CAPITAL UNIVERSITY OF SCIENCE AND TECHNOLOGY, ISLAMABAD

Discovery of Bacterial Strain from Midgut of *Anopheles stephensi* in Pakistan

by

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A thesis submitted in partial fulfillment for the degree of Master of Science

in the

Faculty of Health and Life Sciences
Department of Bioinformatics and Biosciences

2021

Dedicated to Allah Almighty, Hazrat Muhammad (SAW) and to my father (Sagheer Hussain), who taught me that the best kind of knowledge to have is that which is learned for its own sake.It is also dedicated to my mother (Shagufta Sagheer), who 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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Acknowledgement

I am highly thankful to Almighty ALLAH whose bounteous blessings enable me to complete this research work as well as to write this dissertation. He bestowed us with His Holy Quran and Prophet Hazrat Muhammad (Peace be upon him), the most perfect and best among born on Earth, who enlightens the hearts of believers. I would like to acknowledge several key figures who contribute much to my research endeavor. It seems impossible to pay thanks to my kind and worthy supervisor Dr.Shaukat Iqbal Malik and Co-Supervisor Dr.Sahar Fazal for their supervision, advice and untiring guidance from the very early stage to this research as well as sharing with me extraordinary experiences throughout the work. Her knowledge, unending support and logical way of thinking have been of great value for me. It is really honored for me that I completed my work under the supervision of such brilliant mentor. I am very thankful to his/ her for inspiration, reassurance and counseling from time to time. I owe a deep sense of gratitude to my teachers Dr. Arshia Amin and Dr. Muzaffar for their insightful encouragement motivation and extraordinary kindness throughout my studies. I would like to pay very special thanks to Mr. AAmir Hussain for coming in my life and his inspirable support and he always encourage me to solve problems and help me in every step of my life and prayers. Special thanks to my dear friend Shagufta Ashraf and Shahir Bano support me with every step I took . Finally, my parents and siblings (Muqdass, Sibty Hassan, Zahra, Misbah) deserve special mention for their inspirable support and prayer. My Whole family, father, mother and siblings who always provide a solution for my problems, through their prayer and moral support. Thanks to all.

(Sabah Kalsoom Naqvi)

Abstract

Mosquitoes found everywhere are primary vector for transmission of many economic importance diseases like nuisance, systematic and local skin reactions and also for acute death causing disease like dengue, malaria and West Nile Virus. It seems that *Anophelese stephensi* is one of the major vectors of the malaria in Pakistan and these mosquitoes have preferred mammals for their blood meal, including humans. Mosquitoes were collected by using aspirator from Islamabad city. Prevalent species of bacteria were isolated from the midgut of mosquito by dissection of mosquito and were cultured on nutrient agar and on differential media.in this studies we focused on the isolation of prevalent species of bacteria of the mosquito microbiota. The most diversified group of microbes was found in *An.stephensi* are *E. coli*, *Bacillus*, *Klebsiella* and *Staphylococcus*. Biochemical characterization was done by Gram staining,Urease test, Catalase test, Citrate test. The prevalent isolated bacteria were *E. coli*, *Bacillus*, *Klebsiella* and *Staphylococcus* based on biochemical characterization. 16S rRNA sequencing was performed for one of the prevalent strains and suggested its close association with *Staphylococcus* spp, with 96% similarty. Phylogenetic analysis showed that the sequenced strain seems independently evolved and revealed ancestral similarity with *Staphylococcus* spp. The strain antibiotic sensitivity was checked against three antibiotics. The strain showed highly resistance against Imipenem with 93.3% and least with Nalidixic acid with 15.38% and showed 0% insecticide resistance against Allethrin, Resmethrin and Thiacloprid. The computational results of the functional genomic of the whole genome were obtained by the NCBI Genome and RAST server, it was observed that *Lactobacillus* species were not involved in any of the aromatic compound metabolic pathways but 15 subsystems were identified to be involved in the antibiotic resistance pathways.

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Abbreviations

API	Application Programming Interface
AR	Antibiotic Resistance
ART	Antibiotic Resistant
An. Stephensi	*Anopheles stephensi*
BA	Blood Agar
BLAST	Basic Local Alignment Search Tool
E.Coli	*Escherichia Coli*
EMB	Eosin Methylene Blue
IRS	Indoor Residual Sprays
MACC	Macconeky
MCAS	Microbial Control Agents
MDR	Multi Drug Resistant
MRSA	Methicillin Resistant Staphylococcus Aureus
MSA	Mannitol Salt Agar
NCBI	National Center for Biotechnology Information
ONPG	Ortho-Nitro Phenyl Glactosidase
P.flaciparum	*Plasmodium falciparum*
P.vivax	*Plasmodium vivax*
RBM	Roll Black Malaria
SCA	Simmons Citrate Agar
TA	Toxin-Antitoxin
TDA	Tryptophan Deaminase Reaction
TSB	Tryptic Soy Broth
UAB	Urea Agar Base

VP	Voges Proskauer
VRE	*Vancomycin Resistant Enterococci*
WHO	World Health Organization
XDR	Extensively Drug Resistant

Chapter 1

Introduction

1.1 Background

Mosquitoes are responsible for many vector-borne diseases, including malaria [1]. The female *Anopheles* were responbile for transfering diseases. It is a endemic disease in tropical and sub-tropical regions where 300 million people die of one million deaths each year. The mosquito family includes 3,570 extant species which can be classified in two sub families and 113 genera. The two sub families are as follow *Anopheline* which includes three genera, and *Culicinae* which include 110 genera, and these 110 genera can be segregate into 11 tribes [2]. Mosquitoes are considered one of the most primitive organisms whose history trace backs to the ancient history of mankind as they were one of the utmost vital vectors for disease of human. They vectored a large variety of disease from very past to human. The medical importance of mosquitos can be estimated from this that 300 to 500 million people are effected from malaria annually. From which 1 million people lost their lives while the maximum number of mortalities are noted in infants and young children. The region which is mostly effected by malaria is Sub-Saharan African region [3]. In the recent years, dengue virus has expanded its range to 50 to 100 million population annually with thousands of mortalities due to severe form i.e. dengue hemorrhage fever. Mosquitoes (family *Culicidae*) are of great

medical importance that have always been in the focus of worlds research due to their property as vector for medically important diseases of human. The wide range of diseases spread is due to the dual property of mosquito as they can be biological vector as well as they can be the mechanical vector. According to the World Health Organization (WHO) report published in 2010, about 247 million worlds population became ill due to mosquito bite. The distribution of mosquitoes throughout the world is somehow misinterpreted that they occur only in tropical and subtropical environments but to some extent it is not true as mosquitos can cause nuisance and annoyance or can also spread pathogens or viruses in temperate latitudes as well [4]. With the recent out break of dengue fever in Pakistan, the studies on mosquito distribution and role as vector bloomed in Pakistan [3]. Almost 104 species of mosquitoes have been reported in literature present in Pakistan. The correct vector identification for the controlled strategies is very important in case of vector borne diseases [5].

The DNA based approaches are used to study the mosquito presence in a specific ecology. In Pakistan many species of *Culex* and*Anopheles* have been identified. Malaria and Dengue is still a threat to this region. The mosquito *Anopheles* is known globally as a species of mosquito malaria because it is known as primary vector of the disease. It seems that these mosquito species have prefer blood-drinking animals, including humans. Adult *Anopheles* mosquito,s body color is dark brown to black in color and has consisting three parts which are the head, thorax and abdomen. The *Anopheles* mosquito can be found anywhere in the world except Antarcteradicated, there is always the possibility that malaria could spread to the area again.because these mosquitoes can be found in the areas where malaria has been about 430 species of *Anopheles mosquitoes*, but only between 30 and 40 species of mosquitoes are real vectors or spread of malaria. Malaria has been ancient considered to be a disease confined to rural areas. Immigration to in progress cities and urban centers has resulted in large areas of urban agriculture, green space and unplanned urban hydropower [6].

In such areas, features are exchanged with rural sites and, as a result, mosquito vectors are included, including both *An.stephensi* and *An.Gambia* are able to

maintain malaria transmission, in some cases, at a wide rate of 30% to 40% [7]. Unlike native African mosquitoes, Asian malaria vector *Anopheles stephensi* is one of the few species of these *Anopheles* found in central urban areas. This is thought to be a direct result of their ability to find clean water in water storage tanks for laying their eggs [8].

Most temporary larval sites, such as ditches or pits commonly used by dominant African species, are more likely to be littered or contaminated (such as with oil or dirty water) in urban settings, and behind pollutant-sensitive *Anopheles* have to be removed. However, there is growing evidence that some species of African species are increasing their tolerance for dirty water. *An. stephensi* apparently expanding its geographical boundaries.

Thus, an estimated additional 126 million people could be at risk of malaria if these *An.stephensi* is able to continue her attacks throughout the subcontinent. Malaria remains a major health problem and is out of control due to drug parasite resistance, vector pesticide resistance and changes in unplanned land use. Malaria is endemic in 109 countries, including Pakistan.

The most formable and friendly deities in the world. Globally, about 1.2 billion people inside the threat of malarian [9]. Since the discovery of the malaria parasite and initiation of mosquito's role in transmitting malaria, it was considered as non-stop to be a major global health problem [10]. The most important genes that are the main human pathogens in vectors are *Anopheles*(for malaria) [11]. The mosquito belongs to the order *Diptera*, with the family Culicidae. The family of these mosquitoes is a very large and abundant group found in the oceans and tropics of the world. Yet a small fraction of the species of mosquitoes on the planet's vectors cause disease to humans.

The sub family *Anopheline* has three genres and the 110 genes of Coliseum are divided into 11 ethnic groups [12]. The *Anopheles stephensi* mosquito is more active in two times that were before sunrise and after dark. At these times of the day, it is important to control outdoor mosquitoes to avoid malaria mosquito bites. Locations are the oldest and most important vectors for vector-borne diseases in human history. It has been found that the Roman emperor Julius Caesar had to wade

through a swamp in an attempt to control the "Roman fever"(malaria). Malaria affects 300 to 500 million people a year, of which 1 million die. The highest number of deaths among children and adolescents in sub-Saharan Africa is from 300 to 500 million people a year. Of the 1 million deaths, sub-Saharan Africa has the highest number of deaths among children and adolescents [13]. *Anopheles stephensi* is one of the major malaria vectors in Pakistan, India, Iran and Afghanistan.

In some parts of its range, this species has increased both relative and absolute abundance suggesting that large-scale irrigation is a response to human-centered climate change. Pakistan's mosquitoes are rarely studied. But modern technological descriptions and keys to the major genus species found in the country include 23 species, in addition to the regional keys to the female *Anopheles* [14]. About 23 species of *Anopheles* have also been reported in Pakistan. Of these, two *Anopheline* species have been confirmed as malaria vectors i.e. *An.culicifacies* as primary vectors in rural areas and *An. stephensi* as a partially responsible vector in urban areas of the country [15].

