconkey with continuous stirring by magnetic stirrer it was then autoclaved for 15-20 minutes at 121℃. The final media was poured in petri dishes. Under the laminar flow hood total of 10 plates were prepared and were allowed to solidify at room temperature.

3.4.4.2 Eosin Methylene Blue Agar [EMB]

Volume of 250ml of distilled water was added to 9.375g of Eosin methylene blue agar. Magnetic stirrer was used for stirring and proper mixing of media. Prepared media was autoclaved at 121℃ for 15-20 minutes. Media was left for solidification at room temperature after pouring it in petri plates.

3.4.4.3 Mannitol Salt Agar [MSA]

27.75g of powdered MSA was added to conical flask containing 250ml of distilled water. The media was mixed and autoclaved 15 to 20 minutes on 121℃. The media has been left to solidify at room temperature after pouring.

3.4.4.4 Streaking of Culture media

The bacterial colonies grown on Nutrient agar were streaked on the differential media. The criteria for selection of bacteria was color, shape and morphology. Every bacteria taken from nutrient agar plate was streaked on prepared differential media. After streaking plates were incubated 37℃for 24 hours.

3.4.5 Preservation of Purified Stains

Glycerol stock of 100ml was prepared for the preservation of purified strain. 50% of glycerol was prepared by dissolving 50ml of glycerol and 50ml of distilled water. it was autoclaved at 121℃ for 15-20 minutes. 2.5ml eppendorf tubes were taken and autoclaved at 121℃ for 15minutes. The eppendorf tubes were numbered in the laminar flow hood. 1ml of glycerol solution was filled in these eppendorf tubes with the help of 1000μL pipette. Suspension was made with loop full of bacteria picked from each differential media and added into eppendorf tubes containing glycerol stock. Eppendorf tubes with bacteria and glycerol were kept at -4℃.

3.4.6 Gram Staining

3.4.6.1 Preparation of Crystal Violet Solution

Gram staining crystal violet was prepared by dissolving 2g of crystal violet in 10ml of ethanol. Solution was stored in the eppendorf tubes.

3.4.6.2 Preparation of Gram Iodine Solution

0.03g of iodine pearl, 0.667g of potassium iodide and 0.1g of sodium bicarbonate were dissolved in 10ml of distilled water for preparation of iodine solution.

3.4.6.3 Preparation of Safranin Solution

0.1g of safranin was dissolved in 4ml of 95 percent concentrated ethanol for the preparation of stock solution. The working solution was obtained by adding one part of stock solution in the five parts of distilled water.

3.4.6.4 Preparation of Destaining Solution

5ml of 95 percent ethanol was added and mixed with 5ml of acetone for making destaining solution. It was further stored in the eppendorf tube for Gram staining purpose.

3.4.6.5 Gram Staining Procedure

Gram staining procedure was first developed by the Hans Christian Gram in 1844. As a differential staining method, it differentiates gram positive and gram-negative bacteria. A glass slide was cleaned with 75 percent ethyl alcohol then the dilutions was prepared by adding a loop full of purified bacterial culture in the 2ml of sterilized water in the beaker. A drop of bacterial suspension was poured in middle of slide and slide left air dry. After that heat was provided using spirit lamp for 60

seconds to fix. On the heat fixed bacterial stain drop of crystal violet was added and left for 30 seconds; it was rinsed with sterilized water and blot the water with blotting paper around the bacterial stain. After that, 3-4 drops of Gram iodine was added on the slide and was left for one minute. The slide was again rinsed with sterile water for one minute. Decolorizer was used for washing, which contain 95% ethanol, it was run through the stained area so that it decolorizes the stain and washes out the color, the slide was again rinsed with sterile water. Then 3-4 drops of safranin were added and left for one minute after that rinsed. Cover slip was placed on the slide and blot the moisture from sides and slide was observed under microscope at 40X. The gram -negative bacteria shows pink color and gram -positive bacteria shows purple color.

3.4.7 Biochemical Characterization

Different types of biochemical tests were performed for the biochemical characterization of bacteria by Murray [144].

3.4.7.1 Citrate Utilization Test

Bacterial strains with citrate utilization are called citrate positive and those without citrate utilization are called citrate negative. For the execution of this test, 100ml of Simmons citrate solution was prepared. 2.424g of Simmons Citrate was taken and dissolved it in 100ml of distilled water in conical flask. After that, it was autoclaved for 15-20 minutes, at 121℃. Media was poured in the petri plate. Total six plates were prepared for biochemical test. The isolated bacterial strain was inoculated on the Simmons citrate media plates, by taking a loop full of bacteria from each plate. The plates were then incubated in the incubator at 37℃ for 48-72 hours after proper wrapping. Green color of media turned blue is called as citrate positive other that don't cause color change are citrate negative.

3.4.7.2 Urease test

This test basically use for the utilization of urea by the bacterial samples. For this test, the Urea Agar Base [UAB] was weighed 2.5g. Then added it in the conical flask with 100ml of distilled water in it. After proper mixing, the conical flask was properly covered and prevented from the contamination, it was autoclaved for 15 to 20 minutes at 121℃. The media was poured into the six plates. The plates were stored in the refrigerator for future use for one day.

Streaking of isolated cultures was done on the plates containing Urea Agar Base [UAB]. The plates were incubated in the incubator at 37℃ for 48-72 hours. The bacterial strains with pink color are urease positive and other that don't turn the color into pink are urease negative.

3.4.7.3 Catalase test

Catalase is a enzyme, enzyme that decompose hydrogen peroxide into water and oxygen. Hydrogen peroxide forms as one of the byproduct of aerobic carbohydrate metabolism. If this oxidative product remains in the body of bacteria, it becomes lethal for their survival. The reagents that are present in the catalase test contain 3% hydrogen peroxide. A loop full of bacteria from pure culture were taken and placed on the slide. In addition two drops of 3% H2O2 was added on the slide to check the production of hydrogen peroxide in the bacteria.

3.4.7.4 Validation of Biochemical Tests by using API 20E

The bacteria were also characterized biochemically by using API 20E kit. Standard procedure that is undertaken for the biochemical characterization of bacteria it includes 20 miniaturized test for the identification and characterization of bacterias. It contain 20 micro tubes that constitute dehydrated substrates.

Bacteria to be identified was first to be isolated on suitable culture medium according to standard microbiological technique. The bacterial suspension was added in

the microtubes after that they were placed for incubation which lead to the the color change that might be spontaneous or can be observed after some time. First, tryptic soy broth [TSB] was prepared for the API kit test. Therefore, in this context for the preparation of 100ml TSB, total of 3g of TSB was taken and dissolved in 100 ml of water. After the proper stirring, the media was autoclave at 121℃ for 15 minutes. On the other side six test tubes were wrapped after proper washing and covering with the help of cotton and air tight so that no air could entered into the tubes. After this, tubes were autoclaved at 121℃. The 10ml of media was poured in each test tube having proper covering with cotton. Now a loop full of purified bacteria was inoculated and made slant of the media with that of bacteria and incubated it for overnight at 37℃. Then 1ml of media was transferred in the strip hole by using the pipette. All introduced tests except VP, TDA and IND gave the result within five minutes, which was recorded and interpreted. However, in these three tests after 24 hours the reagents were introduced, TDA reagent and VP1 and VP2 reagent in TDA and VP test respectively. The results were recorded after 5 minutes. It takes 3-5 minutes to change color which is an indication of record results [145]

3.4.8 16S rRNA Sequencing

The high throughput the earliest technique to study the microbial ecology is the use of ‘16SrRNA sequence that seems to be the most conserved one. It is cost effective approach in a community for the survey of bacteria [9]. In order to determine the microbiota associated with the houseflies the preserved strains were send for 16S sequencing, the samples were sequenced from Microgen Korea.

