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Resistome Analysis of Gut Microbiota of Mosquitoes

by

Haleema Zeenat

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

in the

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I dedicate this thesis to all the great people came in my life specially my beloved Parents and my Supervisor who encouraged me to stand out in this world with nobility and motivated me to step ahead without any fear.

CERTIFICATE OF APPROVAL

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(Haleema Zeenat)

Abstract

Mosquitoes are medically and clinically significant arthropods which belong to the family Culicidae, order Diptera and notable genera include Aedes, Anopheles and Culex. There are 3500 species of mosquitoes worldwide among which various are also reported from Pakistan. They impact a health threat as they act as ectoparasites which require blood to nourish their eggs and during this process transmit different infectious agents (bacteria, protists etc.) into the human host to cause different diseases such as malaria, dengue and yellow fever. To combat these diseases, reproduction and spread of mosquitoes need to be controlled. There are four important stages in mosquito life cycle; egg, larvae, pupae and adult. It is easier to target mosquitoes at the early stages of their life cycle. One of the prominent methods to control them includes use of insecticides (pyrenoids and organophosphates). The insecticide used may have environmental hazards that affect food chain and contaminate underground water reservoir but they are still used at a large scale. Due to this exposure with insecticides, mosquitoes have developed resistance against these chemicals. Various mechanisms are involved in acquisition of this resistance, amongst which the role of gut microbiota of mosquito cannot be undermined. In this study we have investigated the different bacterial strains, present in selected insecticide resistant mosquito gut that may render resistance against insecticides. The genomes of microbial species were explored for putative insecticide resistant genes/proteins and the docking of a frequently used insecticide i.e., Derris was performed against these proteins. The list of potential insecticide resistant genes within each microbe was found and this study would pave way to understand insecticide resistance with the focus on genetic control to counter mosquito-borne diseases.

Keywords: Mosquito, insecticide resistance, bacterial strains, organophosphates, Mosquito-borne diseases.

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Abbreviations

ABC	ATP-binding cassette
DDT	Dichlorodiphenyltrichloroethane
DHF	Dengue Hemorrhagic Fever
GST	Glutathione S-Transferases
RND	Resistance Nodulation Cell Division
SFV	Semliki Forest Virus
SINV	Sindbis Virus
VGSC	Voltage Gated Sodium Channels

Chapter 1

Introduction

Mosquitoes are one of the most important arthropod vectors which are responsible for diseases transmission. Mosquitoes belong to family Culicidae, order Dipteria and this family comprises of about 3500 species distributed worldwide [1]. The protozoan species such as *Plasmodium falciparum* and *Plasmodium vivax* transmitted by mosquitoes are responsible for causing human diseases including malaria, affecting 500 million people and death of 3 million people annually [2].

Similarly, mosquito-borne viral diseases like yellow and dengue fever are responsible for effecting millions of people worldwide [3]. Due to their dual vector properties i.e., biological vector as well as mechanical vector and disease-causing ability, mosquitoes are with great medical significance. In order to control vector borne diseases, the exact and right identification of vectors is very important. Therefore, study of mosquitoes in specific ecological niche and as well; as use of DNA based approaches are used for their correct identification. Many species of mosquitoes including *Anopheles* and *Culex* are found in Pakistan especially *Aedes* received receive major attraction during dengue epidemics [4]. Use of insecticides and other Pest management techniques are globally conducted not to completely eliminate the population of mosquitoes but to reduce the number of mosquitoes and control the death rate caused due to disease transmission by mosquitoes. By abolishing the breeding sites and killing mosquitoes larvae, population of mosquitoes can easily controlled [5].

Insecticides such as, organophosphate are commonly used which interferes with the action of choline esterase's enzymes(neurotransmitter acetylcholine regulator), causing damage to muscles resulting in muscle cramps, paralysis, and eventually death [6]. In synaptic junctions of mosquitoes, the blockage of central and peripheral nervous system enzymes i.e.,acetyl cholinesterase occurs which in turn results into repolarization of membrane.This enzyme can be inhibited by formation of strong covalent bond between the insecticides and acetylcholineesterase, thus acetylcholine is accumulated in the synaptic junction and transmission of normal nerve impulse is interrupted [7].

Due to effectiveness against agricultural pests, excessive use of insecticides is done resulting in high rate of insecticides/pesticides is accumulation in the environment, residual effect is high both in assimilated (converted in biomass) and dissimilated (converted chemically into other bioactive compounds). Due to gaseous nature the insecticides spread with air currents to the wider range, therefore, insecticides applied to small area are capable of spreading to nearby areas and also to wide spread areas if they get dissolved in water.

Insecticides may have different fates when they spread to the environment e.g., if the insecticides are applied to agriculture plants, they are able to move to the surroundings environment including water bodies and soil. And if the insecticides are directly spread on soil then they could be washed away with rain and reaches to ground water or may seeps down in lower soil through the porous soil layer [8].

This wide spread of insecticides may result in loss of diversity in insect's species from minor to major level. The impacts of long-term persistence of organophosphate insecticide use on environment in general varies from acute fatal effects to long term fatal effects ranging from the contamination of surface water, ground water, food products, soil and air occur due to in the environment. Non-target organisms like natural pollinators, predators and earthworms etc. are greatly affected by the insecticide applied for reducing mosquito species [9]. The species of mosquitoes that are resistant to insecticides have been identified in almost all regions of Pakistan. The list of mosquito's species is summarized in table 1.1.

TABLE 1.1: Table showing the present species of mosquitoes in Pakistan [4].

Genus	**Species**	**Reference**
Anopheles	*gambiae*	Tark K, SchonebergI et al; 2012.
	stephensi	Avortink TJ et al; 1990.
	subpictus	Tark K, Schoneberg I et al; 2012.
	culicifacies	Otranto D et al; 2009.
	annularis	Avortink TJ et al; 1990.
	splendidus	Krzywinski J, Besansky NJ et al; 2003
	pulcherrimus	Krzywinski J, Besansky NJ et al; 2003
Aedes	*aegypti*	Khan MA et al;1971
	albopictus	Khan MA et al;1971
	walbus	Otranto D et al; 2013
	unilineatus	Khan MA et al;1971
Culex	*quinquefasciatus*	lahi I, Suleman M et al; 2013
	theileri	Suleman M et al; 2013
	tritaeniorhynchus	Otranto D et al; 2009.
	bitaeniorhynchus	lahi I, Suleman M et al; 2013
	mimeticus	Suleman M et al; 2013
	fuscocephala	Avortink TJ et al; 1990

The excessive use of insecticides and their long-term impact on environment has resulted in insecticide resistance. The Insecticide resistance may occur due to metabolic changes and genetic mutations in genomes of mosquitoes. For example, various non-identical mutations causes the insensitivity resistance or kdr mutations which ultimately results in insecticide resistance to pyrethroid lamda-cyhalothrin [10]–[15]. Other major contributors to insecticide resistance are the activation of pathways related to degradation of xenobiotics by microorganisms residing on insect [16], [17]. Degradation of delta-methrin and pyrethroids lambda-cyhalothrin was reported by bacteria such as *Pseudomonas stutzeri, Pseudomonas oleovorans, Arthro-bacternicotinovorans, Enterococcus mundtii* and *Klebsiella sp* [16],

[18], [19]. Agricultural pests utilize the xenobiotic degrading potential of their symbiotic bacteria [16], [18], [20], [21].

The gut microbial species present in mosquitoes are usually acquired from environment, and comprise prokaryotes, fungi and parasites. The metabolic and physiological functions in mosquitoes are greatly affected by the type and diversity of gut microbes they harbor. In these gut bacterial species, the insecticide resistant is sometimes intrinsic due to spontaneous mutations and commonly occurring DNA exchange mechanisms. The bacterial strains such as, Staphylococcus and Mycobacterium are most commonly associated with the onset of multiple drug resistance and the spread of the resistance. Figure 1.1 summarizes the process of bacterial acquisition of multiple drug resistance and insecticide resistance. .

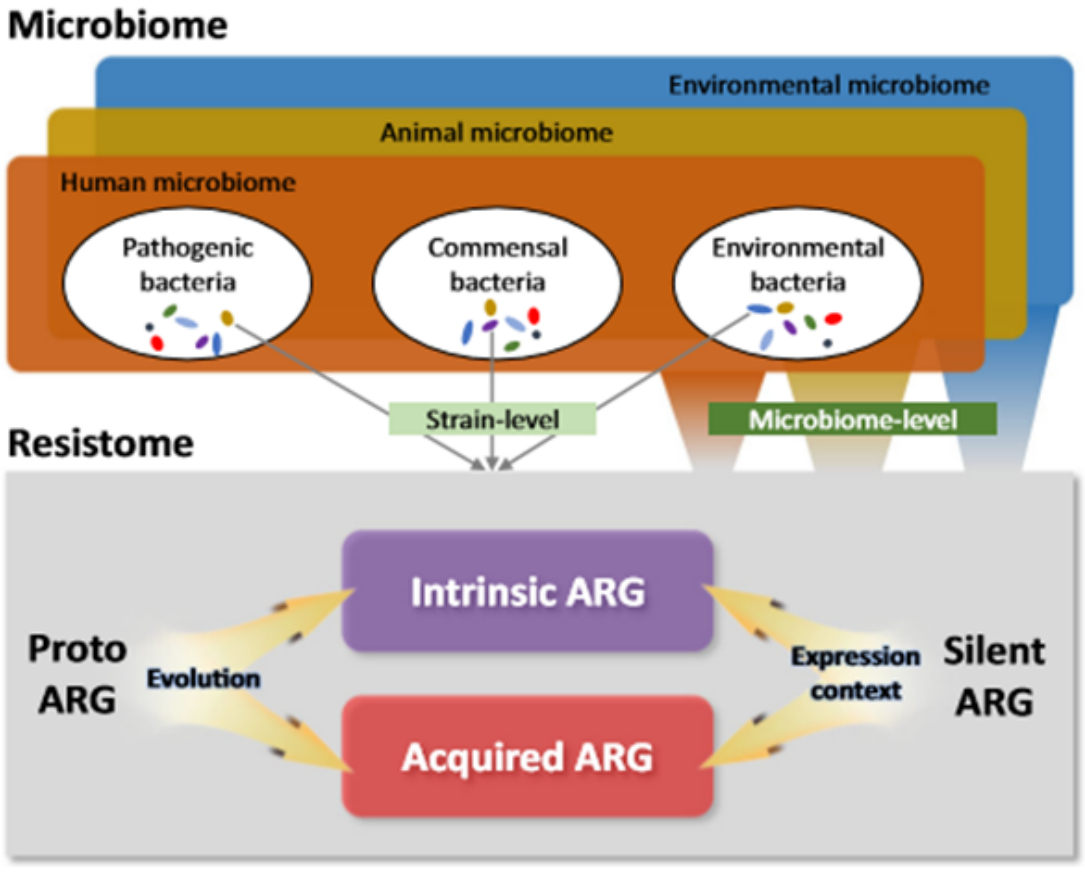

FIGURE 1.1: Various mechanisms by which insecticide resistance is developed in gut microbiome [22].

Intrinsic drug resistance genes present on bacterial chromosome are very common amongst all bacterial species for example, Vancomycin resistant in Escherichia

coli are of intrinsic type. On the other hand, bacterial species sometimes acquire the drug resistant/ insecticide resistant genes due to environmental exposure by horizontal gene transfer mechanism, which also leads to evolution and increase in drug resistance [23]. Understanding of the exchange mechanisms and especially the identification of genes, which are commonly acquired and then exchanges between the microbiota harboring the gut of mosquitoes, can lead to targeting the common genes and development of better control strategies against mosquitoes.

1.1 Aim and Objectives

Mosquito are the most common vector in spread of various infectious diseases and epidemics in human such as Dengue, Malaria, Zika virus, yellow fever and many more. Gut microbiota of mosquito are well reported to play a significant role in increasing the metabolic capabilities and survival strategies of the host and could be an effective target to design control strategies against mosquitoes. But the knowledge, how this gut microbiota facilitates mosquito species is insufficient. The study of insecticide resistance genes of microbiota in mosquito will help epidemiologists to design efficient and effective control strategies focused on the insecticide resistance of microbiota. The project is designed with an aim to identify the insecticide resistance genes in gut microbiota of mosquitoes and to understand their impacts on overall resistance in mosquito. To achieve this aim project is divided into following objectives:

1. Identification of insecticide resistance gut microbiota species in resistance mosquitoes.
2. Identification of key resistance genes in gut microbiota of various mosquitoes' species.
3. Understanding molecular mechanism behind insecticide resistant species.

Chapter 2

Literature Review

This chapter reviews the literature related to insecticide resistance in mosquito owing to this mechanism.

2.1 Mosquito

Mosquito is basically a disease-causing vector. The body of mosquito is small and slender having a length of 3-6 mm. Its species can vary from 2 mm to 19 mm in size. The family Culcidiae includes 41 genera and 3300 species. Toxorhynchitinae, Anophelinae (anophelines) and Culicinae (culicines) are the subfamilies of the major family Culcidiae [24].

2.1.1 Morphology

Head, thorax and abdomen are the three main body regions of mosquitoes like any other insect. These main regions are further subdivided into segments that are not usually distinct except abdomen. The head is of spherical shape having two large compound eyes and it is generally known as body's sensory center. Between head and abdomen, thorax is present containing locomotory organs i.e., legs and wings. Six legs are present in adult mosquitoes. Four wings are technically present

in mosquitoes, out of which front two wings are used for flying and hind two wings are smaller in size and don't appear as wings and called as "halteres". The posterior region that is site for digestion, reproduction and excretion is known as abdomen. Cerci are the copulatory organs protruding from the tip of abdomen and are mostly visible in *Psorophora* and *Aedes* females [25].

2.1.2 Habitat

Except Antarctica and few Islands mosquitoes are worldwide in distribution. They mostly occur near water bodies like rivers, swamps, lakes and other clean as well as marshy places due to their unique adaptive nature. Below sea-level at depth of 1250 m and elevations of 5500 m they are found [24]. *Aedes vexans* have developed the ability to grow in temporary flooded areas. The mosquitoes including *Ochlerotatus communis, Ochlerotatus cataphylla, Ochlerotatus cantans* and some other species have ability to grow in snow-melt, swampy woodlands and marshy by encountering the conditions and make themselves ideal [26].

2.1.3 Life Cycle

The life cycle of mosquito consists of major four stages i.e., egg, larva, pupa and adult stage. Egg, larva and pupa are the first three stages that completes in aquatic environment., Depending on the ambient temperature and the species, with few exceptions each of the stages typically lasts 5 to 14 days [27].

Mosquitoes are able to delay their development for months in areas having freezing temperature, they diapauses their activities in waterless conditions and when there is maximum availability of water, they carry on with normal life activities e.g. In diapauses the eggs of *Aedes* remain unharmed even in dry out conditions, as soon as they are covered by water hatch to become larvae and pupa respectively. As mature pupa floats at the water surface, adult mosquito emerges from it. Life spans of bloodsucking mosquito species vary from week to several months depending upon its species, weather conditions and sex As shown in figure 2.1 [28], [29].

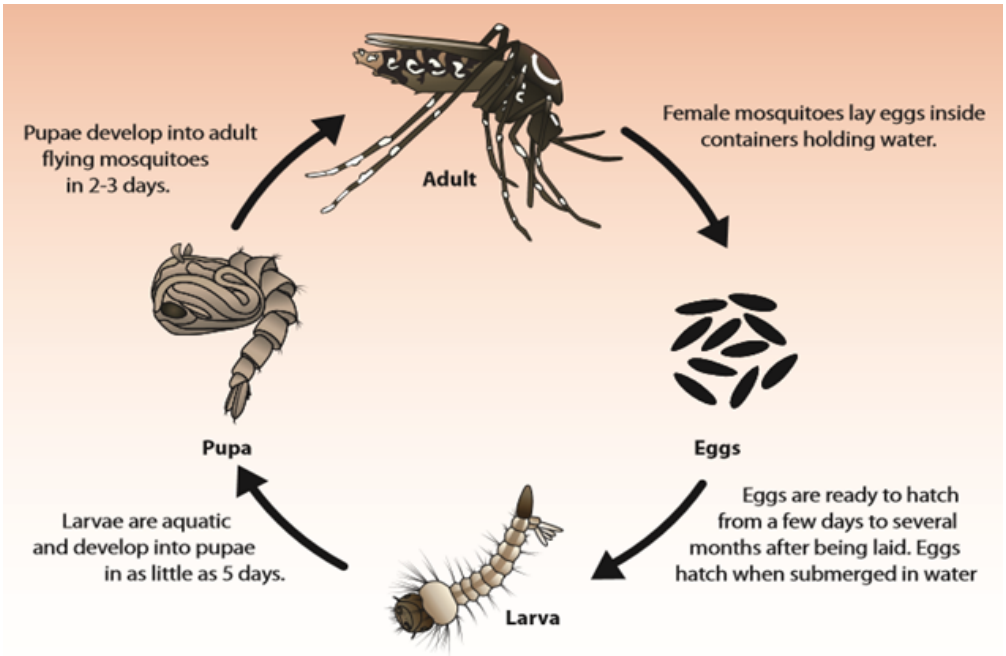

FIGURE 2.1: Life cycle of mosquito showing the stages from egg to adult.

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Eggs when submerged to water are ready to hatch after several months, after hatching larva emerged from these eggs and then pupae is formed after 5 days from that larvae. Finally pupae develop to form the complete mosquito [30].

2.1.4 Classification

Mosquitoes are insects that belong to kingdom Animalia and phylum Arthropoda. The order of insects is Diptera and suborder is Nematocera. The family Culicidae includes 110 genera of mosquitoes. The most important genera of mosquito like *Anopheles*, *Culex*, and *Aedes* include 3600 species [18].