An. stephensi is considered to be of secondary important vector in urban areas and in the city is only partially responsible for the transmission of malaria. [16] [17]. In actual *An. stephensi* is an important vector in other parts of her species, such as the Persian and Arabian Gulf [18]. The growing ample quantity of *An. stephensi* in Punjab may reflect the change in this particular difference and reflect its more ability to adapt to environmental conditions, such as those related with IBIS [19]. The status of the classifiscation of these types is not clear, it is likely that *An. stephensi* is a single, highly variable species, as no hybrid infertility has been observed [20]. In a study in South Punjab, *An. stephensi* urban wastewater has long been found in an unusual habitat for *Anaphylaxis* mosquitoes [21]. He suggested that due to the salinity created by irrigation in this part of Punjab, more favorable conditions could be created for *An. stephensi*, which allows it to be more popular than relatively less salt tolerant vectors [22]. However, the special feature of the *An. stephensi* Pakistani Punjab system is the fear of documented evidence of changes in the Anthropomorphism as well species formation in the middle of the human race [20] [22].

1.2 Problem Statement

An. stephensi mosquito is one of the most important vectors of human diseases like Malaria, Arboviruses and also responsible for large-scale infectious diseases. Given the importance of the microbiome for the survival of pests, especially vectors the microbiota needs to be modeled to achieve an effective target-specific strategy for vector as mosquito and also control strategies of these vector.

1.3 Objectives

The study has the following objectives:

1. To isolate and identify microbiota in which most prevalent bacterial species from gut of mosquito(species *Anopheles stephensi*).
2. To examine antibiotic and insecticide resistance in most prevalent bactarial species in gut of mosquito.

1.4 Scope

The scope of these studies can help people working in this field and other researcher to explore the role or impact of microbiota in mosquito for example physiology, growth and pesticide resistance etc. These findings can help researchers develop more environmentally friendly and effective mosquito (vector) control strategies and vector associated diseases.

Chapter 2

Review of Literature

2.1 Literature Review

2.1.1 Morphology

Mosquitoes are small (up to 15 mm), two-winged insects. *Anophelese mosquito* considered the main vector of the disease and it is recognized worldwide as species of malaria mosquito. These mosquito species seem to have preferred their blood meal to mammals, including humans. *Anopheles* adult mosquito color is dark brown to black and its body consists of three parts, head, thorax and abdomen [23].

The head is exclusive for feeding and for sensory awareness processing and a pair of long, multi segmented antennae and eyes are included. For detection of host odors, the antennae are essential, as are the odors of breeding sites where females lay eggs. The thorax of these mosquitos is specialized for locomotion. The thorax is attached to one pair of wings and three pairs of legs. For food digestion and egg development abdomen is used . When a female takes a blood meal, this segmented body part extends significantly. Over time, after blood feeding it is digested and act as protein nutrient purpose of egg development the abdomen fill steadily [24]. Having long palps, also have long proboscis and distinct blocks of black and white scales on the wings are appeared which can separate *Anopheles mosquitoes* from

other mosquitoes. Near about 430 species of *Anopheles mosquitoes* found but only 30 to 40 species of mosquitoes are true vectors or malaria spreaders.
The most relevant genera in the vector that are the primary human disease causing agents (germs) are *Anopheles* (for malaria) [25]. The mosquito family Culicidae belongs to the diptera order. In two seasons, *Anopheles mosquitoes* are more active; just before the sunrise and just after the sunset, during these times of the day [26].

2.1.2 Habitat

The mosquito *Anopheles* belongs to the family Culicidae and has always been at the center of the world's biological research because the mosquito vector is mostly medically significant disease for human beings. *An.stephensi* is vector of many diseases and spread a number of parasitic, viral and microbial diseases as these mosquitos play both a biological vector and a mechanical vector. Mosquitoes are medically very important because than half of the population of the world is at the risk of mosquito born diseases like Dengue, Malaria, West Nile Viruse, Chikungunya virus yellow fever, Lymphatic filariasis and Japanese encephalitis. About 247 million people became infected with mosquitoes in 2006 according to the World Health Organization (WHO) and infected about 1 million people with these disease in 2008 [27].

Probably it is estimated that all mosquito species are found in tropical (hot regions) and subtropical environments, but up to some extent it is not true that mosquitoes can cause annoyance or spread pathogens or viruses in moderate latitudes [28]. In sub sahara Africa *Anopheles mosquito* are very common as they are highly adaptable in that environment it is very effective vector of malaria.
But in some cases, more effective in transmitting malaria, due to preference for human blood and night bites and relatively high longevity [29]. There is almost not such a place present in the water where mosquitoes breed do not survive. Because of their adaptation, mosquitoes breed can survive in any type of waters,

such as clear water, swamps, large or small pond and or permanent and temporary waters. *Anopheles* survives in different places, butin these most species prefer fresh and clean water. These mosquitoes larvae have been found in freshwater or saltwater swamps, rice fields, mangrove swamps, grasslands, rivers and streams, and small, temporary rainwater ponds. There is hardly an aquatic environment in the world that does not lend itself to mosquito breeding site. In temporary flooded areas, along with rivers or lakes with water alteration, overflow water flooded water mosquitoes, such as *Anopheles* develops in large numbers and many miles away located from their breeding areas [30].

Although about 83% of the habitats had breeding sites for different species of mosquitoes, *Anopheles mosquito* larvae were found in only 49% in the habitats environment. In addition to changing various climate factors or environmental conditions the ability of these mosquitoes are interesting. Once the days are short, the photo intermittently sensitive female mosquito inhabiting at climate which is moderate, lays different eggs from those eggs which these female mosquito lays in long days. The eggs are laid in short days, they are undeveloped and do not survive until the next season, which ensures the survival of these species through winter season [31]. The ability of this mosquito is to adapt temperate climates and the fact that eggs are resistant from dehydration and live for more than a year, including the ability to adapt to artificial breeding sites such as flower pots, tires etc. *Anopheles* make successful species.

This has contributed to the global spread of the *Dracaena* species through international trade in plants. So they can be transported withi n hours or days from one country to another by, airplane, car or transsea container [32]. With the exception of severe cold weather, mosquitoes can survive in almost any climate. Forests, marshes, tall grasses and weeds are preferred, and soil that is at least half of the year wet. Malaria, especially in tropical and subtropical areas, is one of the most common diseases in the world.

Although now malaria is limited to most especially Sub-Saharan Africa, tropical regions and in colder latitudes many species of Anopheles lives. Even in the past,

malaria outbreaks were reported in cooler climates. Since from first world countries *Plasmodium* parasite not the *Anopheles mosquito* has been removed. The habitats of *Anopheles mosquitoes* are classified into two fundamental types: The one is permanent water mosquitoes and the other is flood water mosquitoes.

2.1.3 Permanent Water Mosquitoes

Among the most common permanent water mosquitoes are *Anopheles mosquito*. When the average temperature is over 70 degrees, these mosquitoes are the most active. In order to survive, their eggs need to remain in water and larvae released when eggs are hatched within few days and this make growth process contiue. That collect in containers and retain water, such as buckets or toys left outside, wading pools, may also breed many permanent water mosquitoes.

2.1.4 Flood Water Mosquitoes

Popular species include the floodwater mosquito, also known as the *Anopheles*. Mosquitoes that breed in floodwater environments typically become a concern after heavy rain for about seven to 10 days, and subside in about a week or two. In moist soil, floodwater mosquitoes lay their eggs and these mosquitoes often live in containers.

2.2 Metamorphosis

Mosquitoes exemplify tremendous environmental flexibility in which the following examples of successful reproduction of mosquitoes can be demonstrated. Similar like Diptera, these mosquitoes can also shows metamorphosis complete. All mosquitoes need aquatic environments for growth. Most types of mosquitoes are unautogenous (unnatural), which follows copulation; these female mosquitos to

take blood to complete egg development. Only a small number of mosquito species have populations that are autogenous [33].

2.2.1 Oviposition

The Oviposition means that the egg is released from the ovaries into the external environment and this is a common phenomenon in vertebrates other than eutherian mammals. After feeding on blood, female mosquitoes lay 50 to 500 eggs, within 2 to 4 days or in moderate climates. Generally, due to their egg-laying behavior mosquitoes can be divided into two groups, and that is the of fetal thirst period externally active resting period or deposition genetically defined resting period. The parameters for choosing a site of breeding by females their eggs laying on the surface of the water are unknown still to many species. Factors such as light incidence, water quality, existing eggs, available food and native plants are key factors in choosing a suitable ground of breeding [34].

It is known that in water content of organic matter plays a vital role in attracting the female mosquito to get eggs. Actually, when components of gas such as methane, ammonia, or CO2 released, when organic matter is dissolved, so these substances have creating the effect of attracting female mosquitoes [35]. In the first group, female mosquitoes *Anopheles* laying their eggs either alone or in batches on the surface of the water.

Anopheles laid an egg while standing on the surface of the water or shaving on it. When the development of embryo is complete, the embryos of the first group are not admitted to the distress or depots and hatches. Extraordinary egg-laying species mostly produce each year several species. For the most part in more permanent waters their developmental stages are found and during the breeding season one generation achieves another. The abundance of generations depends on the length of the breeding season as well as the biotic and abiotic environment , and importantly the temperature that slows down the growth [36]. The group, which lays eggs that do not hatch immediately after oviposition is second group.

2.2.2 Adults

Later on hatching the mosquito eggs, it produces larvae that live in water or in damp habitats that develop into larval stage which is the second instar then it goes through a metamorphosis and produces pupae in which the mosquito changes some of its shapes and then completes the metamorphosis in the final stage when between the pupal and the pharate adult cuticl the gas is forced, and goes into its midgut. The pupa swallowing air further increases the internal pressure and straightens the abdomen horizontally. Splits along the ecdysial line of cephalothoracic cuticle of the pupa and from the pupal skin adult slowly emerges. Pupa's cephalothoracic cuticle then separates along the axillary line. To avoid falling to the surface of the water, emerging adult moves cautiously, while its extremities still remain partially in the exuvia. At this stage,from high winds and predators the emerging individual is highly sensitive such as water spiders striders [37].

Stephensi pupa is formed inside the water on the tissues of plants. At the end of the pupal development, they have to rise above the water surface. Therefore, the immature trumpets break tips, from the plant before emergence to release the pupa [38]. Upon emergence, the adult hemolymph increases the pressure that causes the wings and legs to grow. Then they empty the gut immediately. while a few hours later the air is expelled from the gut it drains the droplets. They are able to fly within a few minutes when the soft cuticle has sclerotized. However, respectively male and female mosquito needs one or one and a half days to adjust their metabolism [39].

In the sexual maturity there is also a differenc of a male and a female as they emerge. At the time of emergence male mosquitoes are not sexually mature because they rotate their hypopigmentation through 180^o before they are ready to develops, which takes about 1 day. Hence, in the population males generally emerge 1 or 2 days earlier than females to achieve sexual maturity. Since the pupal of both sexes have the same length, mainly in the larval stage the shortening of the male development. As a result, than the identical female, male pupae and

adults in a population are smaller size after development, the adults are ready to mate, feed and start their life cycle [39] [40].

2.2.3 Mating

The pairing of either opposite-sex or hermaphroditic organisms is mating, for the purposes of sexual reproduction. Once females enter swarms of flying males most mosquitoes mating within the palaearctic region takes place. A swarm may consist of only a few or several thousand male mosquitoes. In general, at low light intensity, males form swarms over a marker, especially in the evening and in the morning time. The markers represent objects that contrast the surrounding such as bushes. When swarming, the male faces in the air and fly backwards and forward. Faces over the marker up and down. By the male wing beat the sound frequency produced is approximately 600. The pairing of either opposite-sex or hermaphroditic organisms is mating, for the purposes of sexual reproduction. Once females enter swarms of flying males most mosquitoes mating within the palaearctic region takes place.