3.5 BLAST and Phylogenetic Analysis

Basic local Alignment Search Tool [BLAST] is the tool available at NCBI that is used for the alignment of sequence with the reference sequence and give the

similarity index according to the matches, mismatches, and gaps. The BLAST results for strain S1–785 that gave 99.43% similarity index with 50% query coverage, MEGAX was used to find out the phylogenetic history of specie. The sequences closest to the strain S1–785 were taken, total of 6 sequences, gave closest similarity to that of S1–785. The sequences were pasted on a separate file. This file was imported in MEGA and then aligned by muscle. After the alignment, the low quality sequences were removed and file was subjected to phylogenetic analysis. The tree was constructed using Maximum likelihood method.

3.5.1 NCBI Submission

After the removal of low quality sequences, sequences were submitted on the NCBI.

3.6 Antibiotic Sensitivity Test

The most important part of disease management is to determine the antibiotic resistance pattern of bacteria for different antibiotics. Kirby and his colleagues A. W. Bauer first developed the disk diffusion method which is alternative of previous broth dilution methods. The test was coined to check the resistance of strains isolated and sequenced that either they are resistant to antibiotics or susceptible. The strains, with less zone of inhibition, show resistant to that specific antibiotic and the strains with more zone of inhibition are susceptible one. Therefore, in this perspective firstly the nutrient broth was prepared, for the preparation of 100ml TSB, 3g of TSB was taken in the flask having 100ml of distilled water. After proper shaking the flask was wrapped with aluminum foil, and autoclaved along with six clean wrapped test tubes at 121℃ for 15 minutes. It was inoculated with bacteria and kept overnight at 37℃ in incubator so that bacteria may grow into the broth.

3.6.1 Kirby Bauer Method Procedure

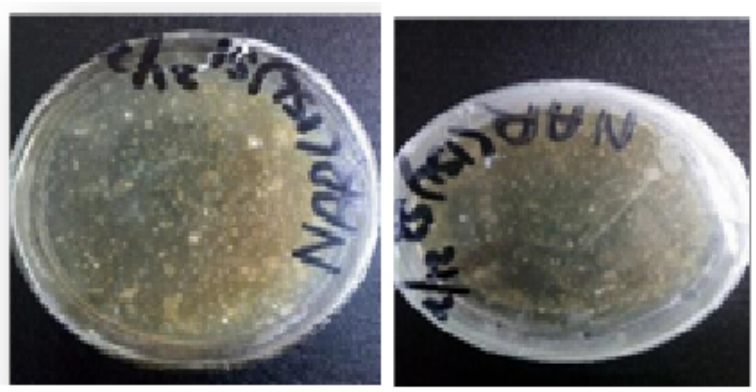
1. Muller-Hinton agar media was set having standardized composition.
2. Muller-Hinton agar media was poured into 150 mm petri dishes at a level of 4mm deep.
3. The agar media was maintained at pH range of 7.2 to 7.4 and broth culture was used for inoculation.
4. The culture plates was made inoculated by streaking a sterile swab passed through broth culture of bacteria.
5. The agar media plates inoculated with bacteria was left for about five minutes to dry.
6. The antibiotics disks were transferred to the inoculated agar plates by using sterilized needles.
7. The discs were gently press by using flame-sterilized foreceps to make sure that each disc is in contact with surface of agar media properly.
8. The plates were incubated at incubation temperature of 37℃ for the night.
9. The zone of inhibition was measured for each antibiotic disc by using scale or screw gauge which determined the effectiveness status of the antibiotic against bacteria.

Chapter 4

Results and Discussion

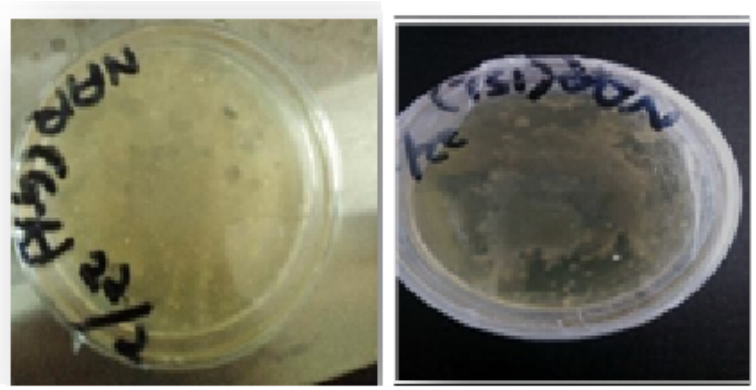
4.1 Nutrient Agar Growth

Bacteria were isolated from the external and internal parts of *M. domestica*. A general purpose nutrient agar media was used to culture the isolated bacteria. It is used for the growth of variety of bacteria and fungi [8, 98]. The nutrient agar is chemically composed of peptone, beef extract and agar. This type of simple formula composition provides the sufficient nutrients to bacteria which are favourable for their growth and their genome replication [132]. Nutrient Agar allows the growth of gram-positive as well as gram-negative bacteria. The culture results showed the growth of variety of bacteria (Fig2.1, 2.2).



A **B**

FIGURE 4.1: A: Nutrient Agar 1, B: Nutrient Agar 2



A B

FIGURE 4.2: A: Nutrient Agar 3, B: Nutrient Agar 4

Fungi and bacteria have been isolated and identified from human and animal premesis such as hospitals, farm houses and food court areas [8]. These growth of bacteria on nutrient agar confirmed the association of bacteria with *M. domestica*.

4.2 MacConkey Agar

The purpose of MacConkey agar used is to isolate the gram negative bacteria extracted from *M. domestica*. MacConkey agar differentiate fermenting gram negative bacteria from lactose non-fermenting gram negative bacteria. Chemically it is composed of gelatin and peptones which is an extraction of meat and casein. These different chemicals provide the source for nutrients and vitamins for the growth of microorganisms. The bacterial pathogens which can grow from MacConkey agar i.e. includes *E. coli, Enterococcus, Aerobacter pseudomonas*. MacConkey media only allows the growth of gram-negative bacteria hence it inhibits the growth of gram positive bacteria. Pathogens were isolated by maceration method from adult houseflies. *M. domestica* collected from 3 locations and were grown on MacConkey agar. MacConkey agar inhibits the growth of gram-positive bacteria. The results showed that all the three locations specimens showed the bacterial growth indicating the presence of gram negative bacteria. MacConkey agar contain Bile salts which prevent most of gram-positive organisms to grow. Neutral red and crystal violet present in this medium are very lethal to bacteria. Gram-negative bacteria

are more resistant to the dyes present in this medium than gram-positive bacteria. Moreover, Bile salts reduces this toxicity for gram-negative bacteria and increase toxicity for gram-positive bacteria. Gram negative bacteria usually shows more significant growth on medium and these bacteria can differentiate due to their lactose fermenting ability. The lactose fermenting bacterial strains shows red or pink coloured colonies and which may be surrounded by a zone of acid precipitated bile. The red colored pattern is just due to the releasing of acid from lactose, when pH of medium drops below 6.8 in the result.