2.2 Medical Significance

The mosquitoes genera like *Aedes, Anopheles, Culex* and many others are of medical significance [31]. Public health authorities and many Researchers focus their

attentions on studying mosquito-borne diseases due to their great emergence rate. Mosquitoes are most dangerous animals for mankind because they are transmitting a number of vector-borne diseases and are also responsible for death of millions of people worldwide. 100 tropical countries are affected due to malaria thus placing about 40% of world's population at risk of mortility.

The transmission of disease can be done by following two ways: either biological or mechanical vectors. In biological vector transmission, the pathogens multiply their number in order to mature into infective stage when they are ingested by mosquitoes. Before becoming capable of entering to the new host these vector takes several days for maturation e.g. (human malaria parasite), these vectors are very complicated due to pathogen's development and parasitic containment by vector.

The causative agents from the contaminated materials are transmitted to the body or food of human in case of mechanical vector transmission method [32]. Lethal diseases that are produced due to valuable parasites and pathogens of mosquitoes are Malaria, Dengue, Yellow fever, Zika virus, Encephalitis etc.

2.2.1 Malaria

Malaria is one the lethal disease that scared almost all of the human life in Europe including Southern and Northern Europe. Two main species of *Plasmodium* i.e. *P. falciparum* and *P. vivax* were commonly found in Europe. 300 million infections and more than 1 million deaths occurred due to Malaria. In malaria endemic regions of Pakistan, about 60 percent population lives.

Each year 3.5 billion suspected and confirmed cases of malaria are reported in Pakistan. In malarial burden of Pakistan, *Plasmodium falciparum* contribute 81.3 %, *P. vivax* contributes 14.7 % and remaining 4 % contribution is due to mixed species. With high malaria transmission and about 100 % of the population living at risk Pakistan is among the six WHO Eastern Mediterranean region countries [33]. Due to variable climates in different provinces and even in different cities the

endemicity of malaria varies. The genotypic and phenotypic plasticity of vectors is difficult to determine that's why to distinguish and recognize the mosquito species modern techniques such as PCR-assays was performed using genetic markers [34].

2.2.2 Dengue

The first epidemic of dengue viruses reported in Asia, Africa and North America from 1779 to 1780. So it is indicated that this virus is distributed worldwide from more than 200 years ago. The dengue is reported as fetal disease in Thailand and Philippines due to its first dengue hemorrhagic fever (DHF). The two main vectors of dengue are:*Aedes albopictus* and *Aedes aegypti*.

Mosquitoes are responsible for causing dengue to human beings. The serotypes of mosquitoes are: DEN-1, DEN-2, DEN-3, DEN-4. Due to crowded cities, unsafe drinking water, low vaccination coverage and poor sanitation, Pakistan is at greater risk of being hit by large epidemics. The infections and outbreaks like dengue virus spread in country due to these endemics. It has been identified by researchers that the 2006 outbreak in Karachi is due to co-circulation of DEN-2 and DEN-3. Marginal association of DHF Cases is shown with DEN-2. The severe outbreaks occurred in Lahore from last two decades in which 20,000 people were hospitalized and 350 died due to Dengue virus. Illness usually starts 5 to 7 days after the intrinsic incubation period. Rashes, headache, pain behind the eyes, muscular and joint pains and diarrhea are the common symptoms of dengue include fever. Dengue patients developed Immunity for the other serotypes that get recovered from one serotype [35], [36].

2.3 Microbiota of Mosquitoes

Plasmodium falciparum is one of the deadliest protozoan's parasite and female anopheles mosquito is responsible for transmitting that parasite into human body causing malaria [37], [38]. *P. berghei* and *P.vivax* are parasitic species responsible

for causing malaria in human beings [39], [40] *Wolbachia* is one of the bacterial intercellular genus found in mutualistic relationship with insects [41]. *Asaia* is also a bacterial genus responsible for causing malaria that was firstly identified in *Anopheles stephensi* [42].

Aeromonas taiwanensis is non-spore forming bacterial genus observed in patients of twains [43]. *Escherichia coli* is one the commonly known rod shaped gram negative bacteria and considered as one the gut microbiota of mosquito [44]. Understanding of the potential microbial functions is developed through the microbiome study in last few decades such as xylane hydrolysis, productions of vitamins in *Glossina palpis*, phenolic metabolism and nitrogen fixation in Pine Beetle, signal mimics in Gypsy Moth species, resistance against antibiotics in Gypsy Moth species.

2.3.1 Diversity in Gut Microbiota of Mosquitoes

Prokaryotes, Fungi and other microbes constitute the gut microbiota of mosquitoes. Varying greatly with species, diet, stage of development of mosquitoes and geography, the composition of gut microbiota is considerably dynamic and this diversity is primarily acquired from the environment [45], [46]. To study of mosquito's microbiota composition, the sequencing of the 16S rRNA or18S rRNA hyper-variable regions [47] is used as a culture-independent tool. The diversity of gut microbiota varies greatly due to feeding habits and environmental changes [45].

2.3.1.1 Mosquito Bacteriome

Through the aquatic larval habitats during the aquatic life stage, mosquitoes acquired a substantial fraction of colonizing bacteria. Bacteria and planktons are consumed by mosquitoes as nutritious resources. The different colonization patterns of mosquito bacteriome depend upon different environmental characteristics[48]. Rather than salivary glands and reproductive tract, bacteria colonize more in midgut of mosquitoes. In different *Aedes aegypti* populations, bacterial

species like *Pseudomonas, Acinetobacter, Aeromonas* and in *Anopheles gambiae* the species like *Wolbachia* and *Acinetobacter* were detected [49], [50].

2.3.1.2 Mosquito Mycobiome

Including bacteria and influenza, eukaryotic fungi is a part of the mosquito gut microbiota mosquito mycobiome. In preserving the ecological balance of mosquitoes its position as commensal, mutualist or pathogenic is inevitable. By ingestion of fungi in sugar meals, or physical contact with conidia mosquitoes are exposed to fungi in the form of mosquito larvae in water during the metamorphic transition[51]. In the mid gut and other tissues of mosquitoes filamentous fungi and yeast are the common fungal isolates. Through culture dependent and culture independent methods in *Aedes* and *Anopheles* mosquitoes different genera of yeast like *Candida*, *Pichia* and *Wickerhamomyces* have been identified [52].

2.3.1.3 Mosquito Virome

For a large group of viruses which is insect-specific, mosquito acts as an exclusive host. By Shi et. al, in two genera of mosquitoes *Aedes* and *Culex* metagenomic approach was used to evaluate viral load [53]. *Aedes* showed a low viral diversity and less abundance than *Culex* thus presenting a striking difference in the virome of mosquitoes. In mosquitoes different viral families such as Orthomyxoviridae, Flaviviridae, Mesoviridae, Bunyaviridae, Rhabdoviridae, Reoviridae, un-classified Chuvirus and Negevirus groups have been identified, using the metagenomic approaches. Due to inability to infect vertebrate cell lines, vertical transmission and prolonged host infection most resident virome act as commensal microbe [53], [54].

2.3.2 Insecticides

To kill the insects, the toxic substances are used called as insecticides. To eliminate disease-carrying insects in specific areas and to control pests that infest cultivated

plants these substances are commonly used.

On the basis of their chemistry, their mode of penetration or their toxicological action insecticides can be classified in any of several ways. The insect inhaled the toxic compounds like fumigants through its spiracles. Chemicals as hydrogen cyanide, naphthalene, nicotine, and methyl bromide are commonly used as insecticides.

There are basically two [56] types of insecticides: one of the synthetic insecticides which are produced by alteration of chemicals e.g. lindane, Chlorobenzilate, methoxychlor, cyclodienes, DDT, parathion and malathion etc and others are Natural insecticides which are extracted from plants e.g. Nicotene, pyrethrum, derris etc. Some common insecticides are as follow: DDT, Parathion, Nicotine, Chlorobenzilate, Pyrethroids, Organotins, Carbamates and Derris etc.

2.3.3 Insecticide Resistance

Excessive use of insecticides is enabling to cause insecticide resistance in mosquitoes. Insecticide resistance can be created in insects by creation of target site modification in mosquito's metabolic system such as *Culex pipiens* and *Anopheles* mosquitoes. In this mechanism target side genes altered and lose their affinity of binding site to bind the insecticides [57].

The enzymes like monooxygenases, transferases and hydrolases are involved in insecticide resistance and they convert the xenobiotics into non-toxic compounds [58]. Bacteria also protect themselves from the insecticides by formation of alternative target site that interrupt the normal pathway of antibiotics by continuing its normal pathway [59].

2.3.4 Mechanisms of Insecticide Resistance

The development of insecticide resistance is due to massive use of insecticides-based control techniques. Due to development of insecticide resistance mechanisms, the

elimination of *Aedes* mosquitoes and controlling the increased risks of dengue fever is an increasing challenge. Some of the insecticide resistance mechanisms are: Target site resistance, metabolic resistance, penetration resistance, knockdown resistance and behavioral resistance etc.

2.3.4.1 Target Site Resistance

The target site for the action of insecticides is genetically modified in such a way that its interactions with neurotoxins are limited thus insecticidal effect is consequently eliminated, when target site resistance in mosquitoes is inferred. These modifications generally involve insensitivity of synaptic acetylcholinestrase (AChE1), Vssc modifications and mutation in GABA receptors as summarized in figure 2.2 [60].

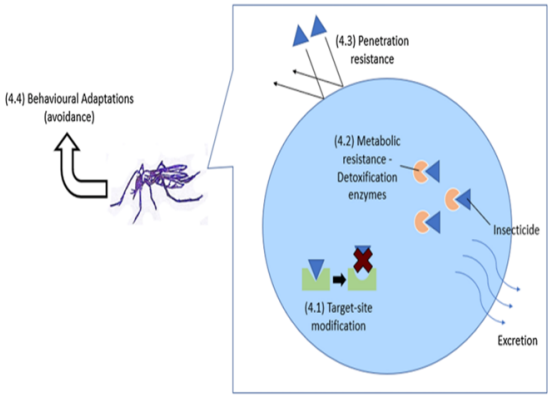

FIGURE 2.2: Insecticide resistance mechanism showing target site modification, metabolic resistance, penetration resistance and behavioral resistance [61].

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2.3.4.2 Metabolic Resistance

Due to conformational changes of or over-expression of enzyme subsequent to point mutation in sis/trans loci of enzyme, resistance strains that detoxify the insecticides are much better than suspected mosquitoes. Three major enzymatic activities are involved in metabolic detoxification such as glutathione S-transferases (GST) activity, esterases and cytochrome P450 mono-oxygenases [60], [62], [63].

2.3.4.3 Penetration Resistance

The insecticide absorption inside the body of mosquitoes becomes slow when the barriers develop at the outer cuticle of mosquitoes resulting in penetration resistance. Likewise, the susceptible strains absorb toxin at much higher rate as compared to resistance strains. Thus, the action of metabolic enzymes is facilitated with more available time due to reduced penetration. Due to their lipophilic property, cuticle resistance is showing its involvement in cross-resistance to multiple insecticides [64].

2.3.4.4 Behavioral Adaptations

Through adaptations mosquitoes can prevent the negative consequences of insecticides. Spatial, trophic and temporal avoidance are the categories of behavioral resistance. In spatial avoidance the mosquitoes escapes from insecticide-treated area and in temporal avoidance mosquitoes reduces risk by mismatching the timings of insecticide exposure, whereas the feeding on hosts in extensively used insecticides is avoided in case of trophic avoidance [65], [66]

2.4 Metabolic Detoxification of Insecticides

The mechanism of the detoxification of insecticides in mosquitoes involves three major metabolic gene families which are: Cytochrome P450s (P450s), esterases and the S-transferases (GSTs) glutathione. In both biochemical as well as the physiological functions of the living organisms, cytochrome P450s are among those genes families which have the most significant role. To activate and to detoxify endogenous compounds as well as the xenobiotics, cytochrome P450s are playing are the most critical and significant role. In the metabolic detoxification and the excretion GSTs [67] are the largest quantity of the exogenous as well as the endogenous compounds having the property of the solubilization. At the transcriptional level the up regulation of the GSTs and the P450s is done which in turn results

in the formation of excessive production of proteins, hence excessive enzymatic activity. With the help of oxidation and also the toxins of plants inside the insects the increases the detoxification of the insecticides occurs which further leads to the tolerance of the insecticides [68], [69].

2.5 Role of Mosquito Genome in Insecticide Resistance

DDT and pyrethroid insecticide targets the insect at molecular level through voltage gated sodium channels (VGSC) and more than 40 species show resistance to these insecticides by changing amino acid sequence [70], [71] By sequencing capillaries of specific exons and introns VGSC gene in mosquito *A. gambiae* two primary resistant variants i.e. L1014F [72], L1014S [72] and one secondary variant that is enhancing the phenotype of L1014F were discovered respectively [73], [74] In case of Ace-1 gene the substitution of G119S found to cause resistance against organophosphate insecticide and carbamate in *Anopheles gambiae* [62], [75] Several kdr mutations have been found in *Aedes aegypti* causing the biodegradation of insecticides through voltage-gated sodium channels [76].

2.6 Significant Genes of Mosquito Gut Microbiome

Kdr gene in *Anopheles gambiae* and *A. arabiansis* causes target site resistant[77] . CYPs, CCEs, ABC-transporters, GSTs genes of *A. gambiae* are involved in insecticide resistance through metabolic pathways [72], [78], [79]. Cecropins, defensins, diptericins and gambicins are the antimicrobial peptides having ability to stimulate the mosquito immune system [80]. CYP6M7,CYP6P9a, CYP6P9b, and GSTe2 genes found in *A. gambiae* found to cause resistance against DDT and pyrethroids[81]. Elevated metabolism levels of Glutathione S-transferases

(GSTs)oxidase, and esterase detoxification are found to cause resistant against DDT and pyrethriods [82].

2.7 Antibiotic Resistance

On our planet recently antimicrobials are practiced in clinics. According to Darwinian's Principle of evolution the antibiotic resistance is normal adoptive response of organisms. Around the globe, the prolonged living span of organisms is due to successful advances in medicines.

Bacteria have creative ability to circumvent the attacks of antibiotics. One of the major threats of 21st century is emergence of antibiotic resistance. Up to now limited research had been done on multidrug resistance (figure 2.3).

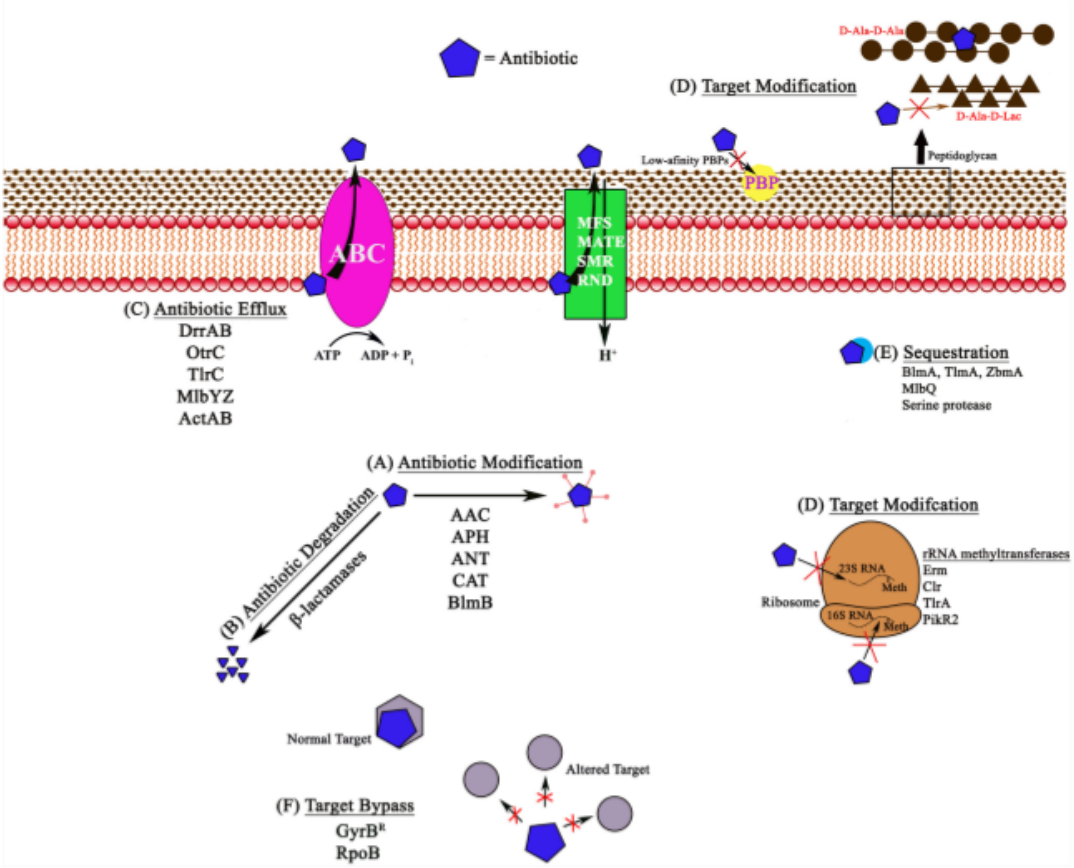

FIGURE 2.3: A schematic representation antibiotic resistance in bacteria showing antibiotic modification, antibiotic degradation, target modification and target bypass [83].