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The male's plumose antennae in particular, respond to the sound produced by females. The flagellums of the antenna begin to move and move Johnston's organ, and that is found within the antenna's swollen portion. Contact pheromones can moreover be included in mating behavior [41] [44]. Once a female enters the swarm, they will instantly seize when the fornication a complex combination requires of female and female regenerative structures. For a man it ordinarily takes

less than half a minute to spermatozoa collect in a bursa copulatrix of the male by a female. Males and females usually come face to face when they move out of the swarm and then in the spermathecae sperm moves.

A substance called matronae present in male accessory gland secretion , after copulation it makes a female unacceptable for the rest of her life. Enough sperms a females store in their spermathecae to fertilize more batches of egg without any copulation. Unlike male, female mosquitoes can mate multiple times. Time of swarming and priority depends on the species. For all species, Change is not necessary and without it some species can adapt. After emergence, usually, the mating takes place immediately as the females take almost no time to bite. Finding a host to receive blood feeding after fertilization the next important step is a female's reproductive life cycle [43].

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2.3 Ecological Importance

Mosquitoes play a significant role in nature and there are 3,500 species. In the ecosystem, mosquitoes have several roles that are ignored. Therefore, mosquitoes still have ecological functions. All adult mosquitoes fundamental food is plant sugar and its related nutrients, most often in the form of floral nectar. Mosquitoes pollinate many of the flowers they visit in the process of looking for nectar. This is one of the most commonly overlooked ecological functions of mosquitoes. For many species, mosquitoes play essential role in many ecosystems, serving as food, pollinating flowers, helping to flourish by filtering detritus for plant life, and herding paths also affecting. Without mosquitoes, thousands of plant species would loose a group of pollinators [44]. Mosquitoes 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. Mosquitoes will affect the ecosystem both positively and negatively. Mosquito larvae remain in water as part of their useful function to food provide for animals and fish, including other species larger larvae such as dragonflies.

Mosquitoes play an important role in the aquatic food chain and their larvae are aquatic insects. Adult mosquitoes, such as butterflies, bats, adult dragonflies and spiders, part of the diet of certain insect-eating animals. When they consume nectar, they also help in pollinating certain flowers. Harming other species of mosquitoes may also play a damaging role, however, it spread most of diseases such as malaria, encephalitis, yellow fever, and dengue. Mosquitoes do not themselves cause infections, but are only carrier like vectors [45]. On the lower range of the food chain, mosquitoes appear to represent a significant food biomass for wildlife. Mosquito extinction may have a negative impact on the ecosystem if it were achievable. Many scientists, however, believe that gradually the ecosystem could regenerate and in the environment another species might take its place.

Livestock and humans get affected by the increase production of the arthropods because of increase in animal production.

2.4 Medical Importance

Medically importance of mosquitoe *Anopheles stephensi* which is responsible for transmitting a number of diseases causing agents and parasites for example viruses, nematodes, bacteria, protozoa, and that cause severe diseases such as malaria, West nil virus and filariasis etc [46]. The transmission can be mechanical or biological. *Anopheles stephensi* involves a necessary period of replication or development of a disease causing agent. Because of their blood-sucking behavior, mosquitoes can transmit parasites or pathogens from one host to another. If physiology and environment of the mosquito for transmission is appropriate. In many tropical countries, microbial control agents played important roles in controlling mosquito larvae. Microbial control agents provide attributes: they have effective control and are environmentally friendly and safe for humans and other species.

A comprehensive vector management plan and the involvement of affected populations can be used to successfully implement the MCAs for mosquito-borne disease control [47]. Extremely actual vectors have to maintain close contact with hosts and enable their longevity to allow pathogens, parasites to increase the number and stages in the vector grow to infectious. For fruitful transmission, many transfusions of blood are necessary. Mosquitoes are the most dangerous animals facing humanity. They pose a threat to billions of people in climate region and tropical and have significantly strengthened the progress of human being not only for social but also politically and economically. Undoubtedly, insect-borne pathogens cause infectious diseases leading to pandemics and epidemics [48]. More than 17% of all vector-borne diseases are human infectious diseases that cause over 1,000,000 deaths a year. Hundreds of various species such as flies, insects, ticks and mites involved in the transmission of various diseases, although mosquitoes are still having medicinal importance. There are various diseases which is caused by mosquitoes are briefly described below.

2.4.1 Malaria

The most important human malaria vector-borne disease caused by protozoa (*Plasmodium species*). More than 100 tropical countries affected by malaria, with 3.3 billions of people at risk by 2006. The disease is responsible for more than 1 million deaths and less than 300 million infections each year, especially under the age of five in children and more than 90% of people affect which live in tropical Africa. Many labour deaths, losses, medical treatment costs, and negative effects on the development of the disease make malaria a economic and social burden. The annual cost of malaria in Africa alone, was estimated at about 2 billion [49]. At least Plasmodium genus four species (*P. vivax, P. falciparum, P. malaria and P. ovale*) cause of human malaria and are spread only by mosquitoes *Anophelese*. There are more than 400 species of *Anopheles* worldwide and about 40 of these species are major vectors of human malaria. The most important vectors in sub-Saharan Africa and the most effective malaria vectors in the world belong to the *Anopheles*. Because of their physical feasibilities and anthropophilic behavior they have a higher vector capacity than other closely related sibling species.

Apparently, during evolution, pathogens, parasites and vectors mosquitoes either adapted to each other, pathogen,parasite and resulting in coexistence, was repelled. This exacerbates difficulties in identifying and effectively monitoring phenotypic and genotypic plasticity vector populations and implementing control and strategies of management. However, new techniques such as PCR techniques. A used by specific genetic markers scientists to differentiate between specific populations and sibling species [49] [50]. In the long fight against malaria, lack of knowledge, lack of resources, and training. As well as chloroquine resistance use of other anti-malarial drugs, pesticides and environmental barriers in Africa especially malaria collectively hinders prevention and control for many years [51].

The global campaign against malaria, led by the Roll Black Malaria (RBM) program, is aimed at addressing these long-standing shortcomings. Adult Vector for rapid case treatment and detection increasing facilities based on the use of long-term pesticide treated bed nets (LLLs), indoor residual sprays (IRS) and epidemic preparations against mosquitoes, these current strategies for epidemic diseases are

the cornerstone of Malaria vector control in Africa and around the world. In their aquatic larval habitats controlling vector-mosquito populations provides an additional opportunity to significantly increase the safety of management strategies of existing vector and malarial prevention. Malaria endangered human life until the first half of the 20th century specifically in Europe. The disease was more severe in southern Europe, it is documented that malaria was also a known threat to life in northern Europe. The two main types of *Plasmodium* in Europe found are *P. falciparum and P. vivax*. While throughout the subcontinent, it first occurred to southern Europe its impact was limited. The parasite in Northern Europe, may have been P. vivax due to its adaptation to the temperate climate. Furthermore during the cold phase of mosquito transmission it is possible that this parasite will survive as hypnozoites in the human liver. *P. vivax* rarely causes a deadly disease nowadays, over the last century indicating that *P. vivax* has evolved into a less virulent [52].

2.4.2 Arboviruses

The group of viruses which tranfer viral diseases from insects known as arthropods to human is called *Arbovirus*. Insect bite is main cause of spreading of virus. When these feeding insects suck blood from infected host they are also infected when circulation of blood comes through peripheral blood vessels and in blood virus increase its number. Infected insect is now able to transfer virus to other healthy people. These arbovirus are also able to transfer from one arthropod to another arthropods via vertical transmission [53]. That is the reason these viruses are susceptible to winter and they live in eggs of mosquito in winter season. Hundred of viruses are transmitted in human and cause viral diseases.

These three families are responsible for most of human viruses transmissio. *Togaviridae* genus *Alphavirus*, *Flaviviridae* and genus are *Flavivirus* and the *Bunyaviridae*. These *Arbovirus* which cause diseases in human are classified by clinical symptoms as hemorrhage fever, febrile illness due to rash. These infections can also cause mild or serious illness and death most in tropical countries [54].

2.5 Microbiota of Insects

Microbiota of insects plays a vital role in many biological processes like development, nutrition, digestion, sexual reproduction and most importantly prevention of pathogens. Microbiota is also involved in insecticides resistance. According to recent researches. Many specific bacterial role for different purposes like *Wolbachia* can shorten lifespan of some mosquitoes [54] [55] and also inhibit spread of some viral mosquitoes [56].

2.5.1 Microbiota Associated with Mosquito

There are not only bacteria in gut microbiota of mosquito but also many viruses, prokaryotes and eukaryotic microbes are part of mosquito gut microbiota. They get their microbiota from their parents but mainly derived from environment where they live that's why microbiota is highly dynamic and varies in different species according to developmental stages and environment they live[56]. To study independent microbiota structure of mosquito gut microbiota there is need to culture these bacteria and then sequencing of 16S rRNA or 18S rRNA is needed [57]. The juvenile stage of mosquito is aquatic and adult is terrestrial. The larvae of mosquito feed on organic matter in water, unicellular organisms, small invertebrates and other debris white adult male and female take nectar from plants but female needs blood of vertebrates for development of eggs. They not only take blood but also responsible for transfer of many pathogens in host. So these food may help them to develop their gut microbiota.

Studies before 20 century shows that larval and adult mosquito harbor communities of extracellular microbes from their gut microbiota to digestive tract. But recent studies shows that the microbes in gut of mosquito have many important role in biology of mosquito and a hot topic for research. Results of many recent studies suggest that gut microbiota of adult female mosquito effect both positively

and negatively on host. It shows that female adult mosquito have ability to maintain and transmit diseases through pathogens. Gut microbiota of female adult mosquito plays vital role in transmission of many diseases.

2.5.2 Acquisition of Gut Microbiota by Mosquitos Larvae

Mosquitoes derive their gut microbiota genetically from parents or they may get it from the environment where they live and feed. Different evidence show that mosquitoes derive their gut microbiota from environment. According to first research evidence that larvae of mosquito obtain their gut microbiota from environment and according to matter in water they do not have extracellular microbes in their gut [58]. According to another research it is indicated that most of microbes in gut of mosquito have structure of microbial community present in water where they live [59].

According to third line of focus mosquitoes have microbes in their gut which cannot be expected from environment these may derived from parents or any other source. Larvae feed on eggshells and other organic matter in water where they live microbes derived from aquatic media where they live their juvenile stages or life. Mosquitoes also transmit intracellular bacteria like *Wolbachia* and other genera in their eggs. Some viruses are also present and have been transmit but these do not make part of that microbes which make gut microbiota of mosquito [60]. According to culture based study larval mosquito expel its gut microbiota into meconium during metamorphosis and adult develop with gut microbiota.

Results show that gut microbiota was developed by using water in larval stage and food resources in aquatic medium which is organic matter and extra floral nectaries.