Absorption of neutral red starts and lateral change in colour of the dye occurs. While lactose non-fermenting bacterial strains like *Salmonella* and *Shigella* shows transparent and colourless appearance which normally do not change the medium appearance. The samples were collected from Domestic Kitchen, some cultured samples showed a shiny pink color colony, some cultured samples showed white appearance and some showed orange.

FIGURE 4.3: MacConkey Agar Plate 1 Chakwal

The results of pathogen isolated showed gram negative bacterial growth. The colorless colonies indicate the probability of presence of Salmonella spp and Proteus vulgaris as shown. To further confirm the presence of gram negative bacteria, gram staining procedure was carried out.

FIGURE 4.4: MacConkey Agar Plate 2 Chakwal

FIGURE 4.5: MacConkey Agar Plate 3 Chakwal

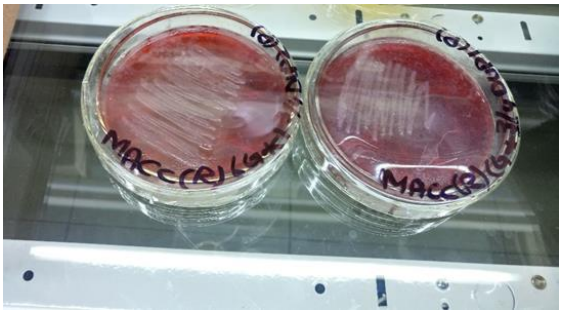


FIGURE 4.6: MacConkey Agar Plate 4 Chakwal

4.3 Eosin Methylene Blue Media (EMB)

In dehydrated premixed form, EMB is available for commercial use. When commercial powder is rehydrated it produces a medium comprising the following components (g/L): peptone (Bacto-peptone or Gelysate) 10.0, lactose 5.0, sucrose 5.0, dipotassium phosphate 2.0, agar 13.5, eosin 0.4, and methylene blue 0.065. Final pH is 7.2 $\pm$ 0.2. Media with these components allows the growth of gram negative bacteria but inhibits the Gram positive bacteria.

Dyes of methylene blue and eosin in EMB inhibit Gram positive bacteria, thus favoring growth of Gram Negative. On the other hand Eosin methylene blue media helps in the identification of *E. coli*, from nonpathogenic lactose-fermenting gram negative rod shaped bacteria [146]. Domestic Kitchen was the source for all the samples isolated from *M. domestica* were grown on EMB media. Samples showed green metallic sheen color which depicts the presence of *E. coli* and *Klebsiella pneumonia* with pink mucoid color. Samples cultured showed the green color and luxuriant growth of *E. coli* on media and *Klebsiella pneumonia* glossy pink color.

Gram negative bacteria with lactose fermentation produce acid as the acid acts upon the dyes it turns the colonies into dark purple.

In addition, bacteria with certain lactose-fermentation produce flat, dark colonies with a green metallic sheen. Larger, mucoid colonies, are produce by other lactose fermenters, often purple only in their center. In EMB agar, most of the strains of *E. coli* colonies have a characteristic green sheen. Rapid reduction in the pH of the EMB agar is the critical factor in the formation of the green metallic sheen observed with E.coli, rapid fermentation of lactose and formation of strong acids. Bacteria without lactose fermentation are either colorless or light lavender. The basic component of EMB are enzymatic digest of gelatin, lactose sugar that majorly help to differentiate lactose fermenter from non-lactose fermenter, it also contain dipotassium phosphate, eosin Y: indicator, agar, and methylene blue. Therefore, the primary purpose for which we use this media is to separate lactose fermenter bacteria from non -lactose fermenter bacteria. EMB gives significant

results to bacteria that was streaked on EMB. Appearance of green sheen indicate the presence of *E. coli* on the media. Dyes present in the EMB are potentially hazardous for the growth of gram-positive bacteria thus EMB inhibits the growth of gram-positive bacteria. The different colors of colonies are obtained.

Dyes with reversible oxidation-reduction potentials, such as methylene blue, are toxic to bacteria. Growth of gram-positive bacteria being inhibited by the EMB media. EMB with the growth depicts the presence of gram negative bacteria. Dyes with reversible oxidation-reduction potentials, such as methylene blue, are toxic to bacteria [147]

FIGURE 4.7: EMB plate 1 Chakwal

FIGURE 4.8: EMB Plate 2 Chakwal

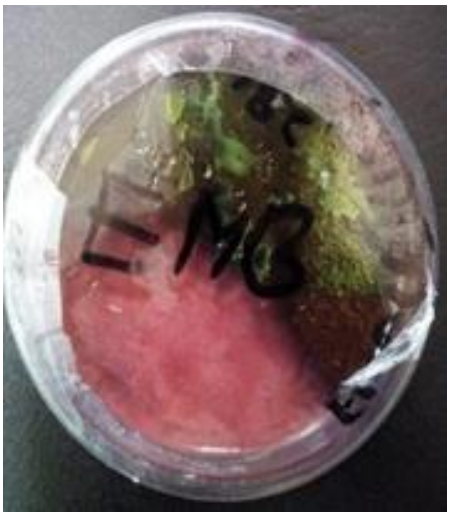


FIGURE 4.9: EMB Plate 3 Chakwal

FIGURE 4.10: EMB Plate 4 Chakwal

4.4 Mannitol Salt Agar (MSA)

For the isolation of *Staphylococci*, Mannitol salt agar (MSA) is used that is both selective as well as differential medium. This medium consists of 7.5% sodium chloride, that's because it is chosen for those bacteria which can bear high salt concentrations. The only carbohydrate in the MSA is sugar mannitol which is used to distinguish bacteria on the basis of fermentation. Mannitol fermentation is demonstrated by changing of media color, not only by colony color. This process is predominantly significant as several *micrococci* are pigmented [148]. All the samples collected from three locations showed growth indicating the probability of presence *Staphylococci* in the isolates. The absence of any bacterial growth also

indicates or confirm the results of MAC and EMB results as MSA inhibits the growth of *E. coli, Klebsiella, Enterobacter* and *Proteus spp.* The change in color of media depicts the growth of *Staphylococci.* This predict that houseflies contain the staphylococci that was to be separated on the MSA agar plates.