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2.7.1 Molecular basis of Antibiotic Resistance at DNA Level

More than 25 years ago in Africa, America and Europe due to a loss of sensitivity of the insect's acetylcholinesterase enzyme to organophosphates and carbamates, resistance to insecticides among mosquitoes like *Anopheles gambiae* and *Culex pipiens* had emerged [84]. A single amino acid substitution in enzyme can cause insensitivity to pesticides. From Tropical Africa and Temperate Europe areas ten highly resistant strains of *C. pipiens* and *A. gambiae* were found. New insecticides can be prepared by visualizing such kind of mutations. By hydrolyzing the neurotransmitter acetylcholine, acetylcholinesterase terminates its synaptic transmission thus leading to paralysis and death. A strong resistance to this insecticide is shown by mosquitoes. Ace-1 and Ace-2 are responsible for causing resistance in *C. pipiens* by encoding different isoforms of acetylcholinesterase [85], [86].

Complete Ace-1 mRNA coding sequences of two *Culex pipiens* strains i.e., one resistant and one susceptible, were analyzed to identify mutations involved in resistance in mosquitoes. From these two selected strains cDNAs vary at 27 nucleotide positions, in resistant mosquitoes amino-acid substitution is generated by only one of these: at position 119the GGC (glycine) codon, [84], is replaced by an AGC (serine) codon.

2.7.1.1 Genetic Mutation

Any accidental change in polynucleotide sequences of a gene can lead to genetic mutation and these mutations either effects one or more nucleotide sequences. In the replication of DNA these mutations are unpredictable changes. Allele are the different forms of genes formed by mutations and they occupy the original gene locus [87]. By using ace-1 genomic sequences of susceptible and resistant (KISUMU) strain, the insensitive acetylcholinesterase emergence in main African malaria vector *Anopheles gambiae* was found [88]. Having two of them being

non-synonymous, at 18 nucleotide positions the coding sequences differed. In the amino-terminal region of YAO strain, replacement of a valine residue by alanine cause did mutation that did not seem to affect the enzyme's catalytic properties and has no equivalent in Torpedo acetylcholinesterase.

As in *Culex pipiens* (results not shown) the other was the same G119S substitution, indicating that this at least three times in the ace-1 gene, single point mutation has occurred independently, once in *A. gambiae* and twice in the *C. pipiens* complex. For insecticide resistance in mosquitoes, the discovery of the ace-1 mutation opens the way to new strategies for pest management.

In overcoming the spread of resistance there is need for the development of new insecticides, in which G119S mutant form of acetylcholinesterase-1 is inhibited that ultimately will be crucial.

2.7.1.2 Horizontal Gene Transfer

A new strain of bacterial pathogen can be formed through foreign DNA acquisition of by horizontal transfer from unrelated organisms [89]. An early indication of importance of horizontal gene transfer to bacterial pathogenesis was discovered in genes carrying antibiotic resistance self-transmissible resistance (R) plasmids [90].

Yersinia pestis containing the antibiotic resistant strains is an obligate parasite alternating between insects and mammalian hosts. The development of stable mating pairs is influenced by conjugation that depends on several environmental and bacterial factors [91]. *Yersinia pestis* containing the antibiotic resistant strains is an obligate parasite alternating between insects and mammalian hosts. The development of stable mating pairs is influenced by conjugation that depends on several environmental and bacterial factors [91].

In Urinary tract, respiratory tract, mammalian intestine and wounds various evidences for conjugative transfer between different bacteria has been reported (figure 2.4), but even in the existence of antibiotic selective pressure, estimated transfer rates are very low [92].

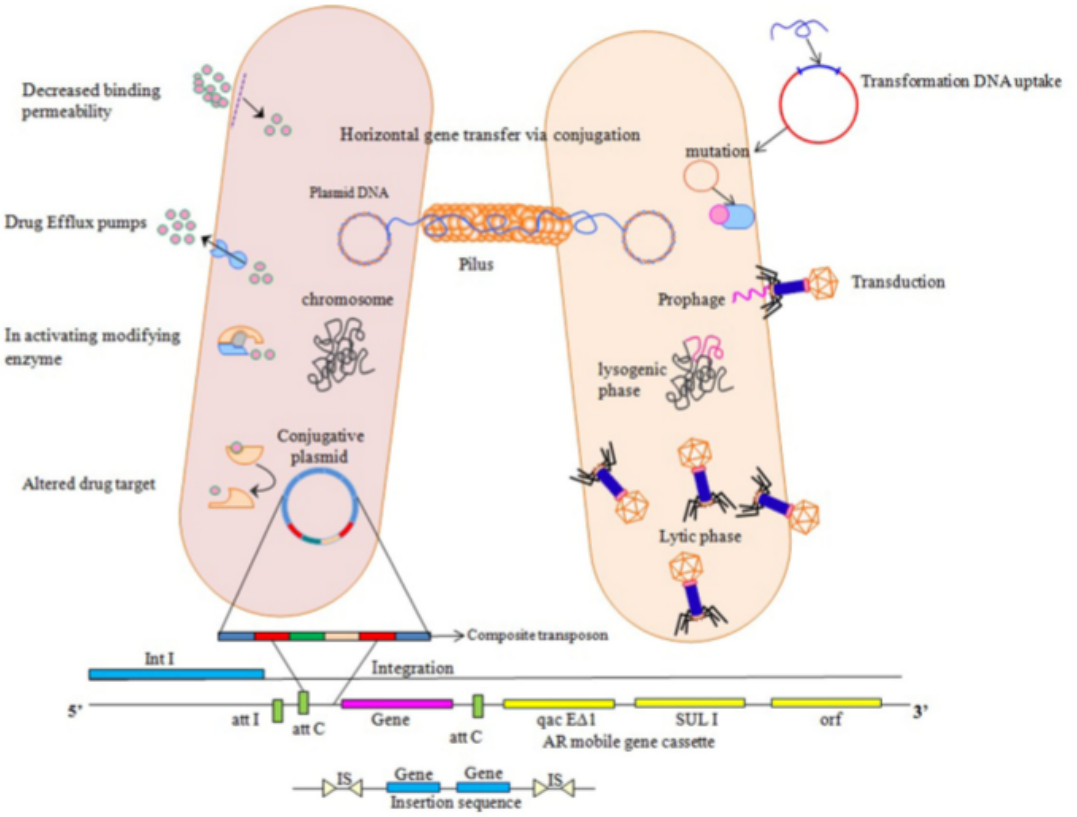

FIGURE 2.4: Horizontal gene transfer in plasmid vectors [93].

2.7.2 Mechanism of Antibiotic Resistance

Insecticide resistance mechanisms (figure 2.5) involves biochemical basis (as in the control of malaria vectors, insecticide avoidance behaviors that is opposed to vectors is important).

Target-site resistance mechanism occurs when the insecticide no longer binds to its target while detoxification enzyme-based resistance is the other biochemical mechanism in insecticides are prevented from reaching the site of action. It is one of the major forms of biochemical resistance while detoxification enzyme-based resistance is the other biochemical mechanism in insecticides are prevented from reaching the site of action due to enhanced levels or modified activities of esterases, oxidases, or glutathione S-transferases (GST). Thermal stress response is an additional mechanism based on biochemical resistance has been proposed [94], but its importance is still unknown.

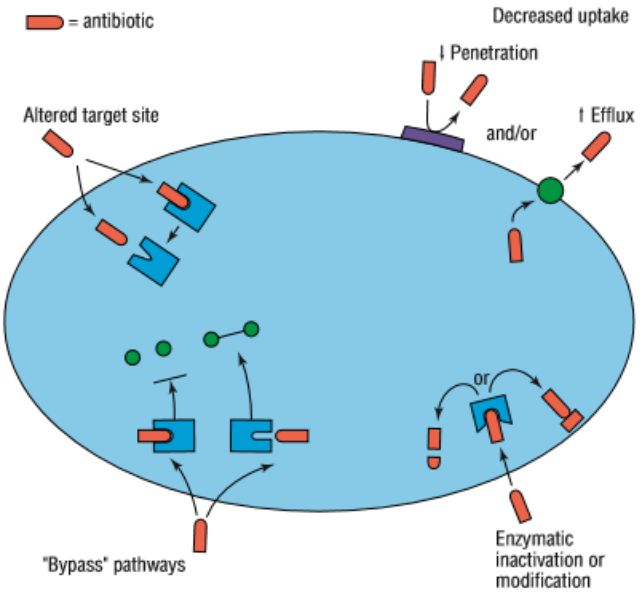

FIGURE 2.5: Four major biochemical mechanisms of antibiotic resistance in bacteria.

2.7.2.1 Structural Modifications of Antibiotics

In antibiotic modification the antibiotic is prevented from reaching the target site although the resistant bacteria retain the same sensitive target as antibiotic sensitive strains. For example, antibiotic can be inactivated in case of lactamases by enzymatic cleavage of four membered lactam ring. Most â lactamases act to some degree against both penicillin and cephalosporin from 200 described types of â lactamase e.g., penicillinases and cephalosporinases. Among many bacterial species Lactamases are widespread and varying degrees of inhibition are exhibited by â lactamase inhibitors, such as clavulanic acid [95].

2.7.2.2 Efflux Pump

For cell-cell communication, biocides, and metabolic products different types of antibiotics and chemicals such as dyes, organic solvents, detergents, molecules are expelled byEfflux pumps that contribute to multidrug resistance [96]–[100]. Five classes of the bacterial multidrug efflux transporters (figure 2.6) are: (1) resistance nodulation cell division (RND), (2) major facilitator superfamily (MFS), (3) small multidrug resistance (SMR), (4) ATP-binding cassette (ABC), (5) multidrug and

toxic compound extrusion (MATE). The major sources to meet the energy demand of these five classes for the active transporting are H+ protons (RND, SMR, and MFS), Na dependent (MATE), or by hydrolysis of ATP (ABC) [99]–[101] The RND efflux pump transporter is present in *Pseudomonas aeruginosa* that is composed of three parts, the linker, the transporter, and the outer membrane pore [102].

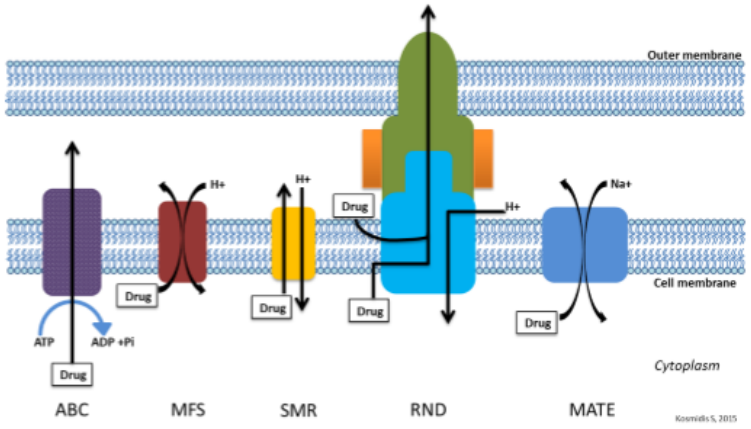

FIGURE 2.6: Structure of efflux pumps families showing channels and transfer of molecules[103].

2.7.2.3 Efflux Mechanism

In *Pseudomonas aeruginosa*, MexAB-OprM is the one of the 12 different types of RND efflux systems that constitutionally expressed accounting for the intrinsic resistance to pathogenicity and flouroquinolones of this organism [100], [104]–[107] The three subunits ofMexABOprM are the antibiotic discharge duct protein and MexA and OprM acting by substrate recognizing energy transfer and connecting the MexB and OprM [108]. Finally MexB entrapped the antibiotics and then transferred this antibiotic to OprM and MexB finally extruded the antibiotic [109]–[111]. Mutations in the genes encoding for efflux pump MexAB-oprM creates higher resistance profiles of *Pseudomonas aeruginosa* to quinolones that regulate the resistance for b-lactams, quinolones, and b-lactamaseinhibitors [112]. MexAB-OprM pump confers resistance to the other non-antibiotic compounds beside its well-defined activity against the known antimicrobial agents, such as tea tree oil and its monoterpene components a-terpineol and the related alcohols [113].

2.7.2.4 Target Side Interference

The competitive inhibitors of acethylcholine (Ach) are OP and CX and enzyme AChE is their target. In the cholinergic synapses of the central nervous system the insecticides prevent the hydrolysis of the neurotransmitter Ach after binding to AChE.

As a result, the death of insects occur by tetany because nervous influx is continued and Ach remains active [114]. The two synaptic enzymes, AChE1 and AChE2 are coded by two described genes, ace-1 and ace-2 in several insects. Persistent insects commonly show five mutations [86]. The most common resistance mutation is G119S in the ace-1 gene located near the catalytic site in mosquitoes, including *Culex pipiens* [115].

From a single point mutation GGC to AGC in ace-1 gene the substitution of glycine by serine results in high insensitivity displayed by *Culex pipiens*. The substitution of glycine by serine results in high insensitivity displayed by *Culex pipiens*. The high insensitivity displayed by is due to the, resulting [115], thus the insecticide allowed a decreased inhibition of the main synaptic enzyme AChE1 [116].

2.7.2.5 Target Side Protection

In some antibiotic resistant bacteria antibiotics are prevented from entering the cell or pumping it out faster than it can flow in to protect the target of action. Through water filled hollow membrane protein known as porin, Lactam antibiotics in Gram negative bacteria gain access to the cell that depends on the antibiotic.

As imipenem cannot penetrate the cell, *Pseudomonas aeruginosa* confers resistance due to lack of the specific D2 porin in case of imipenin resistance. A low-level resistance to fluoroquinolones and aminoglycosides is also seen by this mechanism.

A well-recognized mechanism for resistance to tetracycline is increased efflux via an energy-requiring transport pump that is encoded by related genes such as tet(A) that in Enterobacteriaceae have become distributed [117].

2.7.2.6 Mutation of Target Site

The binding of the neurotoxin products is limited due to point mutations in the target of insecticides, in one of the insecticide resistant mechanism called as the target site modification. Some of the insecticide target modifications are described as: the voltage-dependent sodium channel encoded by the kdr gene, the synaptic acethylcholinesterase (AChE1) encoded by the ace-1 gene and the γ-amino butyric acid (GABA Receptors) encoded by Rdl gene [118], [119].

Across the neural axon in the voltage-gated sodium channel (VGSC) the knock-down resistance kdr gene is the major mechanism that is responsible for resistance to DDT and PYR by reducing the sensitivity of the receptors to these products [120], [121]. Due to the mutations of kdr gene this resistance occurs. At codon many mutations have been reported the mutations like L1014F by the substitution of a leucine (TTA) by phenylalanine (TTT) and L1014S by the leucine (TTA) to serine (TCA) substitution that are associated with knock down resistance in mosquitoes, including *Culex pipiens* [122]–[124] while from China for *Cx.Pipiens* molests the substitution of a leucine (TTA) by cysteine (TGT) in the L1014C mutation by has only been reported [125].

2.7.2.7 Enzyme Alterations of Target Site

Due to structural changes in the molecule, antibiotics are unable to inhibit the activity of target inspite of the fact that the antibiotic penetrates the cell and reaches the target site. Having low affinity, the enzyme responsible cell wall synthesis called as penicillin binding proteins in *Enterococci* are regarded as being inherently resistant to cephalosporins.

Most strains of *Streptococcus pneumonia* that are highly susceptible to both penicillin and cephalosporins, acquire DNA from other bacteria and hence become resistant to inhibition by penicillin by developing a low affinity for penicillin [126]. Now having a different structure, the altered enzyme still synthesis peptidoglycan [127]. In the laboratory mutants of *Streptococcus pyogenes* that express altered

penicillin binding proteins and are resistant to penicillin can be selected, but the cell wall can no longer bind the anti-phagocytic M protein so they have not been seen in patient.

2.7.2.8 Circumvention/ Replacement of Target Site

While continuing to produce the original sensitive target, an alternative target (usually an enzyme) that is resistant to inhibition by the antibiotic is produced by bacteria in final mechanism, by which bacteria may protect themselves from antibiotics. The alternative enzyme "bypasses" allow bacteria to survive the effect of the antibiotic in face of selection. In addition to the "normal" penicillin binding proteins by methicillin resistant *Staphylococcus aureus* (MRSA) the alternative penicillin binding protein (PBP2a), which is produced, is probably the best-known example of this mechanism. The cell has a structurally sound cell wall and continues to synthesize peptidoglycan because PBP2a is not inhibited by antibiotics such as flucloxacillin so protein is encoded by the mecA gene [128]. In a vancomycin resistant MRSA the genes involved can be transferred to *S. aureus* so the appearance in 1987 of vancomycin resistant enterococci has aroused much interest. The alternative target mechanism of resistance is also represented by this mechanism [129]. Further cell wall synthesis is prevented when a penta-peptide that has a d-alanine-d-alanine terminus is prevented to bind vancomycin in case of entero-cocci that is sensitive to vancomycin (cell wall precursor is the normal target of vancomycin). The enterococcus makes an alternative cell wall precursor ending in d-alanine-d-lactate by acquiring the vanA gene cluster that does not bind to vancomycin.

2.8 Pharmacokinetics of Antibiotic Resistance

In the setting of therapeutic failure multiple parameters determine the response to antiretroviral therapy should be considered, while considering the development of drug resistance. Successful virological responses to antiretroviral therapy are

associated with individual Pharmacokinetic parameters, such as virological characteristics (e.g., phenotype, genotype) and trough concentration (Cmin)[130]–[139] Selection and accumulation of drug-resistance mutations are bound to happen in patients on antiretroviral therapy in the absence of maximal suppression of virus replication [140]. With the frequent cumulative acquisition of six or more amino acid substitutions the acquisition of resistance mutations to protease inhibitor (PI) therapy is complex [141]. A given compound has specific primary mutations, whereas secondary mutations that are shared with other drugs within the same family tend to accumulate later [136], [139], [142]

2.9 Pharmacodynamics of Antibiotic Resistance

With bacterial eradication and clinical success PK/PD-linked parameters have shown to be associated and now been applied. To minimize development of resistance and to improve success the principal focus is to optimize these PK/PD achievement targets. The complex relation of resistance is driven by MIC and is affected by acquired and inherent resistance as well as by mutation frequency. The PD target achievement may also be affected by mechanical factors, including such things as stationary growth phase of the organism, biofilm and inoculum effects. Therefore, optimal drug exposure is achieved by minimizing resistance development. When antibiotic exposure was suboptimal the probability of developing resistance during antibiotic therapy significantly increased and is demonstrated by the study evaluating factors associated with development of bacterial resistance [143].