Control group show that gut microbiota of larvae was transmitted to adults. The gut microbiota of adult mosquito can be replace by water, nectar and other food resources containing microbes intake by mosquito. Blood they feed also contains microbes, composition of blood permanently or temporarily alter the structure of gut microbiota of mosquito by altering the metabolism [61].

2.5.3 Composition of Gut Microbiota of Mosquitos

According to many recent researches it is evident that gut microbiota of mosquitoes is largely consist of gram negative anaerobic bacteria mostly belonged to four phyla; Bacteroidetes, Proteobacteria, Firmicutes and Actinobacteria. These genera of bacteria found in gut of mosquito are also members of general community of insects which get their gut microbiota from habitat. Many of bacteria living in gut of mosquito are those which have been isolated and cultured in laboratory. Identification of many viruses is also found in many survey of gut microbiota of mosquito. Most viruses are members of small families and also with small RNA genome like Flaviviridae, which one is also consider as susceptible of many mosquitoes born pathogens. But the phase of virus is not cleared in published studies this suggests that this virus may infects bacteria present in gut of mosquito or the bacteriophages that effects the bacteria in mosquito gut [62].

2.5.4 Gut Microbiota of *Anopheles Stephensi* Mosquitoes

It is understood that the native gut microbiota of *Anopheles mosquitoes* plays a vital role in its host's physiological process. Interestingly, the development of *Plasmodium* in its host mosquitoes can also be affected by this microbiota [63]. For the successful reproduction adult female mosquitoes needs a blood meal as an essential requirement. Mosquitoes the very important ways of the transmission of diseases like Dengue, Chikungunya, Malaria, Yellow fever and West Nile Viruses. *Anopheles stepensi* is main vector of Malaria and Chikengunya.

The gut physiology of mosquitoes undergoes complex modulation immediately after blood meal uptake to promote rapid digestion of blood meals and activation of the egg's development [64]. Feeding of blood also allows the intestinal microbiota to proliferate, eliciting an immune response, and after the digestion of the feeded blood is completed within the first thirty hours, the body defense system tends to stop at the initial stage [65].

The gut immune response of this mosquito may indirectly cause *Plasmodium* . In

Anopheles mosquitoes gut endosymbiosis is considered as potent controller transmission of pathogens for malaria in host and also for development of sex.

This antagonistic association between gut bacteria of many *Anopheles* mosquitoes in the sporogonic development of *Plasmodium*. Introducing *Pseudomonas*, *Serratia* and *E.coli* by a feeding with mouth decrease the burden of gut oocytes in *Anopheles stephensi*. But *Plasmodium's* interrelationship with bacteria remains unclear.

TABLE 2.1: Bacterial species in gut of *Anopheles*

Sr.No	**Genera**	**Species**	**Accession No.**
1	*Pantoea*	*P. agglomerans*	AY849936.1
2	*Pantoea*	*P. stewartii*	AF373198.1
3	*Bacillus*	*B. pumilus*	GQ152134.1
4	*Sphingomonas*	*S. paucimobilis*	D16144.1
5	*Brevundimonas*	*B. aurantiaca*	AJ227787.1
6	*Brevundimonas*	*B. aurantiaca*	AJ227787.1
7	*Brevundimonas*	*B. aurantiaca*	AJ227787.1
8	*Lysinibacillus*	*L. sphaericus*	CP000817
9	*Rahenella*	*R. aquatilis*	U90757.1
10	*Aeromonas*	*A. bivalvium*	DQ504429.1
11	*Pseudomonas*	*P. mendocina*	DQ178224.1
12	*Lysinibacillus*	*L. sphaericus*	CP000817.1
13	*Aeromonas*	*A. punctata*	GQ259885.2
14	*Pseudomona*	*P. sotitidis*	AY953147.1
15	*Pseudomonas*	*P. otitidis*	AY953147
16	*Acinetobacter*	*A.calcoaceticus*	AJ888983.1
17	*Aeromonas*	*A. hydrophila*	FJ768456.1
18	*Rahnella*	*R. aquatilis*	U90757.1
19	*Rahnella*	*R. aquatilis*	U90757.1
20	*Rahnella*	*R. aquatilis*	U90757.1

2.6 Impact of Microbiota on Mosquito Pathogen Transmission and Physiology

Microbiota of mosquito plays a vital role in many mosquito's biology, as well as nutrition, digestion, mating and sexual reproduction, growth, pathogenesis and immune response functions [66].

2.7 Impact of Microbiota on Mosquito Activity, Nutrition, Reproduction and Development

The gut microbiota of mosquito is developed with its environment and also effect on mosquito's nutrition, reproduction and development. This microbiota is depending on variety of species, development and geography. The microbiota specifically colonized in midgut and rarely in salivery glands and also in reproductive organs. Mosquito microbiota plays a vital role in host nutrition, digestion, coupling, sexual reproduction, growth, immune function and pathogenesis [67]. The scientist compared the transcriptome between infected and uninfected adult female mosquito's feed different diets and found that some of the genes involved in digestion and metabolic processes are stimulated by the presence of microbiota [68].

2.8 Antibiotic Resistance

Antibiotics are those kinds of compounds that are produced by certain microorganisms that could destroy or inhibit other microorganism's growth. Nowadays many synthetic compounds with similar functions including beta- lactams, 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 [69]. The rapid development of hospital acquired infections by antibiotic-resistant (ART) pathogens and opportunistic pathogens such as MRSA, *Clostridium di cile*, vancomycin-resistant *Enterococci* (VRE) and Resistant to *Fluoroquinolone*, *Pseudomonas aeruginosa* (FQRP) has become a major public health concern in recent years [70].

Antibiotic resistance was detected in 1930s when sulfonamide-resistant *Streptococcus* pyogenes detected in military hospitals. Subsequently, *Streptomycin* resistant had also been detected. 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. 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. With the passage of time antibiotic resistant bacterias 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 is close to 100,000 per year. 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%). The situation becomes 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 [71]. Many previously affordable and effective antimicrobial treatments became unsuitable for their original usage due to increasing

17 resistances in bacteria. Many resolutions have been proposed, and a number of recommendations have 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. These results need some interrogations about the influences 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 [72]. 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 disease to evolve in more severe form now. This overuse is strongly discouraged, but still a large number of populations are not aware about these phenomena and there remains over-prescription causing more panics.

2.8.1 Antibiotic Resistance Mechanisms

The most common survival strategy of antibiotic resistant(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. 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 [73]. 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 off spring are the conditions when it accumulate mutations, and acquire resistance through horizontal transfer. Compared to the limited cases of intrinsic resistance and the low frequency of mutation around 108-109, horizontal transmission of antibiotic resistant determinants play an important role in the rapid dissemination of AR [74].

2.9 Insecticides Resistant

Numerous studies have shown that the individual mosquito species are involved in multiple mechanisms of resistance [75] [76] [77]. In particular, two mechanisms increased metabolic detoxification of insecticides and reduced target protein sensitivity which is having the most critical part on which the insecticide acts and which is also known as the insensitivity of the target site have been studied very extensively and which have the most wide acceptance due to its extreme importance [78] [79]. The relationship between the genes related to the resistance on the regulation level of genes have provided with a very excellent example show that how precisely these resistances develops in the insects [80]. In the coding region, the overexpression and the amplification of the gene having the mutations results in the structural differences insides the proteins are most often being linked with the resistance of the insecticides in the populations of mosquitoes. The overexpression at the transcriptional level of the genes present in the insects showing resistance to the insecticides, have been proven to be the most common and critical feature for the resistance development in the insects [81]. Collectively, it is very easy for the researchers to conclude that these resistances are not only being transmitted from one generation to the other but also it is being regulated with the help of various regulation levels of the genes, especially the genes responsible for the resistance in the mosquitoes [82].

Chapter 3

Material and Methods

3.1 List of Equipment

1. Autoclave,
2. Magnetic stirrer,
3. Measuring balance,
4. Laminar flow,
5. Incubator,
6. Vortex,
7. Water bath,
8. Microscope,
9. Shaker,
10. Refrigerator,
11. Fishing net,
12. pH balancer.

3.2 List of Apparatus

1. Beakers,
2. Spatula,
3. Conical flasks,
4. Eppendorf Tube,
5. Micropipette,
6. Petri dishes,
7. Spirit lamp,
8. Aspirator,
9. Icebox,
10. Inoculation loop,
11. Glass rod,
12. Dissecting needle,
13. Dropper,
14. Parafilm,
15. Graduated cylinders.

3.3 List of Chemicals

1. Nutrient Agar,
2. Blood Agar,
3. MacConkey Agar,
4. Mannitol Salt Agar,

5. Oxidase test,
6. Catalase test,
7. Urease test,
8. Eosin Methylene Blue Agar,
9. Simmon's citrate Agar,
10. Saline and Distilled Water,
11. Crystal violet and Safranin.

3.4 Methodology Flowchart

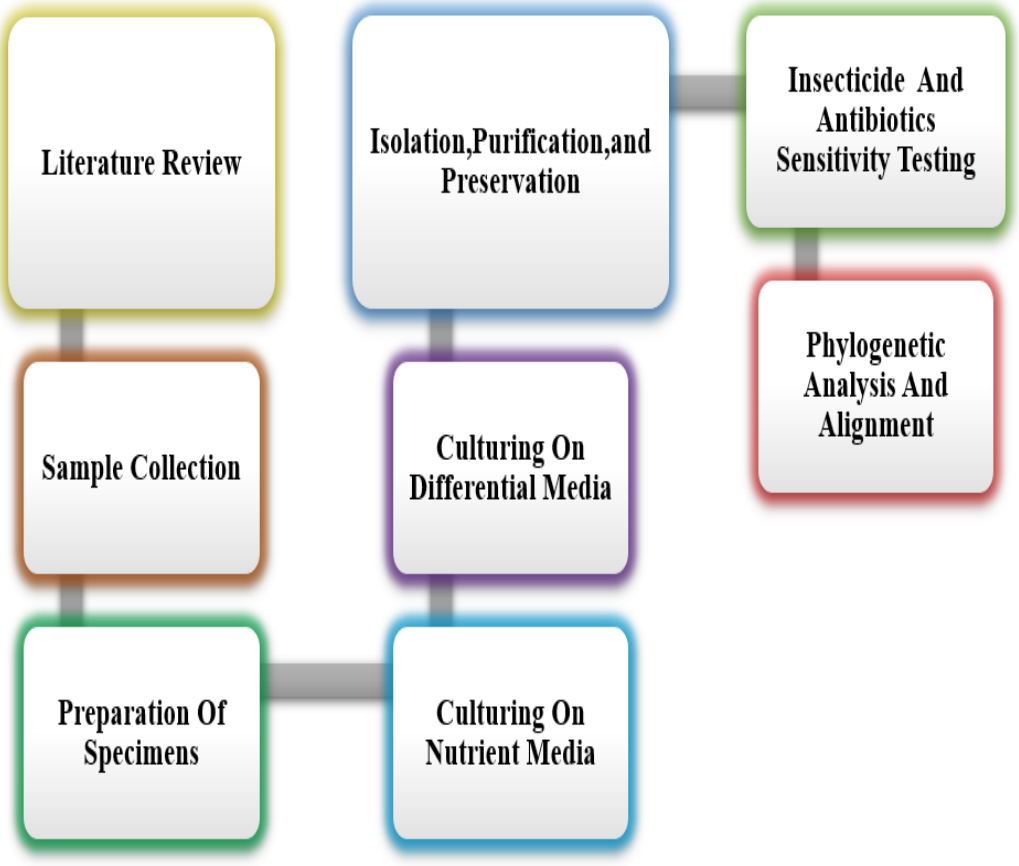


FIGURE 3.1: Methodology of Project

3.5 Sample Collection

1. Mosquitoes were collected from 2 different categories of Islamabad. From houses and from public places.

2. Randomly mosquitoes were collected from house and public parks. In each location 3 points were selected to capture adult mosquitoes. Mosquitoes were collected in dawn and dusk.