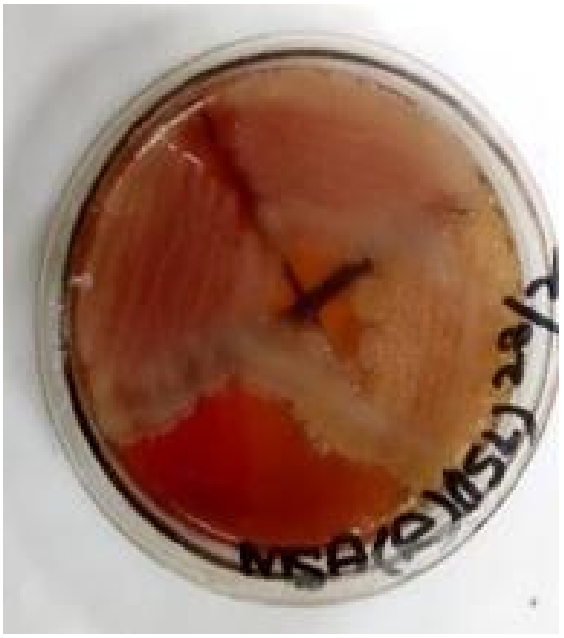


FIGURE 4.11: MSA plate 1 Chakwal

FIGURE 4.12: MSA Plate 2 Chakwal

FIGURE 4.13: MSA plate 3 Chakwal

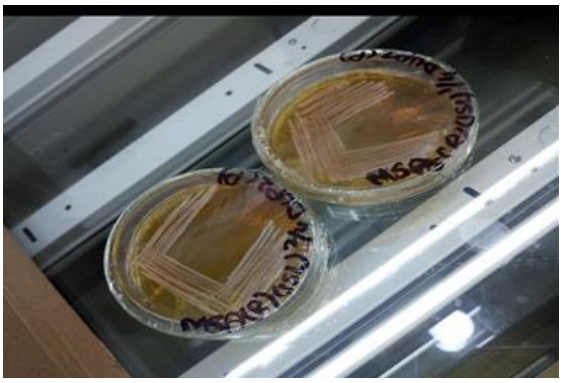


FIGURE 4.14: MSA Plate 4 Chakwal

4.5 Isolation of Bacterial Pathogens

The culture that was obtained on the differential media was streaked further to isolate the bacteria. Different types of bacteria were obtained with different morphology, different color characteristics, different colony characteristics. Bacterial species or genus were categorized based on the color characteristics and morphology on differential media.

Following results including their sample location, colony color, morphology, pigmentation, media, predicted strain name and figure are shown in table (Table 4.1).

Table 4.1: Bacterial Isolates on different Media

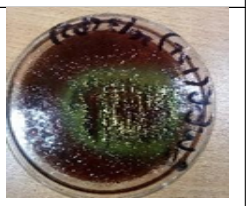
S#	Reference Code	Sample Location	Colony Color	Colony Morphology Form	Pigmentation	Media	Predicted name of strain	Figures
1	EMB (P) 1	Chakwal	Green sheen	Circular	Green sheen	Eosin methylene blue agar	*E. coli*	
2	MSA (P)1	Chakwal	White	Punctiform	White	Mannitol salt agar	*Salmonella* /*Staphylococcus*	
3	MACC (P)1	Chakwal	Off white	Circular	Off white	MacConkey agar	*Salmonella*	

Table 4.1 continued from previous page

S#	Reference Code	Sample Location	Colony Color	Colony Morphology Form	Pigmentation	Media	Predicted name of strain	Figures
4	MSA (P)2	Chakwal	Pink	Circular	Pink	Mannitol salt agar	*Staphylococcus epidermidis*	
5	MSA (P) 3	Chakwal	White	Circular	White	Mannitol salt agar	*Salmonella*	
6	MSA (P) 4	Chakwal	White	Circular	White	Mannitol salt agar	*Staphylococcus*	
7	MACC (P) 2	Chakwal	Pink	Circular	Pink	MacConkey agar	*Klebsiella*	

Table 4.1 continued from previous page

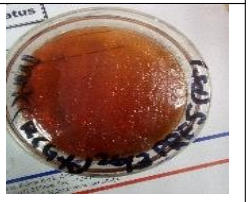
S#	Reference Code	Sample Location	Colony Color	Colony Morphology Form	Pigmentation	Media	Predicted name of strain	Figures
8	MACC (P) 3	Chakwal	Yellow	Circular	Yellow	MacConkey agar	*Shigella*	
9	MACC(P)4	Chakwal	Orange	Puntiform	Orange	MacConkey agar	*Salmonella*	
10	MSA (P)5	Chakwal	White	Puntiform	White	Mannitol salt agar	*Staphylococcus*	
11	MACC(P)5	Chakwal	Yellow	Circular	Yellow	MacConkey agar	*Salmonella*	

Table 4.1 continued from previous page

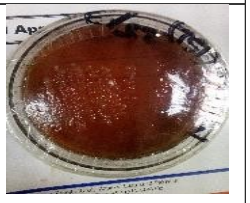
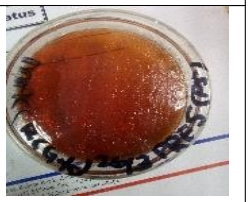
S#	Reference Code	Sample Location	Colony Color	Colony Morphology Form	Pigmentation	Media	Predicted name of strain	Figures
12	EMB (P)2	Chakwal	Purple	Circular	Purple	Eosin methylene blue agar	*Pseudomonas*	
13	EMB (P)3	Chakwal	Green sheen	Circular	Green sheen	Eosin methylene blue agar	*E. coli*	
14	MACC (P)6	Chakwal	Orange	Circular	Orange	MacConkey	*Klebsiella*	

Table 4.1 continued from previous page

S#	Reference Code	Sample Location	Colony Color	Colony Morphology Form	Pigmentation	Media	Predicted name of strain	Figures
15	MACC (P)7	Chakwal	Pink	Irregular	Pink	MacConkey Agar	*Aerobacter*	
16	MSA (P)6	Chakwal	Yellow	Circle	Yellow	Mannitol salt agar	*S. aureus*	
17	MSA (P)7	Chakwal	White	Puntiform	White	Mannitol salt agar	*Staphylococcus*	

4.6 Preservation of Prevalent Strains

The bacterial plates that seems to be more prevalent were further purified by streaking and culturing them repeatedly hence, the purified strains are obtained (Table 4.2). These were further stored in the glycerol stock and put in the refrigerator for future use. These pure strains also contain the duplicates, means one strain contain two copies

TABLE 4.2: Preserved strains from Musca domestica

S#	Reference code	Media	Colony Color	Pig.	Incub. time	Figures
1	EMB Culture Plate 1 Chakwal (DUP1)	EMB	Purple	Purple	24 hrs.	
2	EMB Culture Plate 2 Chakwal (DUP2)	EMB	Purple	Purple	24 hrs.	
3	MACC Culture Plate 1 Chakwal	MACC	Pink	Pink	24 hrs.	

4	MACC Culture Plate 2 Chakwal	MACC	Pink	Pink	24 hrs.	
5	MSA Culture Plate 1 Chakwal DUP1	MSA	Yellow	No	24 hrs.	
6	MSA Culture Plate 2 Chakwal DUP2	MSA	Yellow	No	24 hrs.	