2.10 Resistome

Communities of both pathogenic and non-pathogenic bacteriaconsisting of all the antibiotic resistance genes called as Resistome [144]. In 2006, Gerry Wright's group first coined the term "antibiotic resistome" which means the resistance

determinants present in the soil [145]. In various microbial systems resistome is the assemblage of resistance genes encoding the antibiotic resistance. In different sectors of the One-Health concept, ARGs circulate among the microbiomes of animals, humans, and the environment. To comprehend the complex Resistome with in the microbiome meta-genomics is an essential sequence-based approach. By understanding the mobile resistome, which is genetically associated with MGEs and core resistome which is relatively stable in the microbiome, the classification of ARGs was revealed [146].

Chapter 3

Materials and Methods

The methodology was designed to identify the insecticide resistant mosquito species. The microbial diversity in gut microbiota of selected mosquitoes was determined and then the resistant genes in each microbial species were identified. The prioritized resistant proteins were then docked against Derris to confirm their role.

3.1 Identification of Microbial Diversity

3.1.1 Insecticide Resistant Mosquito Species

Manual search of literature related to resistant mosquito species was performed on various search engines, including "PubMed(https://pubmed.ncbi.nlm.nih.gov/), Science Direct, Elsevier, and Google Scholar(https://scholar.google.com/)". On the basis of these literature surveys species summarized in table 4.1.

3.1.2 Identification of Microbial Diversity Among Resistant Mosquitoes

To perform this step meta-analysis approach was performed. Statistical analysis in which different scientific studies were combined is known as meta-analysis. If

the same question is addressed under multiple studies and each study have an issue of some degree of error, then meta-analysis is performed. Meta-analysis is a systematic way of accessing the previous studies by applying epidemiological, formal and quantitative study designs. The results concluded from meta-analysis were more precised.

3.1.3 Inclusion/Exclusion Criteria

Inclusion criteria were designed to screen the irrelevant references or articles with the information not sufficient to be included in the present study. Studies fulfilling the criteria such as study name, type of mosquito, microbiome, type of microbiome, pathways involved and genes involved were included.

All the articles with incomplete information were excluded. 52 articles were selected and the remaining articles were excluded because they were not fulfilling the inclusion criteria. Of the total 52 selected studies, titles, abstracts, and full text of the papers were read and it was observed that 38 were missing important points, therefore, removed. Studies that were removed from this review will be available on request.

3.1.4 Statistical Analysis

The data analyzed using SPSS software package. Statistical parameters like mean and standard deviation were determined for the data reporting microbiome and genes involved and types of microbiome and gene involved. Paired sample t test was used to access the association between variables. The Meta-Mar server (https://www.meta-mar.com) was used to perform mathematical analysis.

The effect sizes were calculated using the fixed-effect model and the random-effect model, and the forest plot was generated; correlation coefficients, risk ratios, uniform mean differences, weight effect, and the heterogeneity of the studies were also calculated.

3.2 Methodology of Genome Analysis

3.2.1 Retrieval of Mosquitoes and Microbial Resistant Genome and Mosquitoes Proteome

For analyzing the mosquitoes and microbial genomes, the genome of all the selected resistant mosquitoes and microbes was obtained from (https://www. ncbi. nlm. nih. gov/ genome/) for the retrieval of complete mosquitoes' genome. The complete genome of only five selected mosquitoes that were *Aedes aegypti*, *Anopheles gambiae, Anopheles sinensis, Aedes albopictus* and *Culex pipiens* was given in genome browser whereas the complete genome of 5 out of 13 selected microbial species was given that were *Plasmodium berghei, P.vivax, P.falciparum, Escherichia coli* and *Wolbachia spp.* was given. Proteome of all the selected mosquitoes was obtained from Uniprot (https://www.uniprot.org/proteomes/) for the retrieval of mosquitoes' protein sequences.

3.2.2 Analysis of Phylogeny and Taxonomy

Evolutionary development of species or organisms was studied in phylogenetics. The accession number of 5 selected mosquitoes was inserted into genome-to-genome distance calculator (GGDC2.0) (https://www.dsmz.de/services/online-tools/genome-to-genome-distance-calculator-ggdc) and tree only was selected to generate single tree of selected mosquito species.

3.2.3 Genome Comparison and Orthologs Identification

For genome comparison and identification of orthologs, the complete genome of five selected microbes that was obtained through NCBI genome browser (https: //www. ncbi . nlm. nih.gov /genome/) was visualized to see the gene architecture and gene repertoire of resistant mosquitoes and microbes. For this purpose the obtained genomes of all the selected microbe were analyzed in NCBI

Blastn(https://blast.ncbi.nlm.nih.gov/Blast.cgi? PROGRAM= blastn& BLAST-SPEC= GeoBlast&PAGETYPE = BlastSearch) using the FASTA format.

3.2.4 Pan Genome Analysis

The pan genome analysis was performed for microbial species using the microbializer online tool (https://microbializer.tau.ac.il/). The sequences of all the microbes in FASTA format were inserted into mirobializer and results were generated through email.

3.2.4.1 Identification of Key Resistant Genes

For identification of key resistant genes in microbial and mosquito's genome IS-Finder was a dedicated base using online platform of IS-finder (https://isfinder. biotoul. fr/ blast.php) insertion sequences. The genome of microbes and mosquitoes were visualized to find the common insertion sequences.

3.3 Docking

Using the Patch-dock online software (https://bioinfo3d.cs.tau.ac.il/PatchDock/), the structures of resistant genes were found. In this method, nucleotide sequences of 5 microbes were inserted to Patch-dock software and remaining microbes were neglected due to unavailability of their nucleotide sequences or genome sequences. The structure of proteins was visualized using the discovery studio (https://discover.3ds.com/discovery-studio-visualizer-download). The genes with having bumps in their structure were selected as resistant genes.

3.4 Overview of Methodology

The brief overview of methodology adopted is summarized in figure 3.1

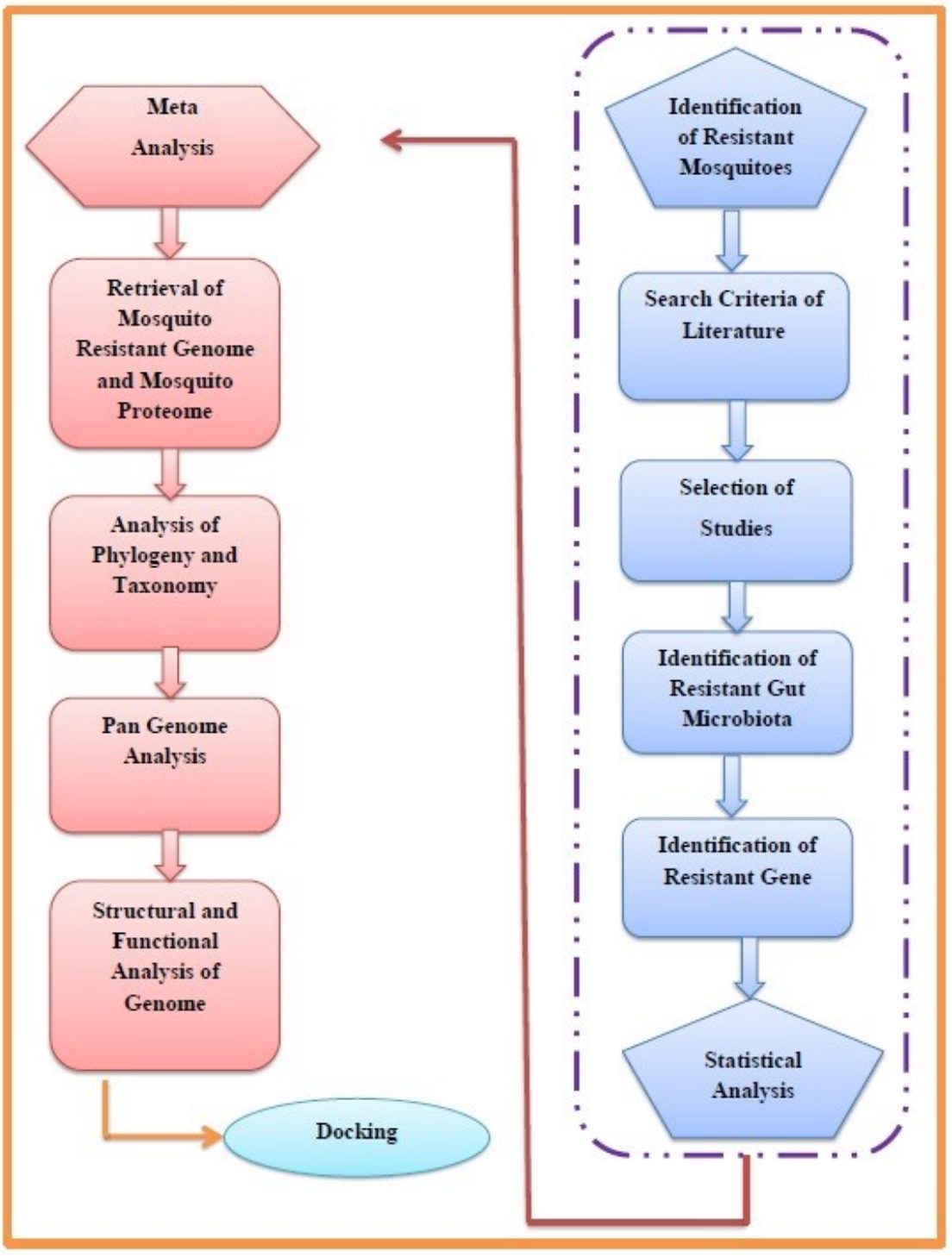

FIGURE 3.1: Methodological steps involved in resistome analysis of mosquitoes' gut microbiota.

Chapter 4

Results and Discussion

The exclusive use of insecticides and pesticides had now resulted in resistance among insects especially mosquitoes against these chemicals. This project was an effort to understood the impact and mechanism underlying the development of this resistance especially focusing on role of gut microbial species of mosquitoes. The results obtained were as follow

4.1 Identification of Insecticide Resistance

In order to determine the insecticide resistance properties in mosquitoes, the strategy of identification of resistance mosquito species to identification of resistant gut microbial species of mosquito to identification of genes in resistance gut microbial species in resistant mosquito was used. To execute this strategy, meta-analysis approach of data mining was exploited.

4.1.1 Selection of Articles

Search engines such as PubMed, Google Scholar, Elsevier and Science direct was used to identify the literature relevant to search topic, keywords used include "Insecticide resistance, Resistance in mosquitoes, Gut microbiota of mosquitoes,

Resistance of mosquitoes and many more. The PRISMA chart summarizing the selection process for studies included were depicted in fig 4.1.

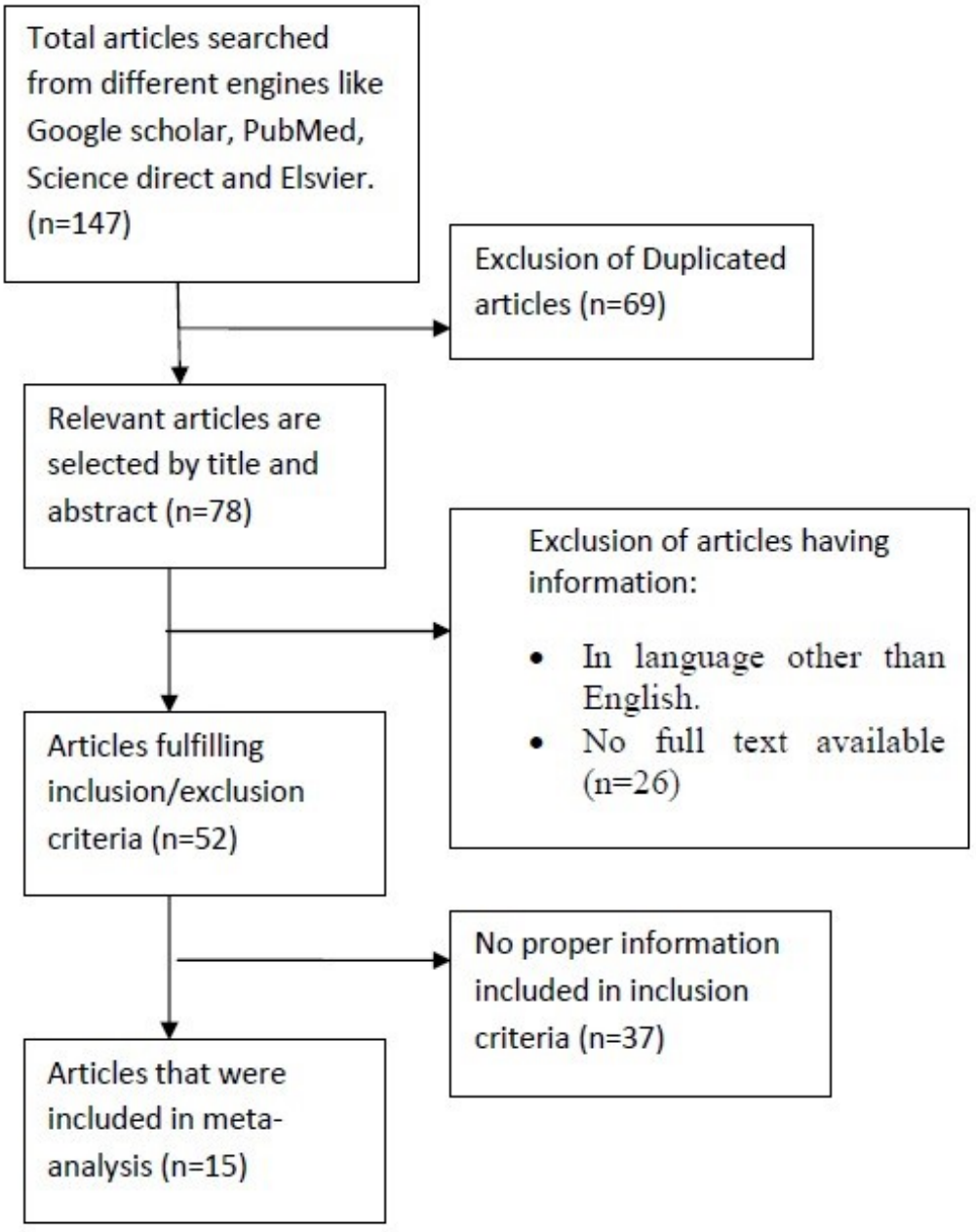

FIGURE 4.1: PRISMA Chart indicating the process of articles selected for metanalysis.

The search identified 147 articles were relevant to keywords. Duplication of articles from different sources was removed. The resultant 52 were screened based on titles and abstracts. Only relevant articles in English language with full text available were considered. The scrutinized 15 articles were thoroughly read and information

required was organized in table 4.1. This table was used as a source table for further data extraction.

TABLE 4.1: Resource table based on articles selected for metanalysis:

Study Name	**Type of Mosquito**	**Micro-biome**	**Type of Micro-biome**	**Pathways**	**Gene Involved**	**Ref.**
Dong et al; 2015		*Plasmodium falciparum*	parasite	IMD pathway	PRRs, FBN9, TEP1, APL1, LRIM1, LRRD7	[1]
	Anopheles gambiae	*SFV*	Virus	Toll Pathway	MyD88 protein, Rel 1, effector gene.	
Seitz et al. 1987	*Anopheles gambiae*	*Plasmodium berghei, Plasmodium falciparum*	parasite			[2]
	Anopheles aquasalis	*Plasmodium vivax*	parasite	Jack-stat pathway	Stat-A , STAT-B	
Jadin et al; 1967	*Anopheles stephensi*	*Pseudomonas*	bacterial species	IMD pathway	PGRPLB protein	[12]
	Anopheles gambiae	*Asaia*	bacterial species		.	
Walker et al; 2006		*Plasmodium falciparum*	parasite	immune signaling pathways	Peptid-oglycan reco-	[14]

					gnition proteins (PGRPs)	
		Wickerh-amomyc esanomalus	Yeast			
Hughes et al; 2011	*Anopheles stephensi*	*Plasmodium falciparum*	parasite	IMD pathway	Nos	[33]
Meister et al., 2009	*Anopheles gambiae*	*Wolbachia spp.*	bacterial species	IMD pathway	NF-kB family, REL2 and of PGRPLC receptor	[20]
Coon et al., 2017	*Aedes aegypti*	*Escherichia coli*	bacterial species	Mitogen activated kinases pathway (MAPK)		[21]
	Anopheles gambiae,	*Asaia*	bacterial species		hypoxia-inducible transc-ription factors (HIFs), insulin/ insulin growth factor	
Garver	*Anopheles*	*Serratia Y1*	bacterial	Toll and	AMP	[24]

et al., 2006	*sinensis*	*and J1*	species	Imd pathways	genes	
Blumberg et al., 2005	*Anopheles gambiae*	*Plasmodium falciparum*	parasite	IMD pathway	(TEP1, REL2, LRRD7, FBN9, defensin 1, and cecropin 1)	[34]
Paradkar et al; 2012	*Culex pipiens*	*Wolbachia spp.*	bacterial species	small interfering RNA (or RNAi) pathway	Vago	[37]
Werren et al; 1997	*Aedes aegypti*	*Wolbachia spp.*	bacterial species	microRNA (miRNA) pathways	AaDnmt2	[39]
Jousset et al; 1967	*Aedes aegypti*	*SINV*	arboviruses	ERK pathway	Sos (AAEL-001165)	[41]
Chen et al; 1997	*Aedes albopictus*	*Aeromonas taiwanensis, Wolbachia spp.*	bacterial species	iMD pathway, toll pathways	antimicrobial peptides	[42]
Bahia et al; 2009	*Anopheles aquasalis*	*Plasmodium vivax*	parasite	JAK-STAT pathway	SOD3A, SOD3B	[55]
Chandel	*Culex pipiens*	*Wolbachia*	bacterial species	jak-STAT	HOP, REL1,	[44]

et al; 2011		pathway, toll , RNAi, TEP1	DCR2, TEP1

4.1.2 Identification of Resistant Mosquito Species

The literature reviewed was summarized in table 4.1 and the data related to resistance species of mosquitoes was extracted. Total seven species of mosquitoes were observed. These species were reported from various regions of Globe including China, Europe, Africa and America. Table 4.2 summarizes the data obtained with relevance to mosquito species, the geographical area, disease association and resistance it imparts against commonly used insecticides. It was observed that most of the mosquitoes show resistance against DDT and pyrethroid which were most commonly used insecticides in agricultural pesticides.