3.6 Sample Collection Method

Sample was collected by mean o Fishing net, Aspirator, Mosquito trapper racket and mosquito landing on hand. Some mosquitoes were collected from walls because they used to rest on walls after feeding. After collection of mosquitoes they were transferred to glass bottles in ice box and transported to the laboratory. They were kept in refrigerator at -4^{o}C.

3.7 Preparation of Sample

A total 300 mosquitoes were separated by mean of their morphology [93]. Prior to the midgut dissection mosquitoes were surface sterilized by washing in 70% ethanol then rinsed four times in 1X PBS. By putting the mosquitoes in a petri dish, they suffer from anesthesia which is kept cold on ice.

FIGURE 3.2: Mosquito before dissection

3.8 Dissection

3.8.1 Protocol

Transfusion of midgut from mosquitoes requires pre-preparation of 1X phosphate buffered saline (1X PBS) solution and anesthesia of mosquitoes subject to a temperature of 4^oC, until immobilized. By putting the mosquitoes in a petri dish, they suffer from anesthesia which is kept cold on ice. Other required materials include: 10x objective in light microscope, equipped with pipette, fine tip top forces, glass slide, needle tip probe.

3.8.2 Procedure

1. 1X PBS drop was placed on the glass slide with mosquito and glass slides under the light microscope.

2. Mosquito was moved to adjust its position on slide under microscope and mosquito's chest was hold with needle probe.

3. Mosquito was held with the probe, by using the straps to grasp the other end of the abdomen and gently pulled the mosquito's abdomen in one motion. The midget was attached to the active thorax.

4. Then mid gut was separated from thorax by using force [94].

5. Then the collection of 50 guts were squashed and incubated in water bath and 3ml of 1X PBS was transferred then stirrer on vortex then again incubated in water bath for 4 hours.

6. Throughout the dissection procedure in laminar flow and observed under microscope dissection needles and forceps were dipped and sprayed in every dissection using 70% of ethanol. Wings, legs and head were removed and gut was separated and confirmed under microscope.

FIGURE 3.3: Mosquito under microscope 1

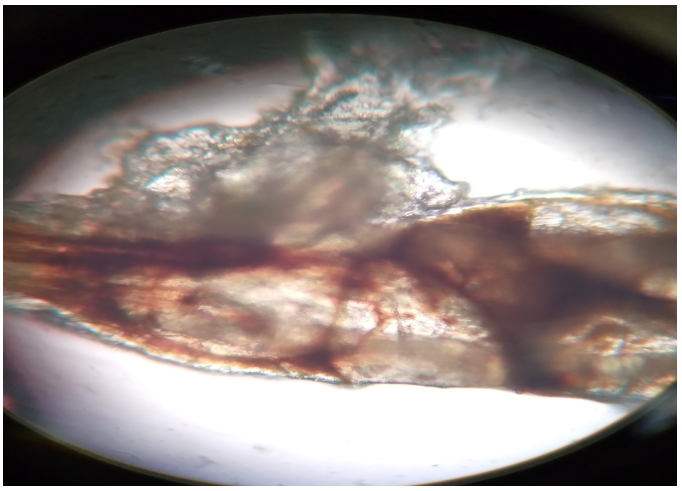


FIGURE 3.4: Mosquito under microscope 2

3.9 Growth on Nutrient Agar

To verify the presence or association of bacterial pathogens in mosquito's gut, 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^{o}C 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 were incubated for 24 hours at 37^{o}C. Plates were incubated in upside down direction to avoid the moisture.

3.10 Growth on Differential Media

Differential media used for identification of aerobic bacteria were MaCconkey Agar (Macc), Mannitol Salt Agar (MSA), Eosin Methylene Blue Agar [EMB], Simon citrate agar [SCA]. Blood Agar (BA) was used for anaerobic flora.

3.10.1 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^{o}C. 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.

3.10.2 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^{o}C for 15-20 minutes. Media was left for soldificationat room temperature after pouring it in petri plates.

3.10.3 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^{o}C. The media has been left to solidify at room temperature after pouring.

3.10.4 Blood Agar

7 gram of blood agar powder was added in 250g of distilled water. Magnetic stirrer was used for stirring and proper mixing of media. Prepared media was autoclaved at 121^{o}C for 15-20 minutes. Media was left for soldification at room temperature after pouring it in petri plates.

3.11 Streaking of Culture Media for Aerobic and Anaerobic Growth

The bacterial colonies grown on Nutrient agar were streaked on the differential media. The criteria for selection of bacteria was color, shape and morphology. Each bacteria taken from nutrient agar plate was streaked on prepared differential media. After streaking plates for aerobic growth were incubated in incubator 37^{o}C for 24 hours and plates of Blood Agar were placed in jar where alka setizer tablet was dissolved in water and jar was air tight for anaerobic bacteria growth.
Culturing on different medias were replaced for 3 times to get the pure strains. From different media growth two bacterial colonies that were prominent and prevalent were selected on preserved.

FIGURE 3.5: Anaerobic bacteria growth in presence of Carbon dioxide

3.12 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^{o}C for 15-20 minutes. 2.5ml eppendorf tubes were taken and autoclaved at 121^{o}C 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^{o}C.

3.13 Gram Staining

3.13.1 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.

3.13.2 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.

3.13.3 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. In this way the safranin solution prepared for the experiment which was accurate.

3.13.4 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.

3.13.5 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 were 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 [96].

3.14 Biochemical Characterization

Different types of biochemical tests were performed for the biochemical characterization of two prevalent selected strains.

3.14.1 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^{o}C 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.

3.14.2 Urease test

This test is 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^{o}C. 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^{o}C 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.

3.14.3 Catalase test

Catalase is an enzyme, enzyme that decomposes 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.

3.15 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 [98]. In order to determine the microbiota associated with the gut of mosquitoes the preserved strains were send for 16S sequencing, the samples were sequenced from Korea.

3.16 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 sequence similarity index according to the matches, mismatches, and gaps. The BLAST results for strain S1–785 that gave 99% similarity index with 0% query coverage, MEGA-X tool was used to find out the phylogenetic analysis of species. The sequences closest to the strain S1–785 were taken, total of 6 sequences, gave closest similarity to that of S1–785. The file was imported in MEGA-X tool 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.

3.17 NCBI Submission

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

3.18 Antibiotic and Insecticide 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 insecticides. 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^{o}C for 15 minutes. It was inoculated with bacteria and kept overnight at 37^{o}C in incubator so that bacteria may grow into the broth.

3.18.1 Kirby Bauer Method Procedures

Muller-Hinton agar media was set having standardized composition. Muller-Hinton agar media was poured into 150 mm petri dishes at a level of 4mm deep. The agar media was maintained at pH range of 7.2 to 7.4 and broth culture was used for inoculation. The culture plates were made inoculated by streaking a sterile swab passed through broth culture of bacteria. The agar media plates inoculated with bacteria was left for about five minutes to dry.

The antibiotics disks were transferred to the inoculated agar plates by using sterilized needles. The discs were gently press by using flame-sterilized forceps to make sure that each disc is in contact with surface of agar media properly.

The plates were incubated at incubation temperature of 37^{o}C for the night. 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.

3.19 Comparison of Computational and Wet Lab Results

3.19.1 Whole Genome Sequencing

In whole Genome Sequences Extraction, we extracted whole genome sequences for the genera extracted from literature mining using NCBI Genome utility to process that WGS for the functional annotation for the pathway identification. The criteria for the selection of whole genome sequences was the total coverage more than 70% if the sequence is less than 50% it was excluded from the search.

3.19.2 Identifying Target Pathway

In systems biology the individual entity or molecule is not considered. The systems are analysis on the basis of systems. The biological systems are so complex that each molecule interact with one and other and produces a combined response. Thus the identification of correct pathway for the interaction studies is necessary in biological process. In our study we used RAST annotation pipeline for the identification of functional pathways. The whole genome sequences were uploaded on RAST with the taxonomic Id. RAST provides a detail view on the systems and subsystems present in that genome.

In our study we selected 64 genomes to be uploaded on RAST. The RAST provided their information and displayed all the information present in those systems [103]. Those systems were selected which were involved in the metabolism of Aromatic compounds. Then they were further cut down to subsystem level and only those microbes were selected which contains Biphenyl degradation system because biphenyl is the core pathway in xenobiotics pathway of degradation [104].

Chapter 4

Results and Discussion

4.1 Nutrient Agar Growth

From the gut of mosquito bacteria were isolated. 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 [83]. 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 [84]. Nutrient Agar allows the growth of gram-positive as well as gram-negative bacteria. 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^{o}C 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 AgarThe culture results showed the growth of variety of bacteria.

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. Plates were incubated for 24 hours at 37^{o}C. Plates were incubated in upside down direction to avoid the moisture. Bacterial colonies with different colours and shape appeared on nutrient agar and these colonies were transferred to differential media.