4.7 Biochemical Analysis

4.7.1 Staining of Pure Cultures

The staining of pure cultures was performed by Gram staining method. A Danish physician, Hans Christian Gram in 1884 performed staining of pure cultures called as Gram staining method, also called differential stain. This procedure differentiates bacteria into, Gram negative and Gram-positive bacteria. Due to different differences in chemical structure of bacterial cell wall, Gram stain reaction give two different colors. The cell wall of Gram positive bacteria is thicker in peptidoglycan layer as compared to Gram negative and also it is surrounded by outer lipid containing layer. Lipid is in high contents in Gram negative forming

large pores causing the leakage of crystal violet, resulting in the decolonization of the bacterium and take counter stain later. The thick and cross-linked peptides in gram positive cell wall causing its dehydration and closure of pores, retaining the primary stain. The bacteria which retain the primary stain appear dark blue or violet and not decolorized when stained with Gram's method are called Gram positive, where as those that lose the crystal violet used counter stain, safranin appear red are called as Gram negative. The Gram stain uses different reagents in the order as crystal violet, iodine solution, alcohol, and safranin. The results were significant that concluded that the bacterial species obtained on MacConkey are stained pink which concluded that the species grown on MacConkey are Gram negative. Moreover, their microscopic examination shows that these are circle. The strains that are obtained on the Mannitol Salt agar are purple in stain, which indicate that these are Gram Positive. The stains that are obtained on the EMB are stained pink, which means they are Gram negative.

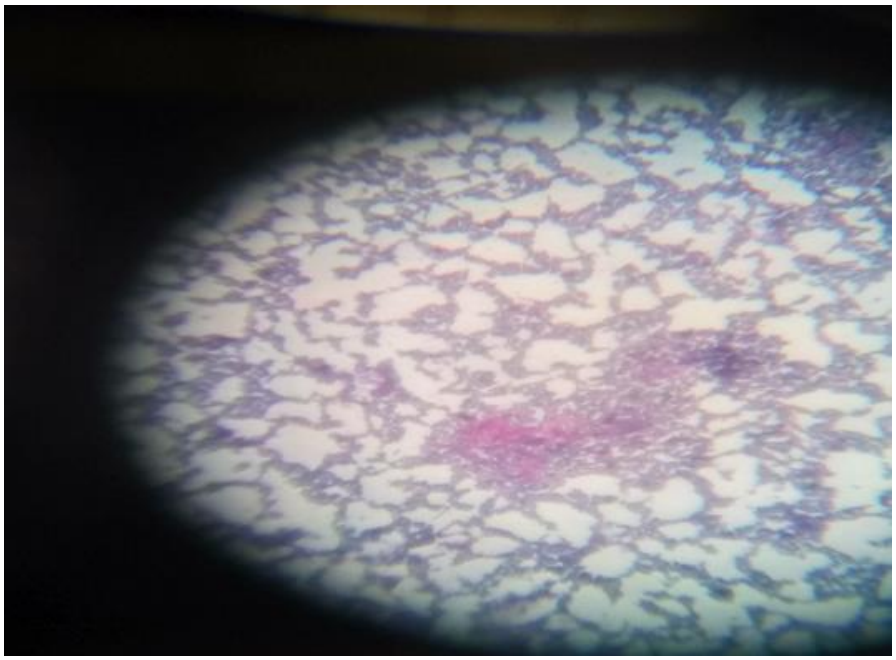


FIGURE 4.15: Staining of Prevalent Strain

4.7.2 Urease test

The urease test that was coined for the analysis that the strains either use the urea or acquire urea after the 2 days examination the strain show positive result with the urease test .The result is considered positive if the yellow color of media is turned into pink after the utilization by the strain culturing in that plate.

4.7.3 Citrate Utilization Test

This test involve Simmons Citrate agar which act as only source of carbon. Bromothymol blue act as an indicator turning its color green to blue when pH increases above 7.6. If it uses citrate,then it produces alkaline products [76]. The results shows that strain give positive result in the media and turned into blue after 4 days. That indicate, this specific strain is utilizing the citrate for metabolic activities.

4.7.4 Catalase Test

This test is performed to differentiate Gram-positive cocci shaped bacteria which are the members of genus *Staphylococcus* that are catalase positive and the members of genus *Streptococcus* and *Enterococcus* which are catalase negative. The use of catalase test is to differentiate between gram positive and gram negative bacteria like aerotolerant strains of *Clostridium* which are basically catalase negative from *Bacillus* spp. which is basically gram positive specie. Another type semi quantitative catalase test is applied for the identification of *Mycobacterium tuberculosis* bacteria which cause tuberculosis in humans. The catalase test is also used for the identification of *Enterobacteriaceae*. The different members of *Enterobacteriaceae* family are basically catalase positive. Based upon the results of MSA catalase test with15% H_2O_2 solution was performed to differentiate the aerotolerant strains of *Clostridium* from Bacillus species. It was observed that instantly the process of bubble formation starts. The bubble formation process indicates the presence of *Staphylococcus* species in all the three samples which

were isolated from *M. domestica* and were collected from Kitchen hence proving the ability of housefly as a mechanical carrier of *Staphylococcus* species.

In few years of last decade much attention has been given to *M. domestica* because these flies have potential in the transmission of bacterial pathogens. Different studies have shown the symbiotic relationship of bacterial pathogens with *M. domestica*. The most common examples of bacteria which can be isolated from the body surfaces of *M.domestica* are *E. coli, Salmonella* spp., *Staphylococcus* spp., Methicillin Resistant *Staphylococcus aureus* (MRSA), *Vibrio cholera* , *Listeria* spp., *Shigella* spp., *Bacillus* spp., *Helicobacter pylori*, *Klebsiella* spp., *Serratia* spp., *Enterobacter* spp. many of these species have been discussed in recent researches [4]. The proboscis of flies contains large number of fine hairs, when the flies sits on the surface of garbage or filthy places they collect harmful bacteria from environmental detritus instantly. It has been demonstrated that when the flies lands on wounds of guinea pigs they carries *Anthrax bacilli*. Number of flies collected from different food processing units and factories and their microbiological analysis of vomitus and waste matter showed the presence of *Bacillus* spp [14]. House flies and bluebottles landed on the surface of food and drop off *Bacillus atrophaeus* spores in food [141]. It has been found that the flying insects have potential to transmit the Gram positive rod shaped, spore forming bacteria so the mechanical transmission of *Bacillus* spp especially spore forming *Bacillus cereus* is possible through flying insects. In another research observed flies from different breeding areas like food halls, food processing units and poultry farms *Bacillus* spp., *Coccobacillus* spp., *Staphylococcus* spp., *Micrococcus* spp., *Streptococcus* spp., *Acinetobacter* spp., *Enterobacter* spp., *Proteus* spp., *Escherichia* spp., *Klebsiella* spp. isolated from the excretory products of house flies [14].

Researchers in Malaysia had isolated different bacterial species from the body of *M. domestica* and *Chrysomya megacephala* from hospitals. Some of the bacterial pathogens isolated are the *Acinetobacter* spp., *Bacillus* spp., *Enterobacter* spp., *Proteus* spp., *Escherichia* spp. and *Klebsiella* spp. Eighteen bacterial species were found to be associated on the body surface of *M. domestica* including *Burkholderia pseudomallei* Gram negative bacteria that caused the disease melioidosis [119].