Table 4.2: Insecticide resistance mosquito species

Sr No.	**Type of Mosquito**	**Geographical Area**	**Disease Caused**	**Resistance Against Insecticides.**	**References**
1	*Anopheles gambiae*	Sub-Saharan Africa, Asia, Africa	Malaria	Deltamethrin, Permethrin fenitrothion	Dennis et al; 2015 Waldo et al; 2012 Carrisi et al; 2015 Gupta et al; 2009 Akira et al; 2006.

					Dong et al; 2009. Takuchei et al; 2006. Uematsu et al; 2006. Dennison et al; 2015. Meister et al; 2005.
2	*Aedes aegypti*	Pennsylvania, Shanghai, China.	Dengue	Pyrethriod and organo-phosphates.	Walker et al; 2014. Bangi et al; 2011. Vogel et al;2017.
3	*Anopheles stephensi*	Paris, France	Malaria	DDT and Malathion	Jadinet et al; 1967 Luckhart et al; 2007.
4	*Anopheles aquasalis*	USA			Bahia et al; 2009. Jadinet et al; 1967
5	*Anopheles sinensis*	China	Malaria	Pyrethroid	Luna et al., 2006. Vogel et al; 2017.
6	*Culex pipiens*	U.S,	Encephalitis,	Pyrethriods	Walker et al; 2012.

		New York	Filariasis	and	Hoffmann et al; 2011.
			Avian	malaria	
7	*Aedes albopictus*	USA, Pacificisland of Maui, Hawai	Dengue virus	DDT	Vogel et al., 2017. Valzania et al., 2018

4.1.3 Microbial Diversity Among Resistant Mosquitoes

Mosquito develop resistance against insecticides using various mechanisms but, in this project, focus was to understand, how gut microbial species contribute in this resistance. For this purpose, gut microbial species present in all seven mosquitos were catalogued in table 4.3.

Table 4.3: Microbial diversity amongst resistant mosquitoes

Sr. No.	**Type of Mosquito**	**Microbiome**	**Type of Microbiome**	**References**
1	*Anopheles gambiae*	*Plasmodium falciparum*	Parasite	Dennison et al; 2015. Dong et al; 2009 Gupta et al; 2006 Waldock et al; 2012.
		Plasmodium berghei	Parasite	Gupta et al; 2009.
		Asaia	bacterial species	Akira et al; 2006. Meister et al; 2005.
		Wolbachia	bacterial species	Dennis et al; 2015.

		Wickerh-amomyces anomalus	Yeast	Akira et al; 2006.
		SFV	Virus	Luna et al; 2006.
2	*Aedes aegypti*	*Escheri-chia coli*	bacterial species	Vogel et al; 2017.
		Wolbachia	bacterial species	Asgari et al; 2012.
		SINV	Virus	Downe et al; 1993.
3	*Anopheles stephensi*	*Pseudomonas*	Parasite	Jading et al; 1967.
		Plasmodium falciparum	Parasite	Luckhart et al; 2007.
4	*Anopheles aquasalis*	*Plasmodium vivax*	Parasite	Bahia et al; 2009.
				Vogel et al; 2017.
5	*Anopheles sinensis*	*Serratia Y1 and J1*	bacterial species	Luna et al; 2006.
6	*Culex pipiens*	*Wolbachia*	bacterial species	Adelman et al; 2001.
				Bian et al; 2010.
7	*Aedes albopictus*	*Aeromonas taiwanensis*	bacterial species	Pang et al; 2016.
		Wolbachia	bacterial species	Zhang et al; 2016.

Gut microbiota of mosquito comprise various organisms including bacteria, viruses and parasites etc.

Mostly parasites were found in gut due to the fact that mosquito is a major biological vector in transmission of parasites from one host to the other.

4.2 Identification of Resistant Genes

Gut microbiota of mosquito play a significant role in insecticide resistance owing the genes they possess. Table 4.4 enlists major resistant genes in microbial species of resistant mosquitoes.

TABLE 4.4: List of Insecticide resistant genes in Gut microbiota of mosquitoes

Type of Mosquito	**Microbiome**	**Pathways**	**Gene Involved**	**References**
Anopheles gambiae	*Plasmodium*	IMD	PRRs,	Dong et al; 2006.
	falciparum	pathway	FBN9, TEP1, APL1, LRIM1, LRRD7	Dennison et al; 2015 Walker et al; 2011 Mesister et al; 2009
	Plasmodi-umberghei	Jack-stat pathway	Stat-A, Stat-B	Gupta et al; 2009.
	Asaia	Immune signaling pathway	Peptid-oglycan reco-gnition proteins (PGRPs)	Akiraet al; 2006. Dziarski et al; 2006.
	Wolbachia	IMD Pathway	NF-kB family, REL2 and of PGRPLC receptor	Dong et al; 2009.
	Wickerha-	Immune	Peptid-	Cappelli et al; 2019.

	momyc esanomalus	signaling pathways	oglycan reco-gnition proteins (PGRPs)	
	SFV	Toll pathway	MyD88 protein, Rel 1, effector gene.	Waldock et al; 2012
Aedes aegypti	*Escherichia coli*	Mitogen activated kinases pathway (MAPK)	Hypoxia-inducible transcri-ption factors (HIFs), insulin/ insulin growth factor.	Vogel et al; 2017.
	Wolbachia	micro-RNA (miRNA) pathway.	AaDnmt2	Zhang et al 2013.
	SINV	ERK pathway.	Sos(AAE-L001165)	Jackson et al; 1993.
Anopheles stephensi	*Pseud-omonas*	IMD pathway	PGRPLB protein	Gendrin et al; 2017.
	Plasmodium	IMD	NOS	Luckhart et al; 2007.

	falciparum	pathway		
Anopheles aquasalis	*Plasmodium*	Jack-stat	Stat-A,	Bahia et al; 2009.
	vivax	pathway	Stat-B, SOD3A, SOD3B.	Kang et al; 2005
Anopheles sinensis	*Serratia*	Toll	AMP	Luna et al; 2006.
	Y1and J1	and IMD pathway.	genes	Waldo et al; 2012
Culex pipiens	*Wolbachia*	Small	Vago,	Stevenet al; 2015.
		interf- ering RNA (RNAi pathway)	HOP, REL1, DCR2, TEP1.	
		Jack-stat pathway, Toll pathway and TEP1.		Hoffmannet al; 2011.
Aedes albopictus	*Aeromonas*	IMD	Antimic-	Pang et al; 2016.
	taiwanensis	and Toll pathway.	robial peptides (AMP).	
	Wolbachia	IMD and Toll pathway.	Antimic- robial peptides	Zhang et al; 2016.

4.3 Statistical Analysis for Number of Microbes and Number of Genes

Data collected was analyzed based on various statistical parameters including mean and standard deviation. For statistical analysis SPSS statistics (https://www. ibm. com/ products/ spss- statistics) was used. For mathematical analysis Meta-Mar server (https://www.meta-mar.com/) was used.

The effect sizes were calculated using fixed effect model and random effect model. Statistical values were summarized in table 4.5, where 'n' refers to number of articles reporting the presence of particular mosquitoes. Mean1 shows the mean value of microbes. Mean was defined statistically as the average value or most common value. It was calculated by dividing total number of microbes reported in particular mosquito species with total number of articles.

SD1 was the standard deviation of microbes. Standard deviation measures the amount of variation of dataset relative to its mean. It was calculated as square root of variance by determining deviation of each data part relative to the mean.

Table 4.5: Statistical analysis for number of microbes

Sr No.	**Types of Mosquitos**	**Number of Articles**	**Number of Microbes**	**Mean M1**	**Standard Deviation SD1**
1	*Anopheles gambiae*	10	35	3.5	3.659
2	*Aedes aegypti*	3	18	6.0	1.000
3	*Anopheles stephensi*	2	9	4.5	4.950
4	*Anopheles aquasalis*	2	18	9.0	0.000
5	*Anopheles sinensis*	2	21	10.5	0.707

6	*Culex pipiens*	2	10	5.0	0.000
7	*Aedes albopictus*	2	17	8.5	4.950

Similarly, table 4.6 summarized statistical values for number of genes in each microbial species. 'n' indicates the number of articles involved. M2 indicates the mean value of gene calculated by dividing total number of genes with the total number of articles. SD2 shows the standard deviation.

TABLE 4.6: Statistical analysis for number of genes

Sr No.	**Types of Mosquitoes**	**Number of Articles**	**Number of Genes**	**Mean M2**	**Standard Deviation SD2**
1	*Anopheles gambiae*	10	28	2.80	1.874
2	*Aedes aegypti*	3	4	1.33	0.577
3	*Anopheles stephensi*	2	2	1.00	0.001
4	*Anopheles aquasalis*	2	4	2.00	0.001
5	*Anopheles sinensis*	2	2	1.00	0.001
6	*Culex pipiens*	2	5	2.50	2.121
7	*Aedes albopictus*	2	2	1.00	0.001

Standardized mean difference i.e., SMD was calculated due to fact that each author was selected articles, expressed or depicted these results differently; similarly,

a different technique was used in each article to determine the diversity among microbial species and number of genes. The same concept was also referred as 'effect size' or Hedges (adjusted g).

It was calculated by dividing difference in mean outcome between groups, with standard deviations of outcomes among participants. The negative 'g' values indicate the difference of microbes in each mosquito species. Standard deviation was summarized in table 4.7.

TABLE 4.7: Metanalysis

Types of Mosquitoes	**Number of Microbes**			**Number of Genes**			**Combined Statistics for Selected Species**			
	N	**Mean 1**	**SD 1**	**N 2**	**Mean 2**	**SD 2**	**g**	**SEg**	**weight (%) - fixed model**	**weight (%) - random model**
Anop- *heles gambiae*	10	3.5	3.659	10	2.80	1.874	-0.230631	0.429867	42.017689	23.230233
Aedes aegypti	3	6.0	1.000	3	1.33	0.577	-4.576341	1.473739	3.574856	11.855786
Anop- heles stephenei	2	4.5	4.950	2	1.00	0.001	-0.571399	0.606088	21.136273	21.348564
Anop- heles aquasalis	2	9.0	0.000	2	2.00	0.001	-565 6.854249	2000 .000082	0.000002	0.000012
Anop- heles sinensis	2	10.5	0.707	2	1.00	0.001	-10.858772	3.881449	0.515362	2.838281
Culex pipiens	2	5.0	0.000	2	2.50	2.121	-0.952525	0.663282	17.648303	20.682323

Aedes albopictus	2	8.5	4.950	2	1.00	0.001	-1.224427	0.716891	15.107516	20.044802

SEg values were standardized g value. SEg values were calculated as standardized g values and represent the same things. In ANOVA and Regression models, the term fixed effect and random effect models were used and applied to various types of statistical model.

Random models show that the 'g' value derived from wider population, whereas the fixed model was supposed to be measures without any error. Both fixed and random models were considered to be measured of 'g' in one analysis were often believed to be same as that of 'g' in another study.

M1 and M2 were random mean differences (RMD) which were effective if the values were calculated as same parameters or the observations were made using same tools or methods. As the calculations or observations were made in each article, related the number of microbes and genes was different Hedge's 'G' value i.e., standardized or normalized mean difference was calculated.

In this regard standard deviation (SD) was used to calculate SMD or G. Standard error for G 'SEG' was calculated which was estimated by samples SD divided by number of samples. Table 4.8 summarizes the effect size calculation for number of microbes measured using both the fixed, random effect model and genes.

TABLE 4.8: Summary of the effect size for number of microbes measured using both the fixed effect and random effect model

	Hedges'g (SMD)	**SEg**	**95% CI**	**z score**	**p value**	**Hetero-geneity**
Fixed Effect Model	-0.79	0.279	[-1.337, -0.244]	2.837	0.004558	I2=74.6%, Chi2=23.58, df=6

Random Effect Model	-1.47	0.694	[-2.829, -0.11]	2.118	0.034 139	74.6%, Tau2= 1.886

Table 4.8 describes effect size of fixed and random effect models and heterogeneity measurement values. In this meta-analysis study, all the number of studies reporting the mosquito species had same result in different forms that's why the standardized mean difference (SMD) Hedges was used as a summary statistic.

The negative SMD values (Table 4.8) of both the fixed and random effect models display the negligible degree of publishing bias that makes the results significant. Standardization of results of the studies becomes compulsory so they could be combined on a uniform scale. Standardization of results of the studies becomes compulsory so they could be combined on a uniform scale.

In each study, the SMD relative to the variable was used to express the size of the intervention effect. In Heterogeneity the experimental degree results that were compatible for all the selected mosquitoes were compatible. If the CI had a low association with the studies, the CI was also defined, suggesting that substantial heterogeneity occurred (Table 4.8).

The heterogeneity in Table 4.8 was calculated to assess the degree to which the findings of all the studies were consistent. The confidence interval (CI) that was mainly used to find correlations among studies was also calculated for all the studies. A significant heterogeneity was presented by Cl having a low correlation values with tests.

Due to various problems in statistically significant values P value of 0.0 was used instead of 0.05A whereas non-significant result should not be taken as evidence of heterogeneity. Using fixed effects and random effect models the threshold value for I2 was obtained as 95% which indicated significant heterogeneity (Table 4.8) among the selected studies. The Meta-Regression results were shown in Table 4.9 and 4.10 respectively.

TABLE 4.9: Calculation of publication bias in selected studies

	Rosenthal (1979)	**Rosenberg (2005)**
	tc(α = 0.05, df = 7) = 1.895	**Zc(α = 0.05) = 1.645**
Fail-N Safe	58.91	8.69

The higher values of both Rosenthal (1979) and Rosenberg (2005) observed in table 4.9. It was confirmed that all the selected studies showed minimum levels of publication bias. The legitimacy of a meta-analysis was perhaps threatened by Publication bias and considered as a challenge to all studies seeking to use the published literature. There was a publishing bias since research was more likely to be submitted and reported with statistically meaningful findings than work with null or non-significant results. Risk of higher bias in publications occurs if this statistic was comparatively minimal. Due to higher number in studies the risk of publication bias was quite minimal not zero.

TABLE 4.10: The meta-regression analysis of all the studies selected for meta-analysis

OLS Regression Model	
Dep. Variable:	G
Model:	OLS
Method:	Least Squares
Log-Likelihood:	-63.063
No. Observations:	7

The meta-regression analysis in Table 4.10 also confirmed significant variability among studies and to predict the behavior of g (dependent variables) the least square method was used, as a result the negative likelihood value (-63.063), thus demonstrating the least chances of similarity in all the studies. The effect size (publication bias) was tested using the Rosenthal (1979) and Rosenberg's Fail-N Safe (2005) file drawer method and shown in Table 4.9.

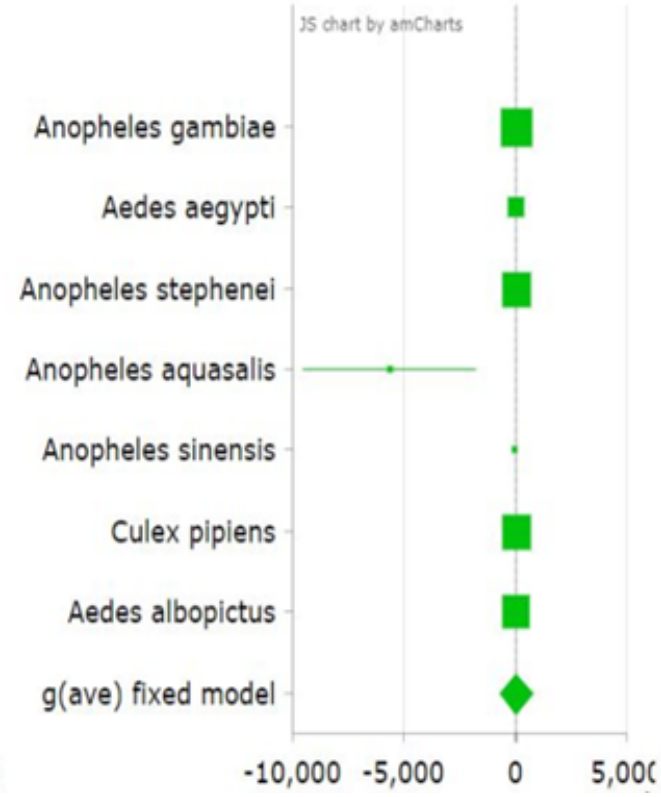

FIGURE 4.2: Forest-Plot for number of microbes.