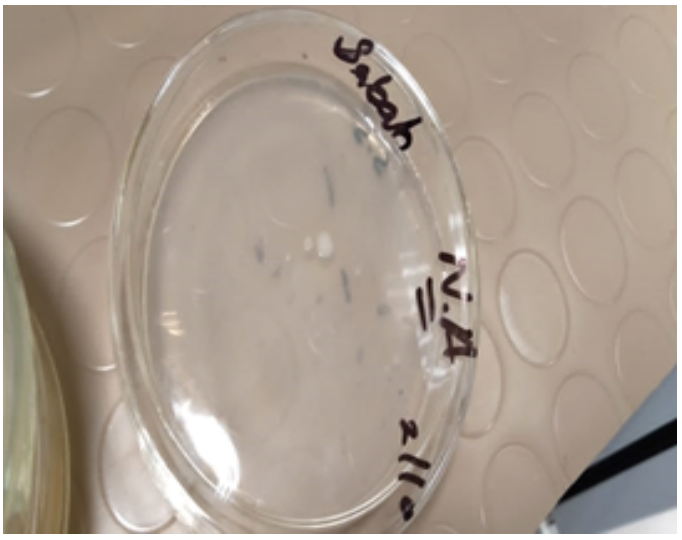


FIGURE 4.1: Nutrient agar plate 1 with bacterial growth

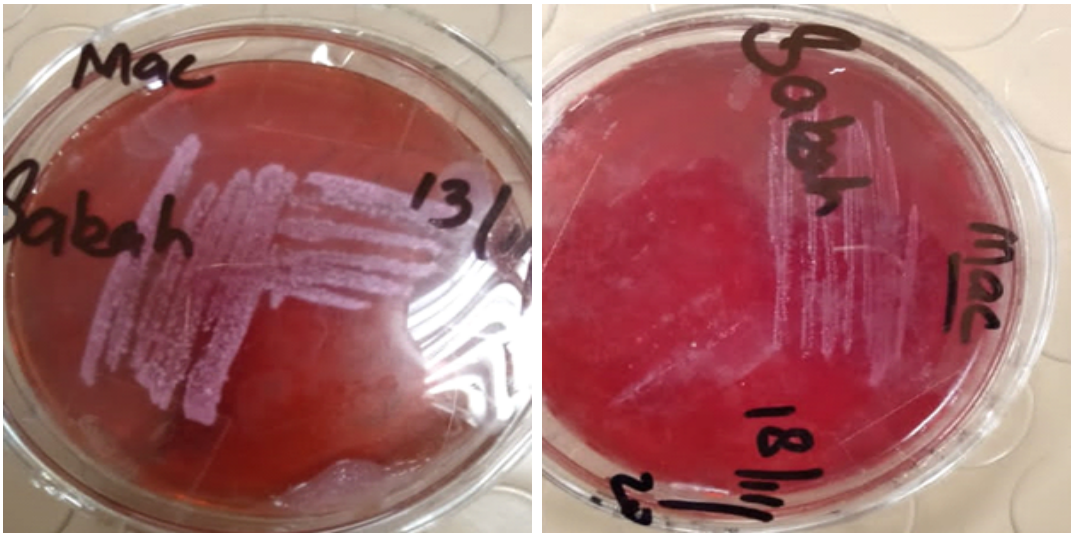
4.2 MacConkey Agar

The purpose of MacConkey agar used is to isolate the gram negative bacteria extracted from gut of mosquito. 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, Klebsiella spp, Citrobacter spp, Pseudomonas*. MacConkey media only allows the growth of gram-negative bacteria hence it inhibits the growth of gram positive bacteria. Bacteria were isolated from gut of *An.stephensi*.

An.stephensi were 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 mosquito, some cultured samples showed a shiny pink color.



(a) (b)

FIGURE 4.2: (a) Growth of bacteria on MacConkey Agar plate 1 (b) Growth of bacteria on MacConkey agar plate 2

Gram negative bacteria usually shows more significant growth on medium and these bacteria can differentiate due to their lactose fermenting ability. The lactose

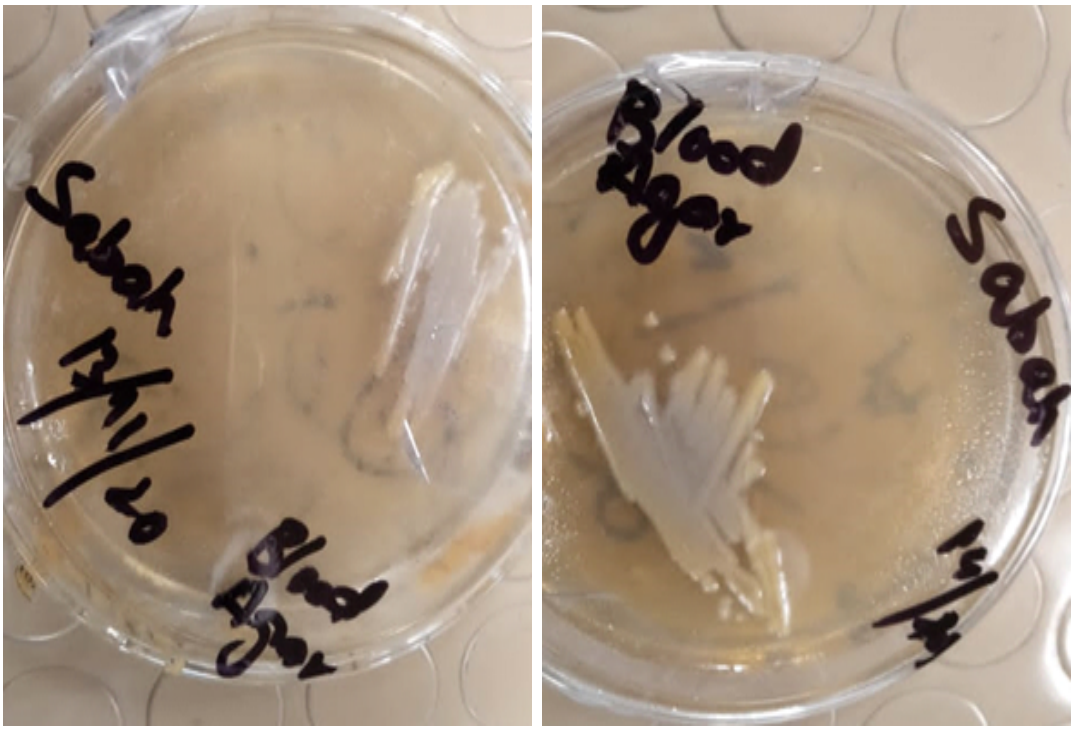
fermenting bacterial strains shows red or pink coloured colonies and which may be surrounded by a zone of acid precipitated bile. The red colored pattern is just due to the releasing of acid from lactose, when pH of medium drops below 6.8 in the result. Absorption of neutral red starts and lateral change 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 mosquito, some cultured samples showed a shiny pink color colony, some cultured samples showed white appearance and some showed orange.

4.3 Blood Agar

Blood agar is moderate with many nutrients and is prepared with the completion of blood. Blood agar is also a basal medium which can be used as it is so no need to grow on other media first. Nutrient agar or a tropic soy agar can also be used as basal medium for blood agar. Blood agar for the cultivation of fasting bacteria. One of the best ways is to use special nutrients and not add too much nutrients to such media. About 5% of defrosted mammalian blood is automatically incorporated into the basal media to prepare the blood agar medium. It is an enriched medium that supports the growth of certain bacteria. In order for these bacteria to grow, inhibitors in the blood must be inactivated by hot blood agar. Blood agar was used for growth of anaerobic bacteria strains present in gut microbiota of *An.stephensi* [85].

Sample was collected from different areas of Islamabad and blood agar was used as medium for growth of anaerobic bacterial species in gut microbiota of mosquito. Blood agar plates were placed in carbon dioxide chamber which was made by use alka setizer dissolved in 500 ml of water. Different bacterial strains were collected from this medium. *Streptococcus*, *Neisseria* and *Clostridium* were commonly found bacteria on blood agar. Blood agar supports development of fastidious organisms and supply nutrients to support their growth. The blood contained in this base provides maximum nutrition to the medium by providing the additional factors

necessary for these bacteria. For the growth of *Streptococci* and other anaerobic bacteria blood agar is especially used as it provides best results. For pH maintenance and osmatic balance control NaCl is added in blood agar. Water is also used as water make the nutrients easier to absorb by bacteria.



(a) (b)

FIGURE 4.3: (a) Blood agar plate 1 with bacterial growth (b) Blood agar plate 2 with bacterial growth

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 [86] [87].

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*, *Stphylococcus* and *Proteus spp.* The change in color of media depicts the growth of *Staphylococci.* This predict that gut of *An.stephensi* contain the *Staphylococci* that was to be separated on the MSA agar plates.

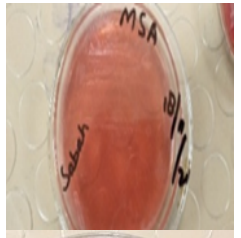
FIGURE 4.4: Growth of bacteria on Mannitol salt agar plate 1

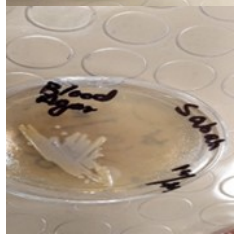
4.5 Isolation of Bacterial Pathogens

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 4.1

Table 4.1: Bacteria isolates on different media

S#	Reference Code	Sample Location	Colony Color	Morphology of Colony	Pigmentation	Media	Predicted name of strain	Figures
1	EMB(P)1	Islamabad	Green sheen	Circular	Green sheen	Eosin methylene blue agar	*Salmonella*	
2	MSA(P)1	Islamabad	Light Orange	Punctiform	Light Orange	Mannitol salt agar	*Staphylococcus*	
3	MACC(P)1	Islamabad	Pink	Circular	Pink	MacConkey agar	*Salmonella*	

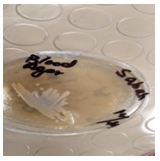
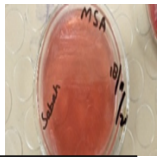
4	MSA(P)2	Islamabad	White	Circular	White	Mannitol salt agar	*S.aureus*	
5	B.A(P)1	Islamabad	White	Circular	White	Blood Agar	*Staphylococcus*	
6	MACC(P)2	Islamabad	Pink	Circular	Pink	MacConkey agar	*Klebsiella*	
7	MACC(P)3	Islamabad	Pink	Irregular	Pink	MacConkey agar	*Klebsiella*	

8	MACC(P)4	Islamabad	Pink	Spherical	Pink	MacConkey agar	*Klebsiella*	
9	MSA(P)4	Islamabad	White	Puntiform	White	Mannitol salt agar	*Staphylococcus*	
10	MACC(P)5	Islamabad	Orange to amber	Pink	Orange to Pink	MacConkey agar	*Salmonella*	
11	MACC(P)4	Islamabad	Purple	Circular	Purple	MacConkey agar	*S. aureus*	

4.6 Preservation of Prevalent Strains

The bacterial plate that seems to be more prevalent were further purified by streaking and culturing them repeatedly hence, the purified strains are obtained. These can use in future use. These pure strains also contain the duplicates, means one strain contain two copies as shown in table 4.2

TABLE 4.2: Preserved strains from Gut Microbiota of *An.stephensi*

S#	Reference Code	Media	Colony Color	Pigmentation	Figures
1	EMB Plate 1	EMB	Purple	Purple	
2	BA Plate 1	BA	White	White	
3	MACC Plate 1	MACC	Pink	Pink	
4	MACC Plate 2	MACC	Pink	Pink	
5	MSA Plate 1	MSA	White	White	
6	MSA Plate 2	MSA	Yellow	No	

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 [88]. 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. . 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 [87] [89]. 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.