House flies are responsible for the transmission of multiple pathogenic organisms and the anatomy of these flies exhibit the sites of contamination. The three most common means are established through which houseflies are able to transmit the pathogens. The mechanism of this transmission is based on the anatomy and as well as on the behavior of fly and their habit of association with the waste products as animal manures and excretion of the humans [140]. The *E. coli* are present on the alimentary canal and on the mouthparts of *M. domestica* [149]. The number of bacteria have been identified which have been isolated from the surfaces as gut of flies particularly *Salmonella enterica* [150]. *Vibrio cholera* bacteria are recognized on the location of abdominal inter segmental membranes in the exoskeleton [148]. It is evident that some of the bacteria have been used to exist on the wings of flies as *M. domestica* wings as *Vibrio cholera*. *Klebsiella spp.* are present in the respiratory tract of human and causes pneumonia and also cause eye infections. It also have ability to produce urinary tract infection. *Klebsiella spp.* also causes nosocomial infections which are associated with the inflammation of upper respiratory tract. *Enterobacter spp.* are also known to cause urinary tract infection [151]. *Staphylococcus spp.* and *Bacillus spp.*, are causative agents for diarrhea and is common in Pakistan. It is a potential in houseflies to carry different pathogenic bacteria that are resistant to multiple antibiotics as been reported in different research works,it show that many of the *Enterococcus spp.* isolates from house flies were resistant to tetracycline and erythromycin. Data from the past studies shows that house flies which grows on animal manure and decaying organic material can play important role in development and dissemination of these antibiotic resistance commensal bacteria in environment. In conclusion, the current study shows that houseflies collected from different locations are all capable of carrying ART bacteria. The free exposure of houseflies to animal farms, poultry farms, slaughter houses facilitates resulted in greater prevalence of Antibiotic resistance bacteria and there is a great capability of houseflies to carry multi-drug resistant bacteria. To control the production of house flies is still an important public health concern in the 21st century especially in developing countries. The conclusion is it is proved that the flying insects act as a mechanical vectors and responsible for

the spreading of diseases. The possible way is to eliminate or reduced the breeding places of flies. To eliminate the breeding sites it is necessary to improve the sanitary conditions and hygienic conditions.

4.7.5 Validation of API 20E Kit Results by Using Media

The most prevalent strain was undergone for API20kit tests. The results of biochemical tests was validated by using API20E, it provided the evidence that the results obtained after the biochemical tests was true with reference to the API 20E strip results. By using the API 20E the change in color was predicted by using the reference guide. It gives the result as follows (Table 4.3)

TABLE 4.3: API20E Kit Results that are Interpreted by Using the Reference Table Used for the Validation of Biochemical Test Results

S#	Name	Results
1	Ortho-nitrophenyl-galactosidase test (ONPG)	Positive
2	Arginine dihydrolase test(ADH)	Positive
3	Lysine decarboxylase test(LDC)	Positive
4	(ODC) Ornithine decarboxylase test	Positive
5	Citrate test(CIT)	Positive
6	Hydrogen sulphide test(H2S)	Negative
7	Urease test(URE)	Positive
8	Tryptophan deaminase test(TDA)	Negative
9	Voges Proskauer test(VP)	Positive
10	Gelatin hydrolysis test(GEL)	Positive
11	Glucose test(GLU)	Positive
12	Mannose test(MAN)	Positive
13	Inositol test(INO)	Positive
14	Sorbitol test(SOR)	Positive
15	Rhamnose test(RHA)	Positive

16	Sucrose test(SAC)	Positive
17	Melibiose test(MEL)	Positive
18	Amygdalin test(AMY)	Negative
19	Arabinose test(ARA)	Negative
20	Indole test(IND)	Negative

Ortho-nitrophenyl-galactosidase test (ONPG test) for β-galactosidase enzyme by hydrolysis of the substrate o-nitrophenyl-b-D-galactopyranoside for bacteria, ADH decarboxylation of the amino acid arginine by arginine dihydrolase, LDC decarboxylations of the amino acid lysine by lysine decarboxylase, ODC decarboxylations of the amino acid ornithine by ornithine decarboxylase, CIT utilization of citrate as only carbon source test , URE test for the enzyme urease, VP the Voges-Proskauer test for the detection of acetoin (acetyl methylcarbinol) produced by fermentation of glucose by bacteria utilizing the butylene glycol pathway, GEL test for the production of the enzyme gelatinase which liquefies gelatin, GLU fermentation of glucose (hexose sugar), MAN fermentation of mannose (hexose sugar), INO fermentation of inositol (cyclic polyalcohol), SOR fermentation of sorbitol (alcohol sugar), RHA fermentation of rhamnose (methyl pentose sugar), SAC fermentation of sucrose (disaccharide), MEL fermentation of melibiose (disaccharide), AMY fermentation of amygdalin (glycoside) and ARA fermentation of arabinose (pentose sugar) were positive. H2S production of hydrogen sulfide, TDA (Tryptophan deaminase) detection of the enzyme tryptophan deaminase Reagent to put- Ferric Chloride, IND Indole Test-production of indole from tryptophan by the enzyme tryptophanase, Reagent- Indole is detected by addition of Kovac's reagent were negative.

4.8 NCBI Submission

The Strain sequence was submitted to NCBI with an accession number MN252579. Strain sequence is as below,

>MN252579.1 Staphylococcus sp. strain RS 4 785 16S ribosomal RNA gene, partial sequence
ATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCCTTCCCCTTCGGGGGACAAAGTGACAGGTGGTGCA
TGGTTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTAAGCTTAGT
TGCCATCATTAAGTTGGGCACTCTAGGTTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCCTTATGATTTGGGCTACACACGTGCTACAATGGACAATACAAAGGGCAGCTAAACCG
CGAGGTCATGCAAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTACATGAAGCT
GGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATACGTTCCCGGGTCTTGTACACACCGCCCGT
CACACCACGAGAGTTTGTAACACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCGTCGAAGGTGG
GACAAATGATTGGGGTGAAGTCTAAAAGGGGGAG

4.9 Phylogenetic analysis

In different clinical laboratories and microbiology laboratories the most common and frequent microorganism isolated is from the genus *Staphylococcus*, the coagulase negative *Staphylococcus* [152, 153]. From as early as 1970s this bacteria CoNS has been known as cause of different infections and are of great importance as pathogens [154, 155]. The infection caused by CoNS occur mostly in patients suffering from neutropenia,in neonates and patients with indwelling foreign devices [156, 157]. They cause infections at different metastastic sites such as the central nervous system, heart, bones and joints, and such infections in these vulnerable populations are difficult to treat [158] .