A forest plot was a graph that compares several clinical or scientific studies studying the sample names and values. The diamond at the bottom of the forest plot shows the result when all the individual studies were combined together and averaged. The squares represent the effect estimate of the individual studies and the the confidence interval horizontal lines indicate; the dimension of the square reflects the weight of each study. In Fig 4.2 all the squares were on the vertical line that shows that all the studies had same level of variations. The heterogeneity was 95%CI having *Anopheles gambiae* -0.231, *Aedes aegypti* -4.576, *Anopheles stephenei* -0.571, *Anopheles aquasalis* -5,656.854 *Anopheles sinensis* -10.859, *Culex pipiens* -0.953 and *Aedes albopictusg* (ave) value -1.224. High variation was observed in *Anopheles aquasalis* that's why study was not linked with the remaining all selected studies which were almost similar. The percentage of heterogeneity was also due to great variation of *A aquasalis*.

There were so many reported mosquitoes but the mosquitoes having almost similar gut microbiota were selected for study. Six out of seven selected mosquito species

show same level of variations in gut microbiota thus achieving our first objective of "Identification of insecticide resistance gut microbiota species in resistance mosquitoes".

4.4 Statistical Analysis for Diversity of Microbes and Genes

Data collected for diversity of microbes was analyzed by various statistical parameters including mean and standard deviation. For statistical analysis SPSS statistics (https://www.ibm.com/products/spss-statistics) was used. For mathematical analysis Meta-Mar server (https://www.meta-mar.com/) was used. The effect sizes diversity of microbes was calculated using fixed effect model and random effect model.

Statistical values were summarized in table 4.5, where 'n' refers to number of articles reporting the presence of particular mosquitoes. Mean1 shows the mean value of diversity of microbes. Mean was defined statistically as the average value or most common value. It was calculated by dividing total diversity of microbes reported in particular mosquito species with total number of articles. SD1 was the standard deviation of diversity of microbes. Standard deviation measures the amount of variation or dispersion of dataset relative to its mean. It was calculated as square root of variance by determining deviation of each data part relative to the mean.

Table 4.11: Statistical analysis for types of microbes

Sr No.	**Types of Mosquitos**	**Number of Articles**	**Number of Microbes**	**Mean M1**	**Standard Deviation SD1**
1	*Anopheles gambiae*	10	21	2.10	1.370
2	*Aedes aegypti*	3	10	3.33	0.577

3	*Anopheles stephensi*	2	4	2.00	1.414
4	*Anopheles aquasalis*	2	2	1.00	0.001
5	*Anopheles sinensis*	2	6	3.00	0.000
6	*Culex pipiens*	2	6	3.00	0.001
7	*Aedes albopictus*	2	6	3.00	0.001

Similarly, table 4.11 summarized statistical values for types of genes in each microbial species. 'n' indicates the number of articles involved. M2 indicates the mean value of gene calculated by dividing total number of genes with the total number of articles. SD2 shows the standard deviation.

TABLE 4.12: Statistical analysis for types of genes

Sr No.	**Types of Mosquitos**	**Number of articles**	**Number of genes**	**Mean M2**	**Standard Deviation SD2**
1	*Anopheles gambiae*	10	28	2.80	1.874
2	*Aedes aegypti*	3	4	1.33	0.577
3	*Anopheles stephensi*	2	2	1.00	0.001
4	*Anopheles aquasalis*	2	4	2.00	0.001
5	*Anopheles sinensis*	2	2	1.00	0.001
6	*Culex pipiens*	2	5	2.50	2.121

7	*Aedes albopictus*	2	2	1.00	0.001

Standardized mean difference i.e., SMD was calculated due to fact that each author was selected articles, expressed or depicted these results differently; similarly, a different technique was used in each article to determine the diversity among types of microbial species and types of genes. The same concept was also referred as 'effect size' or Hedges (adjusted g). It was calculated by dividing difference in mean outcome between groups, with standard deviations of outcomes among participants. The negative 'g' values indicate the difference of types of microbes in each mosquito species. Standard deviation was summarized in table 4.12.

TABLE 4.13: Meta-analysis of diversity of microbes

Type of Mosquitoes	**Types of Microbes**			**Types of genes**			**Combined Statistics for Selected Species**			
	n 1	**Mean1**	**SD 1**	**n 2**	**Mean 2**	**SD 2**	**G**	**SEg**	**weight (%)-fixed model**	**weight (%)-random model**
Anopheles gambiae	10	2.10	1.370	10	2.80	1.874	0.408431	0.433158	44.380321	26.984187
Aedes aegypti	3	3.33	0.577	3	1.33	0.577	-2.772964	1.033172	7.800789	21.472808
Anopheles stephenei	2	2.00	1.414	2	1.00	0.001	-0.571515	0.606102	22.666925	25.639808
Anopheles aqualis	2	1.00	0.001	2	2.00	0.001	571.428571	202.031317	0.000204	0.002266

Anopheles sinensis	2	3.00	0.000	2	1.00	0.001	-1616.244071	571.428857	0.000026	0.000283
Culex pipiens	2	3.00	0.001	2	2.50	2.121	-0.190505	0.575384	25.151711	25.900365
Aedes albopictus	2	3.00	0.001	2	1.00	0.000	-1616.244071	571.428857	0.000026	0.000283

SEg values were standardized g value represent the same things in microbial diversity of mosquitoes. In ANOVA and Regression models, the terms that were generally applied to statistical models were fixed and random models. Random models show the derivation of 'g' value from wider population, whereas the fixed model was assumed to be measures the statistical values without any error. The measurements of 'g' value in both fixed and random models were often considered to be same as that of 'g' in another study.

M1 and M2 were random mean differences (RMD) which were effective if the values were calculated as same parameters or the observations were made using same tools or methods. As the calculations or observations were made in each article, related the number of microbes and genes was different Hedge's 'G' value i.e., standardized or normalized mean difference was calculated. Table 4.14 summarizes the effect size calculation for number of microbes and genes. It calculated P value.

TABLE 4.14: Summary of the effect size for number of microbes measured using both the fixed effect and random effect model

	Hedges'g (SMD)	**SEg**	**95% CI**	**z score**	**p value**	**Heterogeneity**
Fixed Effect Model	-0.21	0.289	[-0.778, 0.353]	0.735	0.462183	I2=81.6%, Chi2=32.55, df=6

Random Effect Model	-0.68	0.962	[-2.562, 1.208]	0.704	0.481284	81.6%, Tau2=3.24

The measurement of heterogeneity and the effect size of fixed and random models were described in Table 4.14. In different forms, all the number of studies reporting the mosquito species had same result for this meta-analysis study, so the standardized mean difference (SMD) Hedges 'was used as a summary statistic. The results were meaningful because of reason that the negative SMD values (Table 4.14) of both the fixed and random effect models display the minimal degree of publishing bias. For merging the results on uniform scale, the standardization of results of the studies became mandatory. In each study, the SMD relative to the variable was used to express the size of the intervention effect. The threshold value for I2 was obtained as 81.6% using fixed effects and random effect models, indicated significant heterogeneity (Table 4.14) among the selected studies. The Meta-Regression Results were shown in Table 4.15 and 4.16 respectively.

TABLE 4.15: Calculation of publication bias in selected studies

	Rosenthal (1979)	**Rosenberg (2005)**
	tc ($\alpha = 0.05$, df = 7) = 1.895	Zc($\alpha = 0.05$) = 1.645
Fail-N Safe	5.62	-5.95

TABLE 4.16: The meta-regression analysis of the studies for meta-analysis

OLS Regression Results	
Dep. Variable:	G
Model:	OLS
Method:	Least Squares
Log-Likelihood:	-56.769
No. of Observations:	7

The meta-regression analysis in Table 4.16 also confirmed significant variability among studies. In all the studies the least chances of similarity can be demonstrated by the least square method that was used to predict the behavior of g (dependent variables) and the negative likelihood value (-56.769). Using the Rosenthal (1979) and Rosenberg's Fail-N Safe (2005) the effect size (publication bias) was tested using file drawer method and shown in Table 4.15.

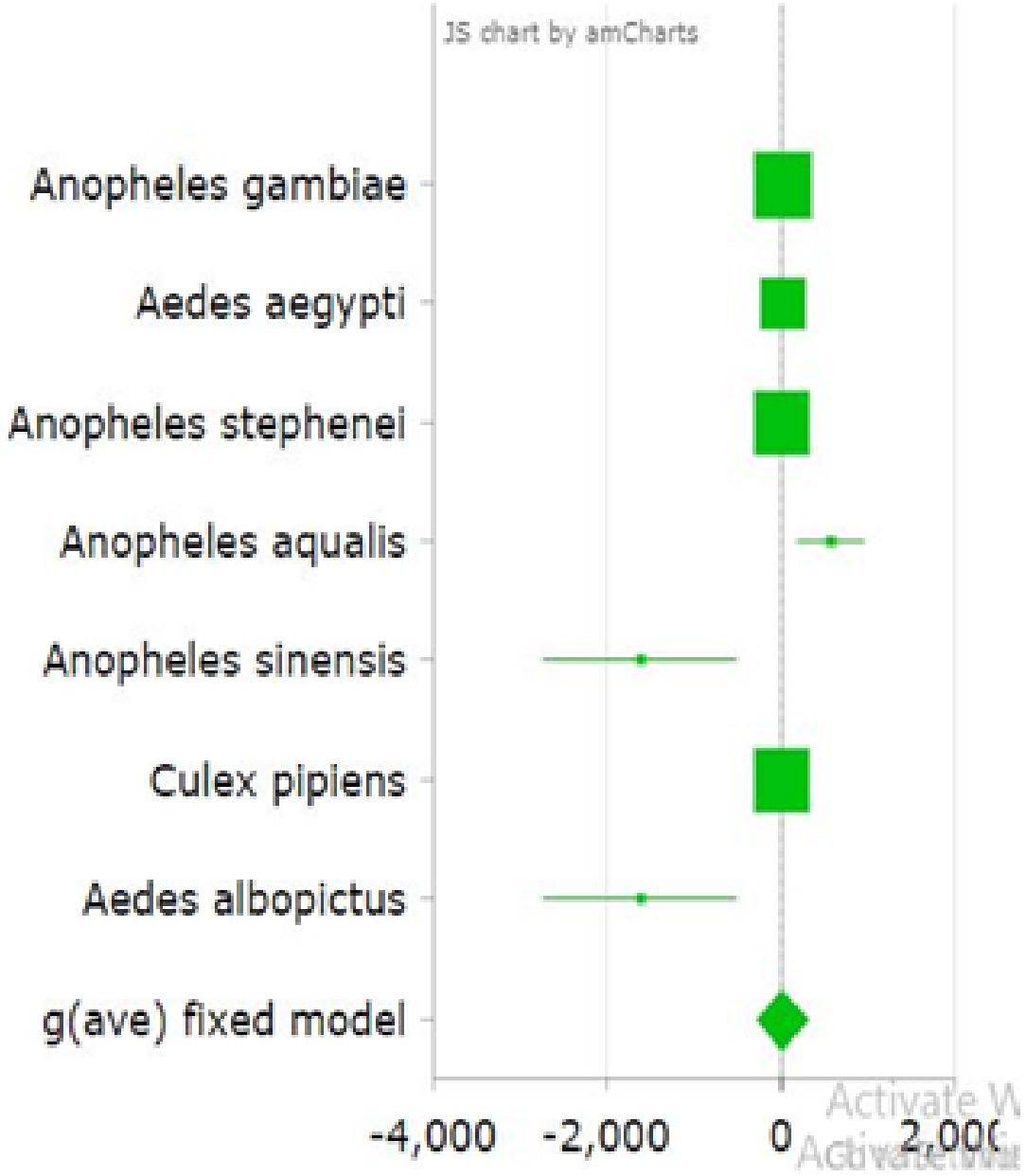

FIGURE 4.3: Forest-Plot for types of microbes.

A forest plot was a graph that compares several clinical or scientific studies studying the diversity of microbiota in selected mosquitoes. The diamond at the bottom

of the forest plot shows the result when all the individual studies were combined together and averaged. The squares represent the effect estimate of the individual studies and the horizontal lines indicate the confidence interval; the dimension of the square reflects the weight of each study. In Fig 4.3 all the squares were on the vertical line representing *Anopheles gambiae, Aedes aegypti, A.stephensi* and *Culex pipiens* shows that all these studies had same level of variations. The heterogeneity was 95% CI having *A.gambiae* 0, *Aedes aegypti* 0.408, *A.stephenei* -0.572, *A.aquasalis* 571.429, A.sinensis -1616.244, *Culex pipiens* -0.191 and *Aedes albopictus* g(ave) value -1616.244. High variation or poor level of variations were observed in *A.albopictus* and *A.sinensis* whereas *A.aquasalis* was significantly similar that's why these studies were not linked with the remaining all selected studies which were almost similar. The percentage of heterogeneity was also due to great variation of these three species.

In seven reported mosquitoes there was diversity among the gut microbiota. Four out of seven selected mosquito species show same level of variations but the other three species had high level of variations.

4.5 Genome Analysis of Insecticide Resistance

For Genome analysis, the microbial species were analyzed for complete genome sequences. From the 13 found resistant microbial species, the species having complete genome were selected. Five resistant microbial species had complete genomes were selected for further analysis. Genome analysis was done for determining the genome size and identification of genomic features.

4.6 Phylogeny and Taxonomy

To study the evolutionary relationship among the organism's phylogenetic analysis can be done whereas taxonomy was used for naming and classification of

organisms into groups and sub-groups. Relationships among broad groups of organisms especially in reference to lines of descent, the history of the evolution of a species or group was known as Phylogeny. Most phylogenies were based on indirect evidence and therefore were hypothesis. Using the same evidence different phylogenies often emerge. The input nucleotide matrix comprised 16 operational taxonomic units and 1585 characters, 717 of which were variable and 374 of which were parsimony-informative. The base-frequency check indicated a compositional bias (p = 0.00, $\alpha = 0.05$).

ML analysis under the GTR+GAMMA model yielded a highest log likelihood of -6023.64, whereas the estimated alpha parameter was 1.88. The ML bootstrapping did not converge; hence 1000 replicates were conducted; the average support was 68.00%. MP analysis yielded a best score of 1060 (consistency index 0.87, retention index 0.80) and 2 best trees. The MP bootstrapping average support was 89.38%.

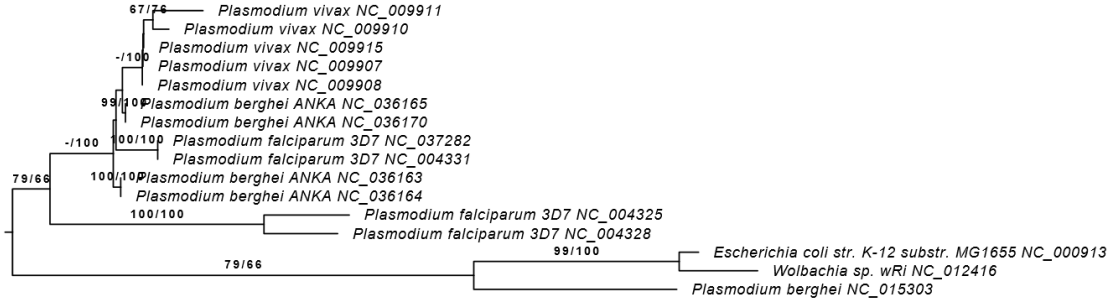

FIGURE 4.4: Phylogenetic analysis of microbes.

Fig 4.4 showed that the *Plasmodium vivax* NC_ 009911 and NC_ 009910 were closely related whereas the *P.vivax* NC_ 009915, NC_ 009907, NC_ 009908 was differently related to former ones. *P.berghei* ANKA NC_ 036165, NC-036170, NC_ 036163, NC_ 036164 sequences and *P.falciparum* 3D7 NC_ 037282, NC_ 004331 were more closely related to *P.vivax* as compared to *P.falciparum* 3D7 NC_ 004325, NC_ 004328. The other microbial species belongs to totally different branch, like *Escherichia coli* and *Wolbachia* were closely related which means that they had common origin. The *P.berghei* NC_ 015303 was also related to their branch which means that *P.berghei* had some association with *E.coli* and *Wolbachia*.

4.6.1 Genome Comparison and Orthologs Identification

The general purpose nucleotide search and alignment program was "blastn" program that can be used to align genomic DNA sequences, tRNA or rRNA sequences as well as mRNA. By speciation the evolution of genes in different species from a common ancestral gene were known as orthologs that retains the same function during the course of evolution. In newly sequenced genomes for reliable prediction of gene function identification of orthologs was a critical process. With reference to genome or gene structures the similar orthologs were found in different organisms. In case microbial genome, gene or protein structure was not predicted then the same orthologs having the predicted gene was used as a model genome for predicting the structure of our required microbial protein sequences.

Using the BLASTN-based software (https://blast.ncbi.nlm.nih.gov/Blast.cgiPAGE_TYPE) whole genome comparison was performed and visualized to show gene repertoire and architecture of resistant genomes of selected mosquitoes. Whole genome comparisons were performed to identify the core and dispensable genomes among the different mosquitoes.

The orthologs of selected mosquitos' species was found using Blastn. By speciation, from a common ancestral gene in different species had evolved were Orthologs and, in general, during the course of evolution orthologs retain the same function. In newly sequenced genomes, identification of orthologs was a critical process for reliable prediction of gene function.