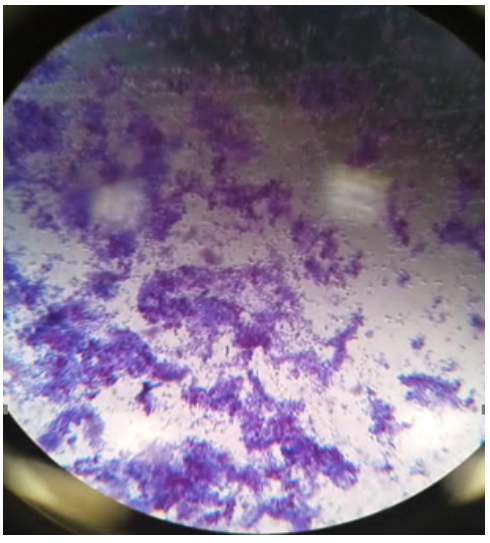


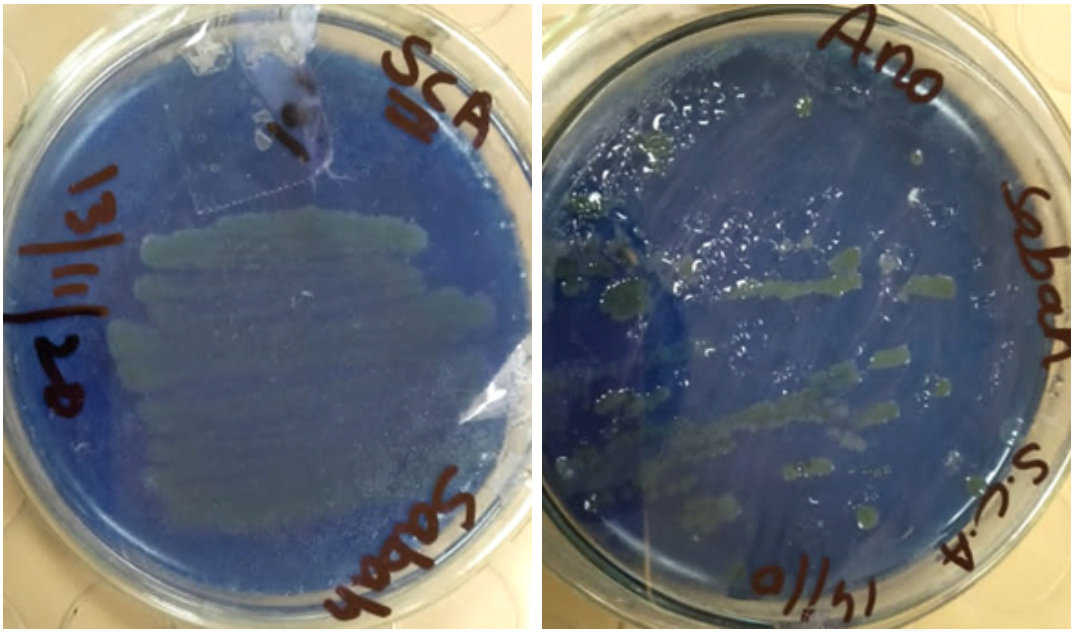
FIGURE 4.5: Staining of Prevalent Strain

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. 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.

4.8 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 [100]. 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.



(a) (b)

FIGURE 4.6: (a) Growth on Simmons Citrate Agar 1 with bacterial growth (b) Growth on Simmons Citrate Agar 2 with bacterial growth

4.9 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. Based upon the results of MSA catalase test with15% H2O2 solution was performed to differentiate the 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 gut of *Anophelese stephensi* and were collected from field hence proving the ability of housefly as a mechanical carrier of *Staphylococcus* species [88] [90]. The most common examples of bacteria which can be isolated from the gut of *Anophelese stephensi* [91].

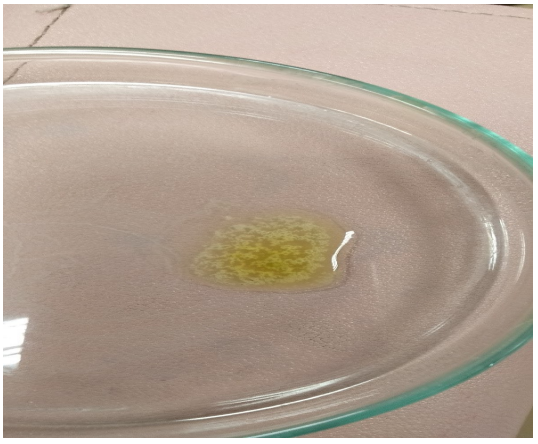


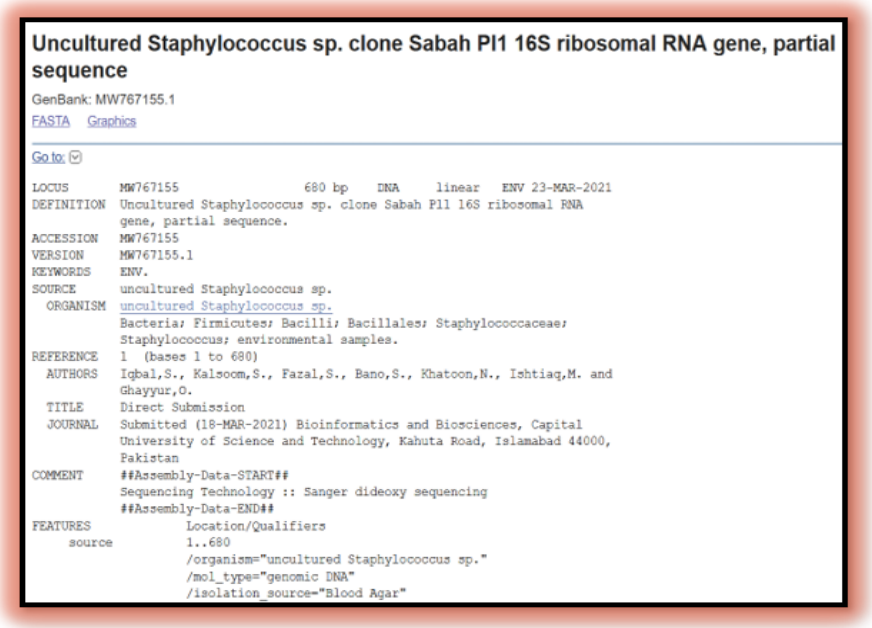
FIGURE 4.7: Bubble formation in catalase test

TABLE 4.3: Characteristics of *Staphylococcus*.

Sr.No	Basic characteristics	Properties
1	Shape	Spherical shape
2	Type	Aerobic
3	Gram staining	Positive
4	Urease	Positive
5	Citrate test	Positive
6	Catalase test	Positive

4.10 NCBI Submission

The Strain sequence was submitted to NCBI and the accession number was GenBank: MW767155.



Uncultured Staphylococcus sp. clone Sabah Pl1 16S ribosomal RNA gene, partial sequence

GenBank: MW767155.1

FASTA Graphics

Go to:

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VERSION     MW767155.1
KEYWORDS    ENV.
SOURCE      uncultured Staphylococcus sp.
  ORGANISM  uncultured Staphylococcus sp.
            Bacteria; Firmicutes; Bacilli; Bacillales; Staphylococcaceae;
            Staphylococcus; environmental samples.
REFERENCE   1  (bases 1 to 680)
  AUTHORS   Iqbal,S., Kalsoom,S., Fazal,S., Bano,S., Khatoon,N., Ishtiaq,M. and
            Ghayyur,O.
  TITLE     Direct Submission
  JOURNAL   Submitted (18-MAR-2021) Bioinformatics and Biosciences, Capital
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      541 gctacggtga ataccttccc gggtcttgta cacaccgccc gtcacaccac gaaagtttgt
      601 aacacccgaa gccggtggag taaccattta tagatcggag tggtcaaaag ggagacatgt
      661 gaatggtgag aagcaaaaag
//
```

(b)

FIGURE 4.8: (a)Submission on NCBI (b) Sequence on NCBI

4.11 Multiple Sequence Alignment of Sequence

The results of multiple sequence alignment made it clear that there were variations of 28 base pairs between the new *Staphylococcus* species and those strains which are closely related to the new strain of *Staphylococcus* (i.e., *Staphylococcus saprophyticus* strain RCB140 16S ribosomal RNA gene and the *Staphylococcus* species strain BB32-1 16 S ribosomal RNA gene).

```
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KT261259.1                          GTGTTAGGGGGTTTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACT
                                    **************  **.****************.************

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KT261259.1                          CCGCCTGGGGAGTACGACCGCAAGGTTGAAACTCAAAGGAATTGACGGGG
                                    ***** ********* * ***.*** ************************

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KT261259.1                          CCTTACCAAATCTTGACATCCTTTGAAAACTCTAGAGATAGAGCTTTCCC
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FIGURE 4.9: Multiple sequence alignment of sequences sequence with the name of SABA with ID "H210205706

```
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                                       **************************************************

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KT261259.1                             GAAGGTGGGGATGACGTCAAATCATCATGCCCCTTATGATTTGGGCTACA
                                       **************************************************

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H210205-030_I04_NB_785F.ab1     70     AAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTA
MN314859.1                             AAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTA
KT261259.1                             AAATCCCATAAAGTTGTTCTCAGTTCGGATTGTAGTCTGCAACTCGACTA
                                       **************************************************

H210205-030_I04_NB_785F.ab1     70     CATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA
MN314859.1                             CATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA
KT261259.1                             CATGAAGCTGGAATCGCTAGTAATCGTAGATCAGCATGCTACGGTGAATA
                                       **************************************************

H210205-030_I04_NB_785F.ab1     70     CCTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAAAGTTTGTAAC
MN314859.1                             CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
KT261259.1                             CGTTCCCGGGTCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
                                       * ***********************************.**********

H210205-030_I04_NB_785F.ab1     70     ACCCGAAGCCGGTGGAGTAACCATTTATAGATCGGAGTGGTCAAAAGGGA
MN314859.1                             ACCCGAAGCCGGTGGAGTAACCATTTATGGAGCTAGCCG-TCGAAGGGTG
KT261259.1                             ACCCGAAGCCGGTGGAGTAACCATTTATGGAG-----------------
                                       **************************.**
```

FIGURE 4.10: Multiple sequence alignment of sequences sequence with the name of SABA with ID "H210205706

4.12 Phylogenetic Analysis

The phylogenetic tree consists of the 2 major clades i.e., clade A and clade B and the sequences of 28 bacterial strains were retrieved from the NCBI genbank and the sequence of new strain is also included in the tree. By doing the phylogenetic

analysis, it was made confirm that the new strain of *Staphyllococcus* was obtained during this research.

This bacterial strain was isolated from the mid gut of *Anopheles stephensi*. From the phylogenetic tree it can be seen that the sequence with the name of SABA with ID "H210205706" is diverging as the separate sub group from the Clade A sub group 6(a) and according to the tree, it has the resemblance with the *Staphylococcus saprophyticus* strain.