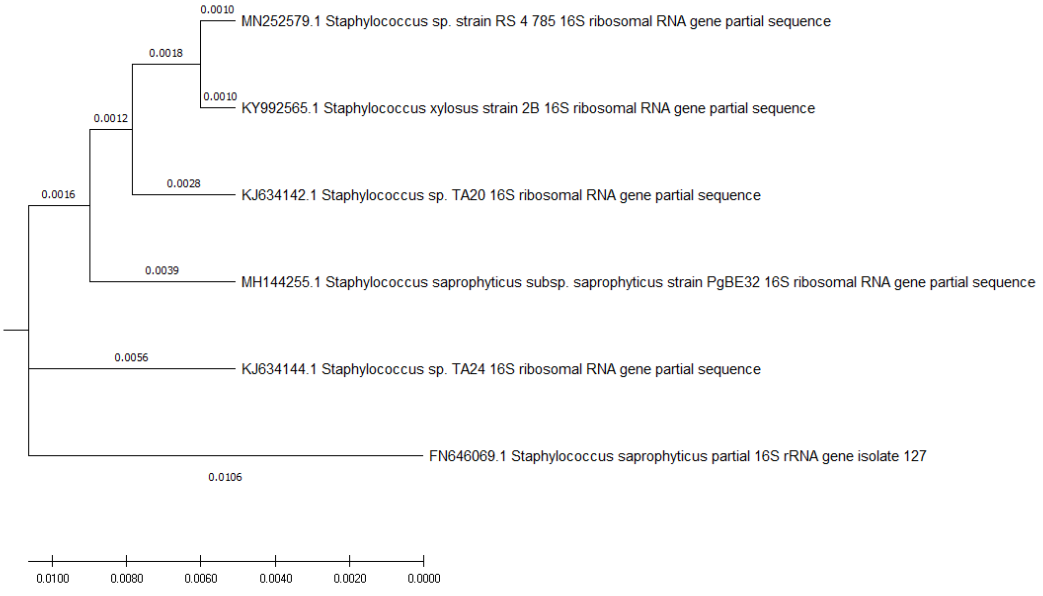


FIGURE 4.16: Phylogenetic Analysis

Using NCBI, BLAST of the *Staphylococcus ssp.* Strain RS 4 785 16S is performed and the species were selected which have the minimum difference and maximum sequence coverage. From BLAST 6 species were selected based. For the selected 6 sequences from BLAST, alignment is performed using MUSCLE in MEGAX tool. After the alignment phylogenetic tree is constructed using Unweighted Pairwise Groups with Arithmetic Mean (UPGMA). The *Staphylococcus sp.* Strain RS 4 785 16S with accession code MN252579 closely resembles with *Staphylococcus Xylosus* (KY992565) and grouped together with only 0.10% difference. There is a 0.28% difference of MN252579 with KJ6341142 and the difference of 0.39 % exists between MN252579 and MH144255, MN252579 has the maximum difference of 1.06% with FN646069 as shown in above figure.

4.9.1 Antibiotic Sensitivity Test

The most prevalent strain of bacteria was isolated and undergone for antibiotic sensitivity test. Total 9 antibiotics were used for this purpose. The Disk diffusion method was used to check the antibiotic sensitivity.

Zone of inhibition for the nine antibiotics is mentioned in appendix 1 and the percentage of resistance is mentioned in table. Highest percentage of resistance i.e, 93.30% was recorded in Gentamycin with a least resistance for Tazobactam. Where as interms of sensitivity Tazobactam was found to be hughly sensitive with 86.6% and no intermediate value was recorded. Cefotaxime and Nalidixic acid showed 0% sensitivity for *S. xylosus* where as 0% intermediate values were recorded for Chloramphenicol, Ciprofloxacin, Fusidic acid, Gentamycin with resistance value of 53.3%, 86.60%, 40% and 93.3%.

Table 4.4: Percentage of Resistance of Antibiotics for *Staphylococcus* Specie

	Cefotaxime	Chloramphenicol	Ciprofloxacin
Resistant	12	8	13
Intermediate	3	0	0
Sensitivity	0	7	2

Table 4.4 continued from previous page

R%	80%	53.3%	86.6%
I%	20%	0	0
S%	0	46.6%	13.3%
	Fusidic acid	Gentamycin	Imipenem
Resistant	6	14	0
Intermediate	0	*0*	1
Sensitivity	9	1	14
R%	40%	93.3%	0
I%	0	0	6.6%
S%	60%	6.6%	93.3%
	Nalidixic acid	Norfloxacin	Tazobactam
Resistant	11	10	2
Intermediate	4	1	0
Sensitivity	0	3	13
R%	73.3%	66.6%	13.30%
I%	26.6%	6.6%	0
S%	0	20%	86.60%

Staphylococcus xylosus proved to be resistant to Cefotaxime, Chloramphenicol, Ciprofloxacin, Fusidic acid and Gentamycin. *Staphylococcus aureus*, involved in the *Micrococcaceae* family is a Gram-positive bacteria. *Staphylococcus* species are the most common bacteria and present in all environments. *Staphylococcus aureus* strain is gram positive and coagulase negative. These are commonly present and had developed resistance against the environment and many aseptic chemicals. These are vector to many diseases causing skin diseases and severe infections so it should be removed from sites [161, 162].

The *Staphylococcus* genus contain different species which are disease causing and live in commensals to skin of animals. Strains including *S. aureus* is a pathogen resistant to methicillin mostly called as methicillin resistant *Staphylococcus aureus*

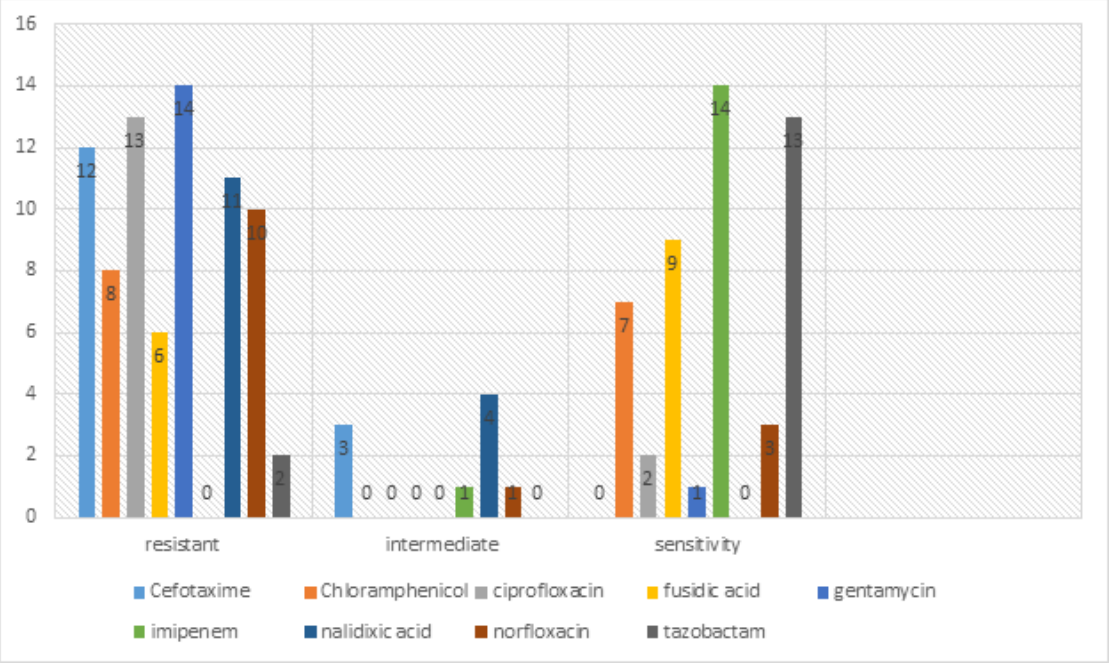


FIGURE 4.17: Drug Resistance Profile of *Staphylococcus xylosus*

and to vanomycin mostly called as vanomycin resistant *Staphylococcus aureus* and this antibiotic is also termed as "drug of last resort" [163, 164]. Moreover, from the last decade, these methicillin resistant *Staphylococcus aureus* has changed their location from hospitals to now being commonly present in living societies and restaurant places [165, 166]. Community-acquired strains have been isolated from areas such as day-care centers, fire stations and educational institutes [167–170].