4.6.1.1 Inclusion/ Exclusion Criteria

From the 13 selected microbes only 5 were analyzed for orthologs using the blastn because the remaining 8 microbes don't had any given genome sequences. The microbial species having complete genome were analyzed. Blastn was performed on the five microbes whose full genome was available. The Blast e-value was the expected hits number of similar score that could occur just by chance. The E-value of *Plasmodium bergei*, *P.falciparum*, *Escherichia coli* and *Wolbachia* sp. was 0.0,

which means that all these results were highly significant but the values of *P.vivax* were 9e-07, 4e-05, 0.006, 0.008 and 1e-04 respectively. The ortholog of selected microbes is shown in table 2 (ref to Appendix).

4.7 Pan Genome Analysis

Pan genome analysis was performed using the microbializer tool to identify number of ORF in all selected microbes. ORF was portion of DNA which start with start codon and had no stop codons and can be translated into amino acids. Some ORF had known genes whereas others had unknown genes. There was a chance that unknown genes or uncharacterized genes were causing resistance.

Pangenome is a broad term that basically constitutes all the genes found in analyzed dataset. It further divided into core genome and variable genome respectively. The core genome constitutes the set of homologous genes in analyzed dataset whereas the variable genome constitutes the gene families shared by two or more organisms. The detailed results were shown in figure 4.5.

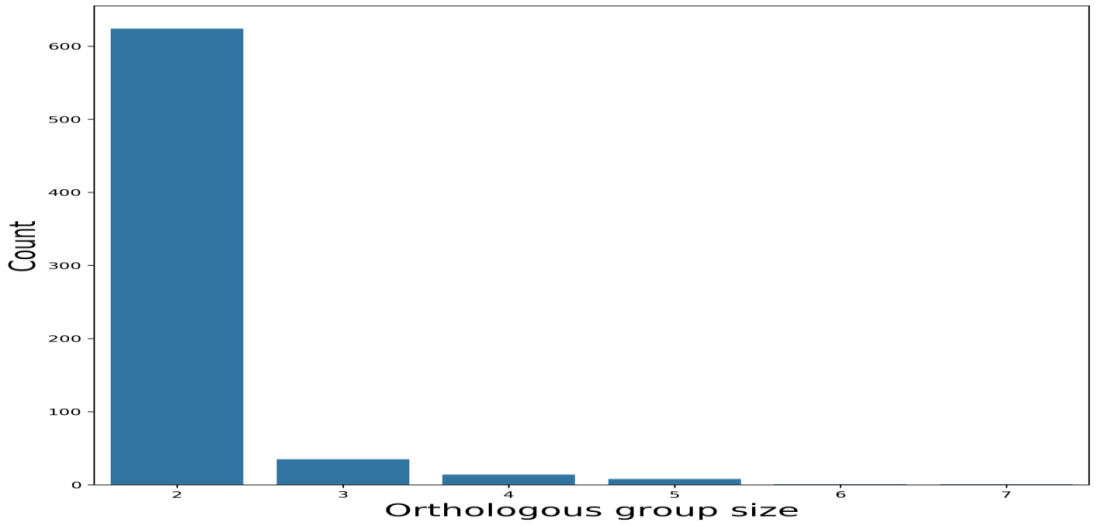

FIGURE 4.5: Ortholog groups.

Sequence 2 shows the maximum number of orthologs that were above 600 and sequence 7 shows the minimum number of ortholog groups having number approximately equal to zero. As shown in figure many genes were shared by all

the selected microbes that's why the line on the left side count above 600 but when closely related genomes were analyzed the count number was very low and upto seventh group it approximately reaches to zero. The reason behind such a lower number was that we had variety of microbial species containing bacteria and different parasites.

4.7.1 Structural and Functional Analysis of Genome

For gene prediction and functional annotation of bacterial genome NCBI prokaryotic genome annotation pipeline was applied. Using online platform of IS finder Insertion sequences and transposases were detected. Insertion sequences were found in mosquitoes and microbial genomes. Insertion sequences were basically the sequences of genome that were inserted from the microbial genomes to mosquito genome.

Insertion sequences were small pieces of DNA that had ability to jump from one part of DNA to the other [147]. When insecticides were given to the insect/-mosquitoes they engulf that insecticide that ultimately damages the gut microbiota of mosquitoes. But microbes of mosquito gut received minimal exposure to that insecticide and inspite of getting damaged or killed, they began to form insecticide resistance genes/ insertion sequences against that insecticide. As a result, these insertion sequences get inserted into mosquito DNA to make it insecticide resistance. We checked which families of insertion sequences were common along the mosquitoes, from which common insertion sequences families were selected. Insertion sequences were the possible cause of insecticide resistance in mosquitoes because they were inserted into mosquito genome from the microbial genome.

Table 4.17: List of insertion sequences and their microbiota

Group	**Mosquitoes**	**Microbiota**	**Common Family**
1.	*Aedes aegypti,*	*Escherichia coli,*	IS200/IS605,
	Culexpipiens,	*Wolbachia,*	IS3, IS4, ISAs1,
	Anopheles gambiae,	*Plasmodium*	IS481,IS30 ,IS5,

	Aedes albopictus	*berghei,*	ISNCY, IS630,
		plasmodium	IS110, IS256,
		falciparum,	IS1182, IS21,
		Plasmodium	IS1595,1S1634
		vivax	
		Escherichia coli,	
		Plasmodium vivax,	
		plasmodium berghei,	
		Plasmodium	
		falciparum	
		Escherichia coli,	
		Wolbachia,	
		plasmodium	
		berghei,	
		Plasmodium	
		falciparum	
		Wolbachia,	
		Plasmodium	
		falciparum,	
		plasmodium	
		berghei	
		Escherichia coli,	
		Plasmodium vivax,	
		plasmodium	
		falciparum	
2.	*Aedes aegypti,*	*Escherichia coli,*	ISkra4
	Anopheles gambiae,	*Plasmodium vivax,*	
	Aedes albopictus	*Wolbachia,*	
3.	*Aedes aegypti,*	*Wolbachia,*	IS6
	Aedes albopictus,	*plasmodium*	
	Culex pipiens	*berghei*	

4.	*Aedes albopictus, Anopheles gambiae*	*Escherichia coli, Plasmodium vivax, Wolbachia, plasmodium berghei*	IS66
5.	*Aedes aegypti, Culexpipiens*	*Wolbachia, Plasmodium falciparum, plasmodium berghei Plasmodium falciparum*	IS982, ISLre2
6.	*Aedes aegypti, Aedes albopoictus*	*Plasmodium falciparum, Plasmodium berghei.*	ISH3
7.	*Anopheles gambiae*	*Escherichia coli, Plasmodium falciparum, Plasmodium vivax, Wolbachia, Plasmodium berghei*	IS1
		Escherichia coli, Plasmodium vivax	IS91

One of the enzymes conversed super family was IS200/IS605 that contains conserved amino acids. For coordination of divalent metal ion it provides two out of three required ligands [148]. The other inverted repeats were IS3,IS4 and IS30 that contains DNA binding domains[149]. Tn3 was involved in cleavages and strand transfer reactions. The elements like IS1, IS2 and IS5 were involved in regulations of other genes expressions. IS66 family composed of 12 members including the

partial elements and several other elements having partially sequenced data. IS91 was composed of only 8 members having imperfect terminal IRs that on insertion does not generate direct target repeats. IS110 family was relatively short having single long well conserved open reading frame. IS256 family represent the unique members having single long open reading frame some of which generating 9-bp duplication and 8-bp direct target repeats. IS630 family contains 12 members and duplication of an invariant target TA dinucleotide was generated after insertion procedure but a very little information was known about this family. IS1380 had only 6 identified members and carry a long single ORF. In its host, Acetobacter pasteurianusIS1380 was present in enormously high copy number. ISAs1 was restricted to gram negative bacteria containing 13 members and H-repeats were also present in this family. 1% of chromosome was represented by this family. ISL3 contain 21 known members and this family contains AT-rich regions. The remaining sequences had unknown classification and description respectively [147].

4.8 Molecular Docking with Derris

Nucleotide sequences of all the selected microbes and mosquitoes were converted into amino acids using ExPASy translate tool (https://web.expasy.org/translate/) and compact format was selected. The ExPASy translate tool generated the amino acid sequences along with highlighted open reading frame (ORF). The longest ORF was selected for further analysis. Now for the predictions of protein structures and functions Phyrre2(http://www.sbg.bio.ic.ac. uk/ phyre2/ html/page. cgi?id=index) was used. The amino acid sequences generated by ExPASy were uploaded into Phyrre2 and normal mode was selected for structure prediction and protein analysis.

4.8.1 Derris Insecticide

It was a colorless, odorless, crystalline isoflavone commonly used as insecticide. In several plants rotenone occur in seeds and stems of several plants. It had ability

to interact with various systems of organism. The structure of Derris insecticide is given in fig:

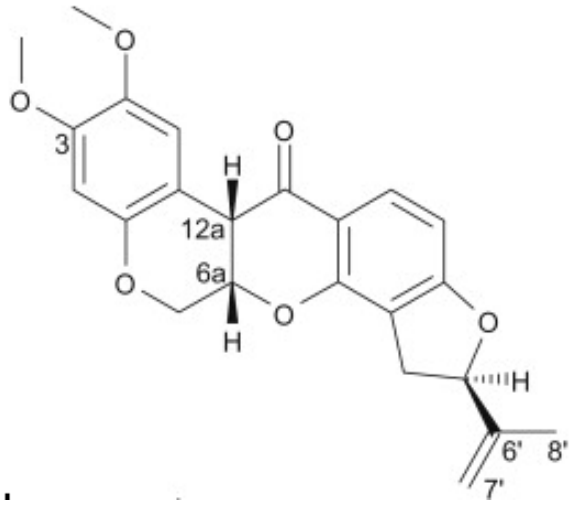

FIGURE 4.6: Derris (Rotenone) a common insecticide[150].

To check the insecticide resistance in given protein structures, the several sequences of protein were docked with insecticide. The insecticide selected for this purpose was Derris insecticide. Derris insecticide is one the most common insecticide used against mosquitoes. Rotenone was a Derris based insecticide that was commonly extracted from the roots of derris plants and cube plants respectively. The purpose behind the selection of Derris insecticide was that it was one of the most commonly used insecticides against insects and it causes no damaging effects on food crops or other plants. Derris was basically a stomach poison that damages the gut/stomach of mosquitoes after chewing its doses. In this way the interaction behind the insecticide resistance of mosquitoes can be easily visualized using the docking procedure of insecticides and proteins of mosquitoes and microbes[151].

Using the Patch-dock online software (https://bioinfo3d.cs.tau.ac.il/PatchDock/), we found the structures of resistant genes by docking of proteins with ligand which was insecticide. The structure of proteins was visualized using the discovery studio. The receptor ligand interactions were visualized in discovery studio and 3D and 2D structures of proteins were predicted. Unfavorable bumps mean there was no interaction between the ligand and that nucleotide of receptor, so here insecticide resistance sequences were visible with red circles.

Following were the figures and tables showing the results of protein docking.

4.8.2 First Group

4.8.2.1 IS200/IS600

The IS200/IS600 was the first family common among the four selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, Aedes albopictus and it was also common among the selected microbes such as *Escherichia coli*, *Wolbachia*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*. Docking was performed for this insertion sequence containing family ISCpe2 against Derris. Only *Plasmodium berghei* ISCpe2 family and *Escherichia coli* ISSen6 family and IS609 family shows unfavorable bumps for this insertion sequence. The binding affinity of this sequence was -238.36. The results were shown in figure.

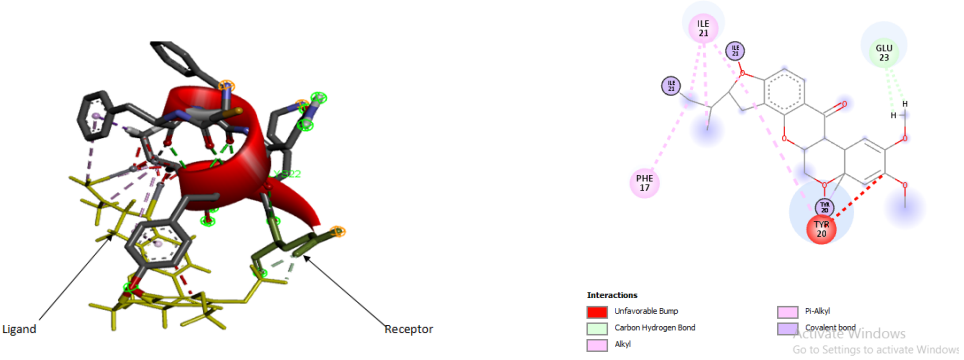

FIGURE 4.7: 3D and 2D representation of *Plasmodium berghei* ISCpe2 family

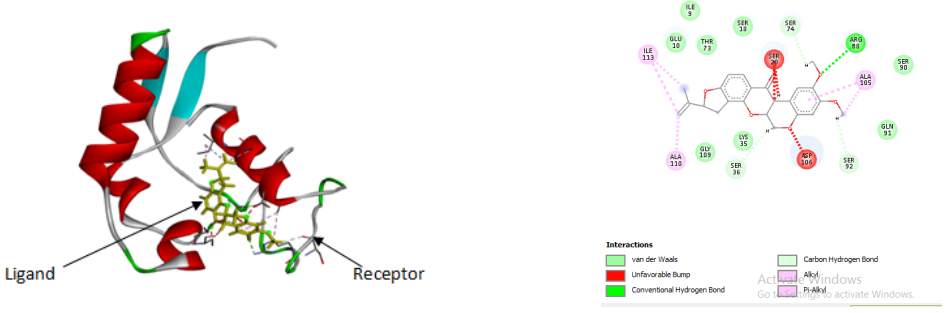

FIGURE 4.8: ISSen6 Family of *E.coli*

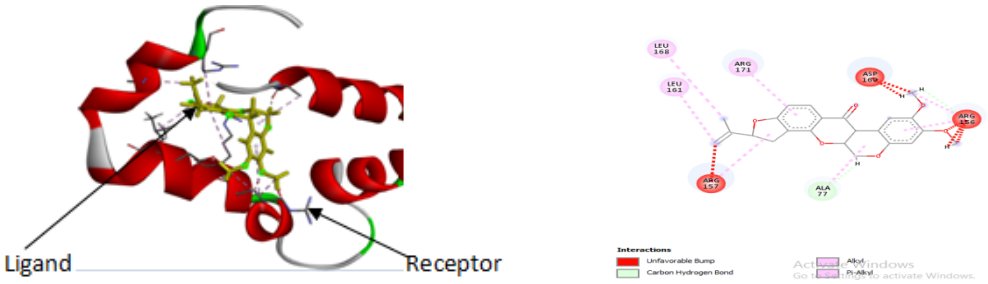

FIGURE 4.9: IS609 Family of *E.coli*

Ligand-receptor interactions of ISCpe2 Family of IS200/IS605 along with derris as a ligand were shown in figure. Dotted lines in 2D figure show the hydrogen bonding between nucleotides and red circles show the unfavorable bumps. TYR was unfavorable bump in this figure. GLU show carbon hydrogen bond, ILE presents the covalent bond whereas ILE and PHE show the alkyl and Pi-Alkyl groups respectively.

4.8.2.2 IS3 Family

IS3 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*. Docking was performed for this insertion sequence containing against Derris. Only *Escherichia coli* containing different families like ISEc16, IS2, IS3F, IS103, ISEc17, ISEhe3, ISKpn8, ISPeat2, ISSF110 and IS150 shows unfavorable bumps for this insertion sequence. The binding affinities of these sequences were -376.92, -183.80,-271.39, -75.67, -271.39, -280.25, -75.67 -179.52, -219.04, -27.36. The results were shown in figure.