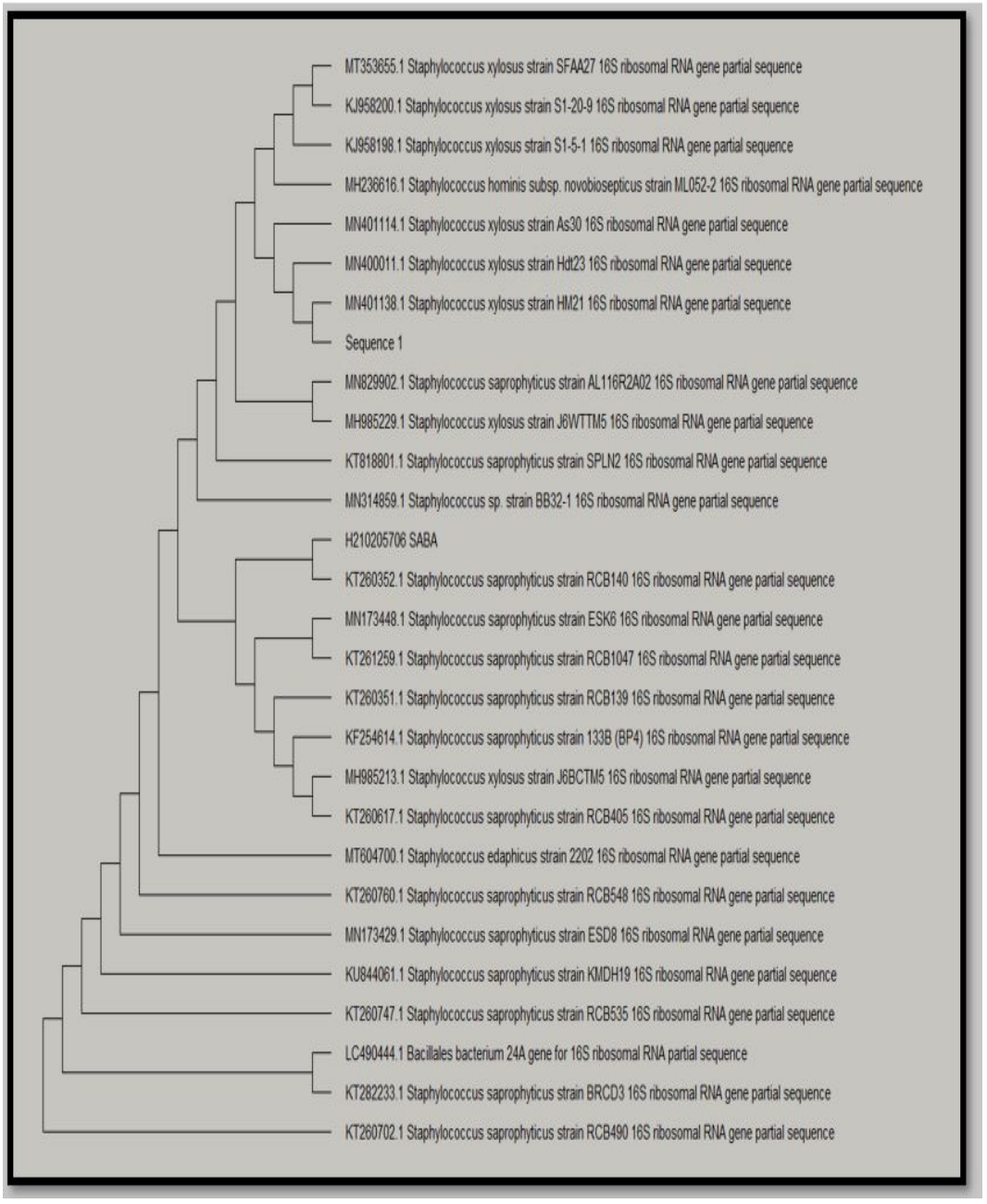

FIGURE 4.11: Phylogenetic Analysis of the Bacterial Strains of *Staphylococcus Species*

4.13 Antibiotic and Insecticide Sensitivity Test

The most prevalent strain of bacteria was isolated and undergone for antibiotic and insecticide sensitivity test. Total 3 antibiotics and 3 insecticides were used for this purpose. The disk diffusion method was used. Ceftriaxone, Imipenem and Nalidixic acid were used for antibiotics and Allethrin, Resmethrin and Thiacloprid were used for insecticides. Zone of inhibition for the three antibiotics and three insecticides are mentioned in appendix 1,2 and the percentage of resistance is mentioned in 4.4 and 4.5. Highest percentage of resistance i.e, 80.5% was recorded in Nalidixic acid with a least resistance for 66.66% for Ceftriaxone. Imipenem showed 0%sensitivity for *Staphylococcus* spp,. Insecticide resistant sensitivity tests were also performed and they showed very less or no resistance to this bacteria.

TABLE 4.4: Percentage of resistance of antibiotics for *Staphylococcus spp,*.

Sr.No	**Antibiotics Name**	Imipenem	Ceftriaxone	Nalidixic acid
1.	**Resistant**	11	10	12
2.	**Intermediate**	4	4	1
3.	**Sensitivity**	0	1	2
4.	**R%**	73.3%	66.6%	80.5%
5.	**I%**	26.6%	26.6%	6.66%
6.	**S%**	0%	6.66%	13.5%

TABLE 4.5: Percentage of resistance of insecticides for *Staphylococcus spp,*.

Sr.No	**Insecticides Names**	Allethrin	Resmethrin	Thiacloprid
1.	**Resistant**	0	1	0
2.	**Intermediate**	0	0	0
3.	**Sensitivity**	0	0	0
4.	**R%**	0%	1%	0%
5.	**I%**	0%	0%	0%
6.	**S%**	100%	99%	100%

4.14 Comparison of Computational and Wet Lab Results

When we compare the computational results of the functional genomic of the whole genome obtained by the NCBI Genome and RAST server, it was observed that *Staphylococcus spp* were not involved in any of the aromatic compound metabolic pathways but 20 subsystems were identified to be involved in the antibiotic resistance pathways. *Staphylococcus specie* was involved in the antibiotic resistance and showed miner relation in insecticide resistance as shows in 4.12, 4.13

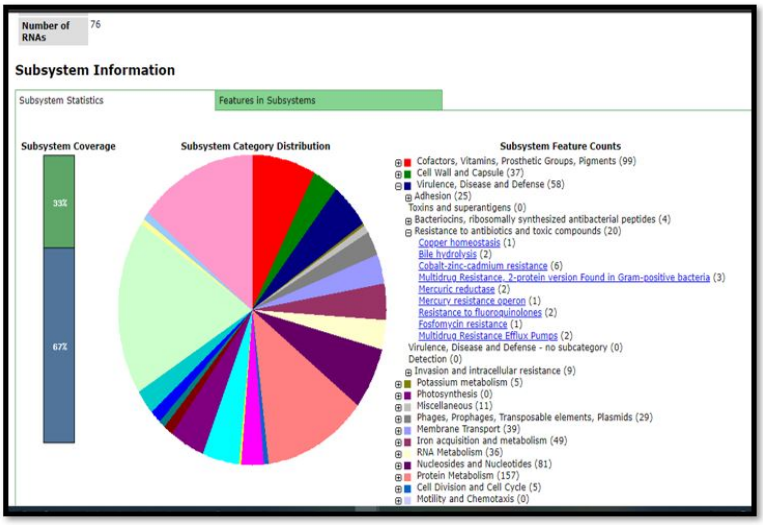

FIGURE 4.12: Shows the subsystems involved in antibiotic and insecticide resistance(1)

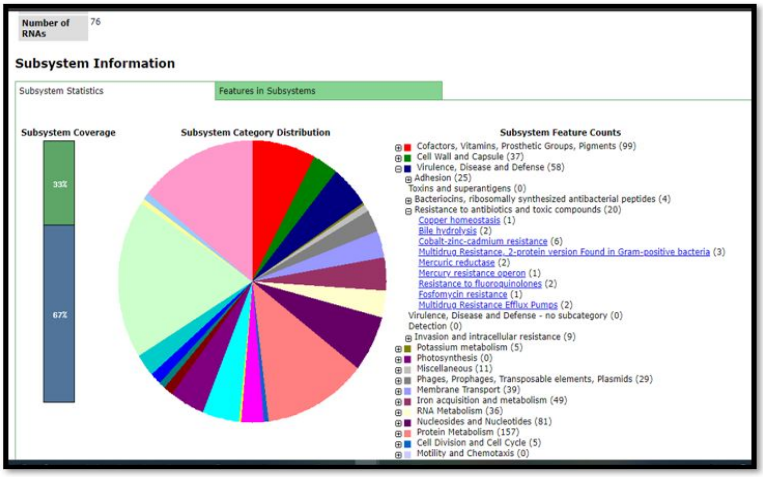

FIGURE 4.13: Shows the subsystems involved in antibiotic and insecticide resistance(2)

4.15 Discussion

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 [92] [93].

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* and to vanomycin mostly called as vanomycin resistant *Staphylococcus aureus* and this antibiotic is also termed as "drug of last resort" [94]. 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 [95]. Community-acquired strains have been isolated from areas such as day-care centers, fire stations and educational institutes. 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 [100]. CoNS isolated from animals have developed resistance against gentamycin, macrolides, tetracycline, streptomycin, trimethoprim, sulfamethoxazole and fluoroquinolones [96]. 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 [97] [98]. 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 *Staphy lococcus 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 [99]. The spreading of antimicrobial was MDR, and only 3.7% of the samples were susceptible to all antibiotics tested in the study [103]. 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 [103]. 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 intra mammary 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 [104].

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 [105]. The resistance against antibiotics in *Staphylococcus xylosus* is reported in world wide. In present the infections which were caused by *Staphylococcus xylosus* has been increasingly problematical due to the production of resistance in bacteria. So aim of this study was to find out

the most prevalent species of bacteria found in the gut of *Anophelese mosquito* and find the antibiotics sensitivity and insecticide resistance in those bacterias. For this purpose mosquitoes were collected from field. Mosquitoes were wild species of *Anophelese* and their gut was removed in highly sterile environment so we can find the bacteria found in gut of *Anophelese* and their relationship with insecticide resistance. [106].

Chapter 5

Conclusion and Future Perspective

Mosquitoes are primary vector for transmission of many economic importance diseases like nuisance, systematic and local skin reactions and also for acute death causing disease like dengue, malaria and West Nile Virus. It seems that *Anopheles stephensi* is one of the major vectors of the malaria in Pakistan and these mosquitoes have preferred mammals for their blood meal, including humans. Mosquito microbiota has an important effect in the host characteristics like development, nutrition, reproduction, growth, vector competence, interactions with parasites and the now our recent studies provide an evidence that microbiota are also in the mosquito resistance to insecticides. The results presented shows differential composition and function of microbiota. The microbiota in *Anopheles stephensi* is abundant and controlling many functionalities in the mosquito. In present research one of our aim was to check the resistance against insecticides. For this purpose, prevalent bacterial species are isolated from midgut of *Anopheles stephensi* collected from Islamabad. The frequency cultured bacteria includes *E. coli*, *klebsiella* and *Staphylococcus* based on different media results. Different biochemical tests are implemented on these strains and results are also proved with 16s rRNA sequencing.

These are most common bacteria isolated from the midgut microbiota of *Anophelese stephensi* and antibiotic sensitivity tests are also performed against these prevalent strains of bacteria. These bacteria susceptibility is checked against the three antibiotics Imipenem, Nalidixic acid,and Ceftriaxone. The most frequent genus of bacteria that was isolated from mid gut microbiota of *Anophelese stephensi* collected from different areas of Islamabad is *E.coli, Bacillus, klebsiella and acinetobacter*. It was further confirmed by biochemical and molecular characterization. Antibiotic test was also performed for this strain. It was shown highly resistant against Imipenem and Nalidixic. These findings suggest that these bacteria can cause resist against antibiotics. Insecticide resistant was also checked against these bacteria and these showed minimum resistance against insecticides so these bacteria are resistant against insecticide chemicals. The study must be expand in other areas.

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Appendix A

Table 1: Appendix 1

Imipenem	**Ceftriaxone**	**Nalidixic acid**
43	19	15
40	13	13
37	15	19
19	12	10
12	9	7
12	18	6
18	7	18
20	25	20
23	33	8

Table 2: Appendix 2

Allethrin	**Resmethrin**	**Thiacloprid**
0	1	0
1	0	2
0	2	0
0	0	0
2	0	0
0	1	2
2	1	0
0	0	0