These resistant bacteria cause diseases in human and animals mostly in horses, with high treatment expenses, morbidity and mortality.

Both groups of *Staphylococcus*, coagulase positive *Staphylococcus* (CoPS) and coagulase negative *Staphylococcus* (CoNS) are pathogens causing many serious infections. All the species of this CoPS are coagulase positive and have the ability to develop resistance against many antibiotics that are used for different treatments of animals and human [171]. CoNS isolated from animals have developed resistance against gentamycin, macrolides, tetracycline, streptomycin, trimethoprim, sulfamethoxazole and fluoroquinolones [171, 172].

The high levels of antimicrobial resistance observed in this study is consistent with the observations in humans in South Africa that were up to 95.1% of the samples

were MDR, and only 3.7% of the samples were susceptible to all antibiotics tested in the study [173].

It has been reported that the variety of bacteria became resistant against the antibiotic. It is very serious issue worldwide. The extensive use of antibiotics in the field of medicine producing resistance in different Gram positive bacteria against the antibiotic. It has been reported in different studies the *Staphylococcus* which have develop resistance against many antibiotic drugs is found in vegetables, poultry, egg, milk and raw meat. In another research it is reported that the *Staphylococcus* with a highest percentage of resistance against was from chicken (23.3%), vegetable salad (20%), raw meat (13.3%), raw egg-surface (10%) and unpasteurized milk (6.7%).

Staphylococcus xylosus is the Gram positive bacteria and most common pathogen in humans. So now a day's antibiotic resistance in *Staphylococcus xylosus* is the main concern because it is responsible for number of infectious diseases like it is the main cause of nasal infection, common cause of hospital-acquired infections. The resistant *Staphylococcus xylosus* bacterial strains transmit the antibiotic resistance determinants to other strains of *Staphylococcuus*, and it is reported in different studies that the resistant *Staphylococcus* have ability to transmit the antibiotic resistant causing bovine intramammary infection.

It has been observed that the fruits and meat contains large number of *Staphylococcus spp.* These bacterial strains extracted from the patients who consumed contaminated fruits and vegetables. In contrast the persons who consumed sterile diet have lower number of *Staphylococcus xylosus* in their clinical tests reports of feces. The bacteria which passed alive through digestive tract to colon are often transient. The resident flora having a protective effect against intruders. The bacteria which are responsible for the transmission of antibiotic drug resistance is still possible, so if our consumed food contains resistant bacteria it could be an important source of creating resistance in gastrointestinal tract.

It is suggested that it is possible that the bacterial populations spreading the resistance from one ecosystem to other [175]. The spreading of antimicrobial

resistance among different bacterial species is a major problem in worldwide and this problem is increasing day by day.

The antibiotic drugs are mostly used for the treatment of infected persons against different infections. The number of findings recommend that poor selection of antibiotics may lead to create resistance in various bacteria and in the result the treatment against the bacterial infections become more difficult [149]. The resistance against antibiotics in *Staphylococcus xylosus* is reported in worldwide. In present the infections which were caused by *Staphylococcus xylosus* has been increasingly problematical due to the production of resistance in bacteria.

Hence the aim of this research was to find the antimicrobial sensitivity pattern of *Staphylococcus xylosus* that was isolated from the *M. domestica* which were collected from domestic kitchen of Chakwal Pakistan.

Chapter 5

Conclusions and Recommendations

The house flies (*M. domestica*) plays a significant role in public health. It is involved in the spreading of different food-borne diseases. These common house flies (*M. domestica*) act as a vector because when these flies sits on the garbage and waste materials enormous variety of bacteria attached on their body surface. In this way when these flies sits or visits on different fruits, vegetables and meat the bacteria drop off from their body surfaces, so as causes contamination. In the present research the isolated bacteria was from *Staphylococcus*. These are pathogenic bacteria and responsible for different infectious diseases in humans as well as Bovine intramammary diseases.

In current research the flies were collected from domestic Kitchen from Chakwal to investigate the nature of pathogens associated with houseflies. Altogether four pathogens belonging to bacteria were isolated and cultured. The frequently cultured bacteria includes *E. coli, salmonella* and *staphylococcus* based on different media results. The present research suggest that washing fruits and utensils before use. Improvement in environmental health conditions through the use of an appropriate waste disposal system. In order to attain the good hygienic practices it

is necessary to prevent the contamination of fruit, vegetables and cattle products. And also disposed the waste products properly.

Furthermore in the present research the microbial activity against the antibiotic drugs were tested. Now a days the bacteria creating resistance against the antibiotic drugs is a challenging problem in worldwide. In this research work the most common isolated bacteria from the *M. domestica* are *Staphylococcus*. In this study antibiotic sensitivity test are performed. The *Staphylococcus xylosus* susceptibility is checked against the nine antibiotics Cefotaxime, Chloramphenicol, Ciprofloxacin, Fusidic acid, Gentamycin, imipenem, Nalidixic acid, Norfloxacin and Tazobactam. The most frequent genus of bacteria that was isolated from domestic kitchen samples of houseflies collected from three different location was *Staphylococcus* it was further confirmed by biochemical and molecular characterization .the phylogenetic analysis showed its close association to Staphylococcus xylosus with similarity of 99.9%. Antibiotic sensitivity test was also performed for this strain of *staphylococcus*. It was found to be highly resistant against Gentamycin and least resistant against Imipenem and Tazobactam. These findings suggest the potential role of houseflies in the transmission of pathogenic bacteria with the antibiotic resistance in households. Exposure of houseflies to animal farming and human habitats has led to greater prevalence of antibiotics resistant bacteria. This study also suggest that direct exposure antibiotics is not required to detect resistant bacteria. Houseflies also plays a potential role in the dissemination of antibiotic resistant to various environments. This study must be expanded to other food localities to inquire the fauna of bacteria frequently occupying in these area. This can help to identify the most commonly associated functions and causes. Moreover, due to shortage of time, it was difficult to identify all microorganisms associated with housefly. Microorganisms found and identified on the basis of differential media and biochemical characterization must also be identified at molecular level.

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Appendix A

Cefotaxime	Chloramphenial	Ciprofloxacin	Fucidic acid	Gentamycin
15	25	19	21	15
14	19	13	19	13
11	13	15	22	19
9	12	12	12	10
10	19	9	23	8
21	27	8	25	9
11	31	3	18	12
13	12	20	23	8
9	19	14	29	8
21	19	17	27	7
11	17	15	30	11
12	13	13	23	10
7	13	13	24	9
8	11	10	19	9
9	11	9	18	5

Impenem	Naldixic acid	Norfloxacin	Tazobactum
43	19	19	30
40	21	17	22
37	18	12	17
15	17	10	33
28	21	8	25

Table 5.2 continued from previous page

30	16	11	25
16	19	10	21
21	22	9	19
27	25	8	18
32	26	12	26
33	27	13	21
23	21	9	22
27	19	7	17
31	18	20	19
29	19	19	21