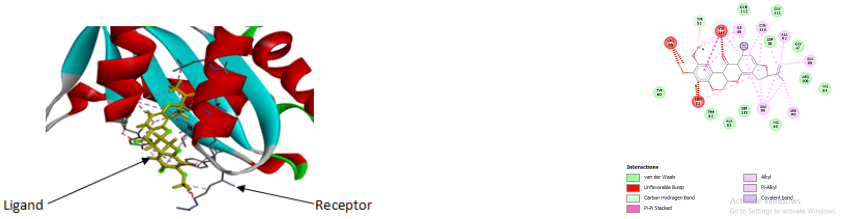

FIGURE 4.10: ISEc16 family of *E.coli*

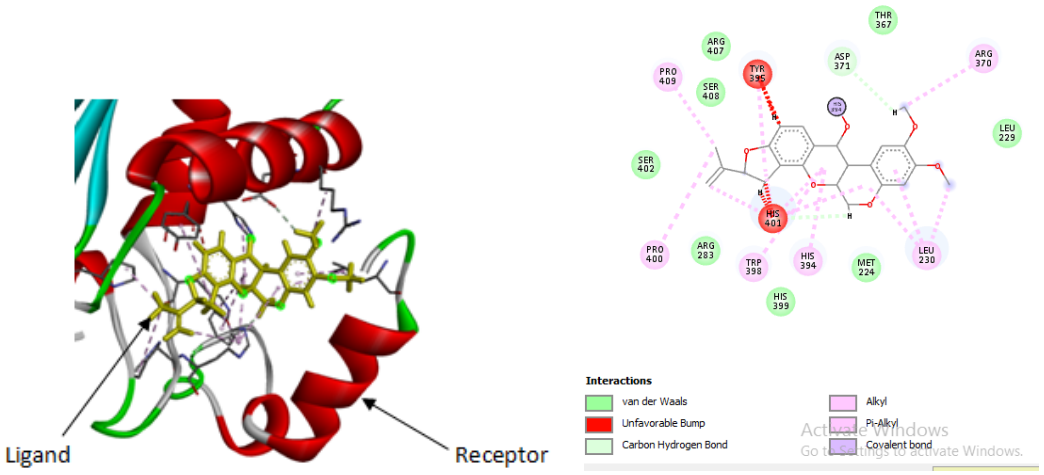

FIGURE 4.11: IS2 family of *E.coli*

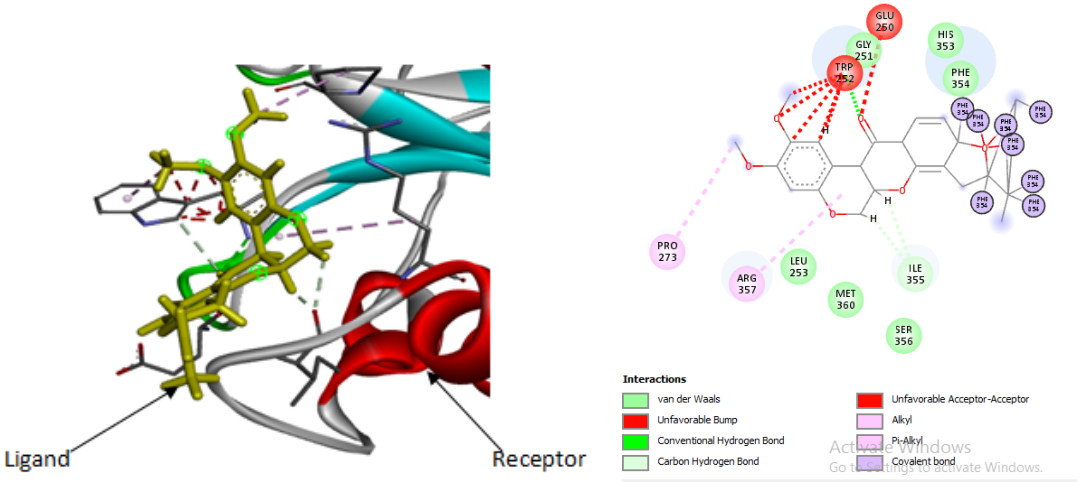

FIGURE 4.12: IS3F family of *E.coli*

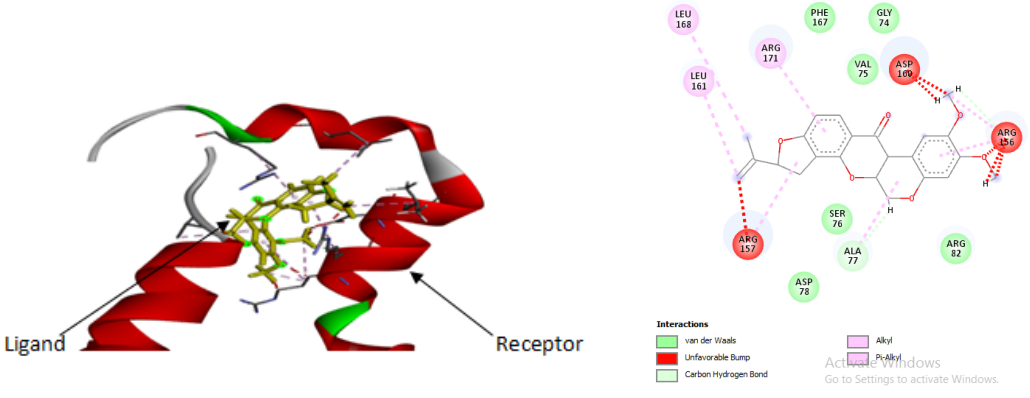

FIGURE 4.13: IS103 family of *E.coli*

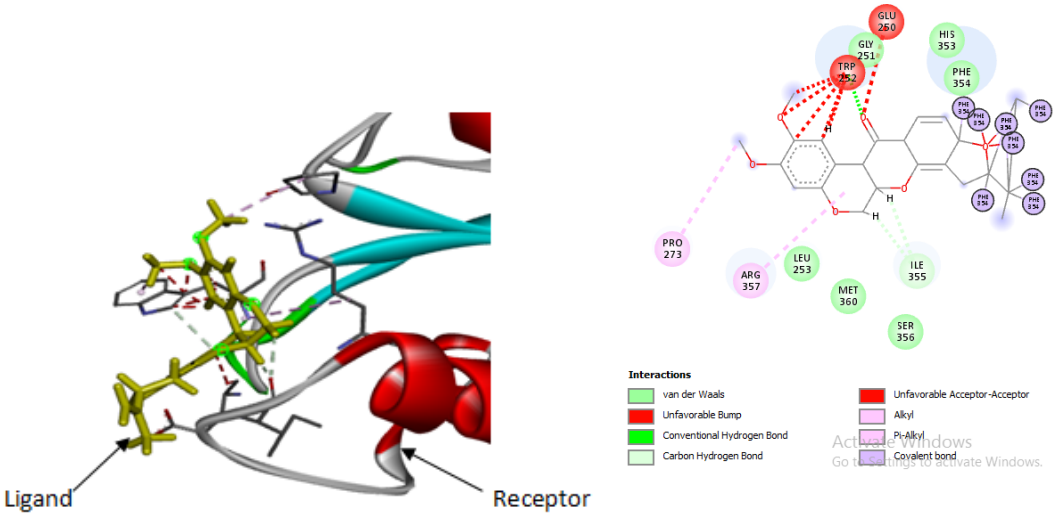

FIGURE 4.14: ISEc17 family of *E.coli*

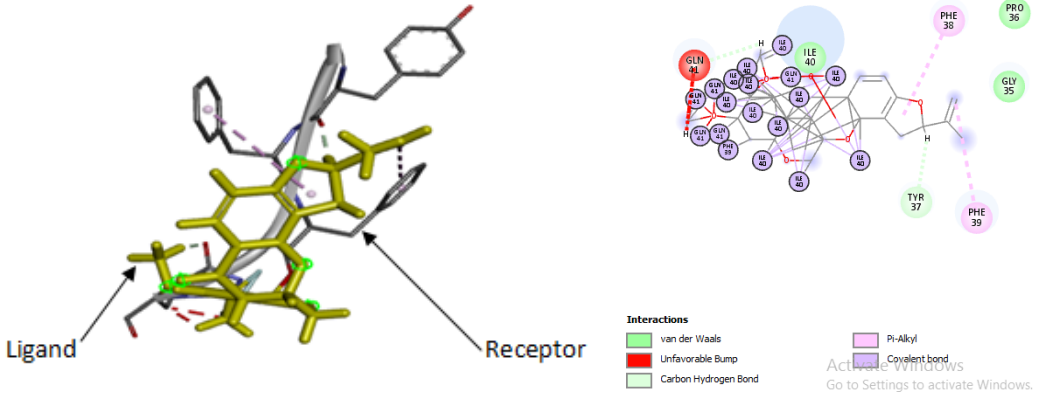

FIGURE 4.15: ISEhe3 family of *E.coli*

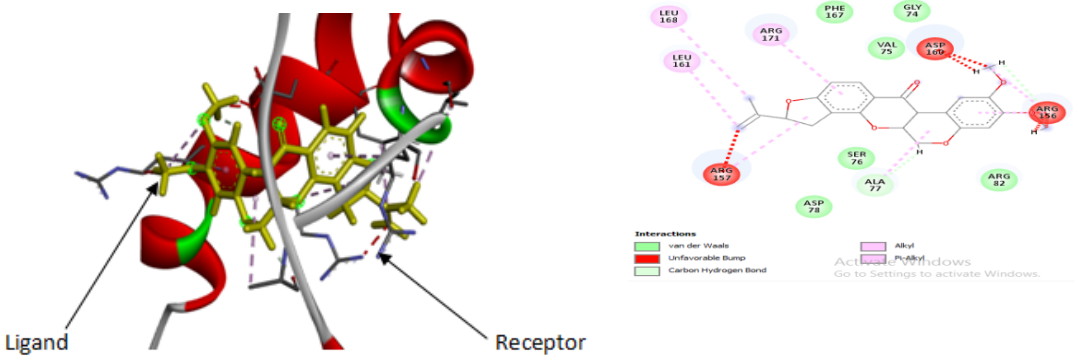

FIGURE 4.16: ISKpn8 family of *E.coli*

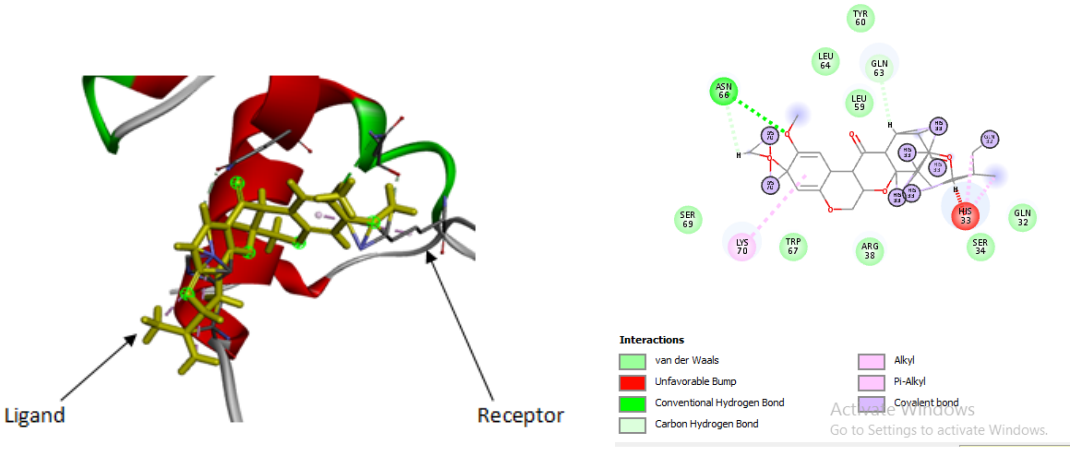

FIGURE 4.17: ISPeat2 family of *E.coli*

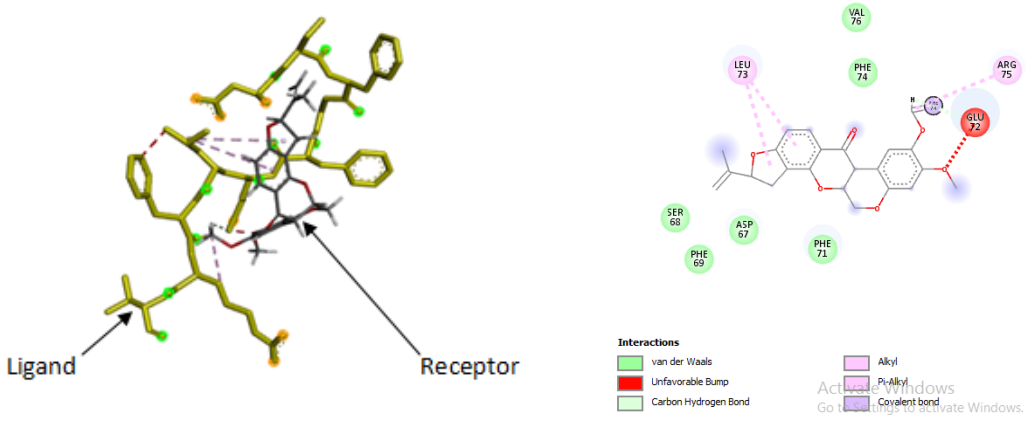

FIGURE 4.18: ISSF110 family of *E.coli*

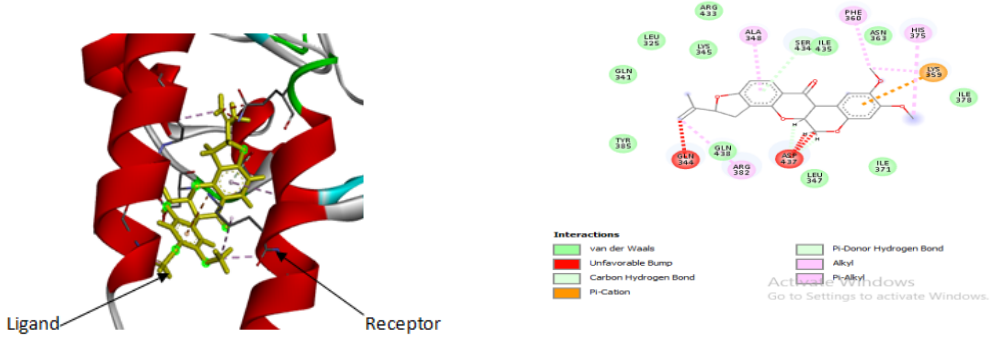

FIGURE 4.19: IS150 family of *E.coli*

4.8.2.3 IS4 Family

IS4 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Aedes albopictus*, *Anopheles gambiae*, and it was also common among the selected microbes such as *Plasmodium falciparum*, *Wolbachia*, *Escherichia coli*, *P.vivax* and *P.berghei*. Docking was performed for IS4 insertion sequence against Derris.

Only *Wolbachia* and *Escherichia coli* containing different families shows unfavorable bumps for this insertion sequence. *Wolbachia* contain ISWen1, ISWosp8, ISWosp9, ISWosp5 and ISWpi18 in IS4 family whereas the *Escherichia coli* contain IS4, ISCro3 and ISPcc3 families in IS4 insertion sequence. The binding affinities of the sequences of *Wolbachia* were -118.90, -274.89,-131.00, 217.47 and -162.10 whereas the binding affinities of *E.coli* were -241.02, -309.27 and -255.02. The results were shown in figure.

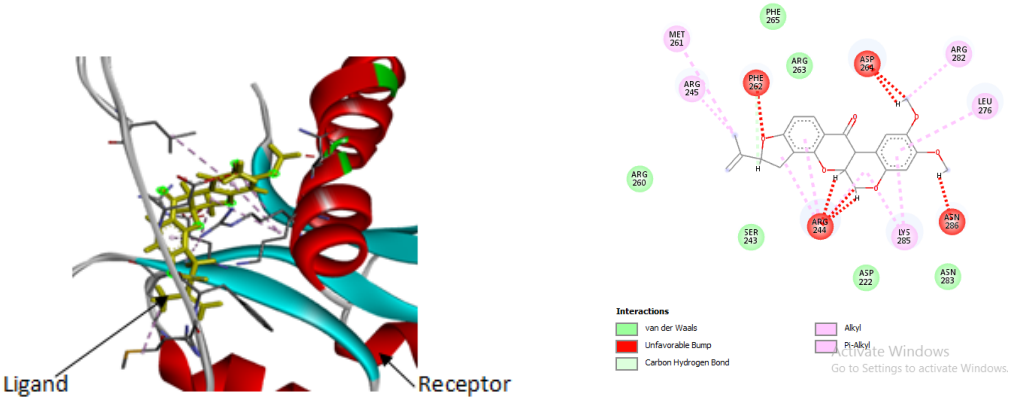

FIGURE 4.20: ISWen1 family of *Wolbachia*

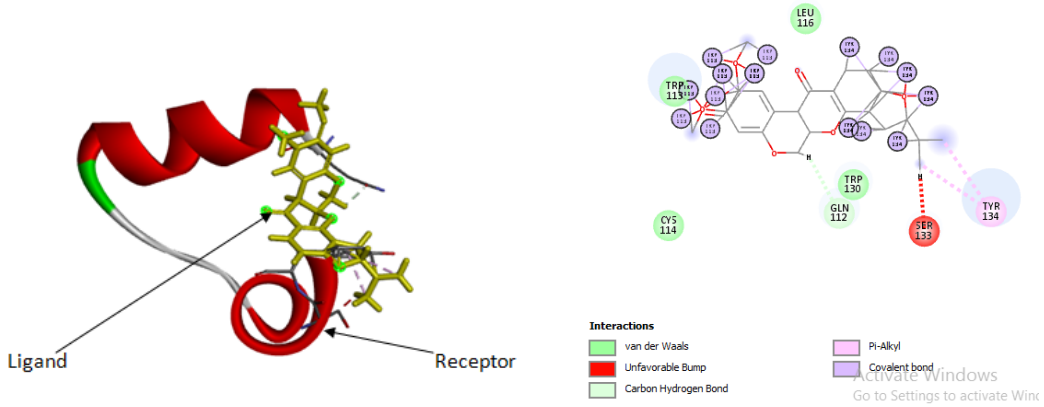

FIGURE 4.21: ISWosp8 family of *Wolbachia*

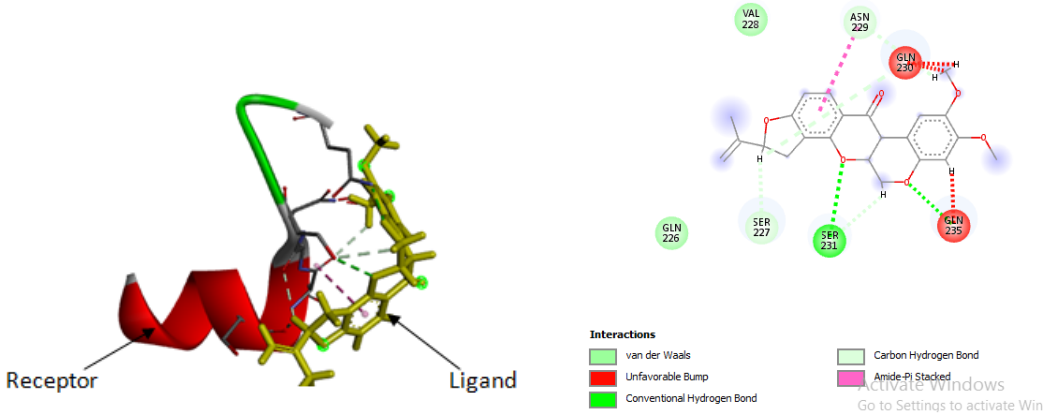

FIGURE 4.22: ISWosp9 family of *Wolbachia*

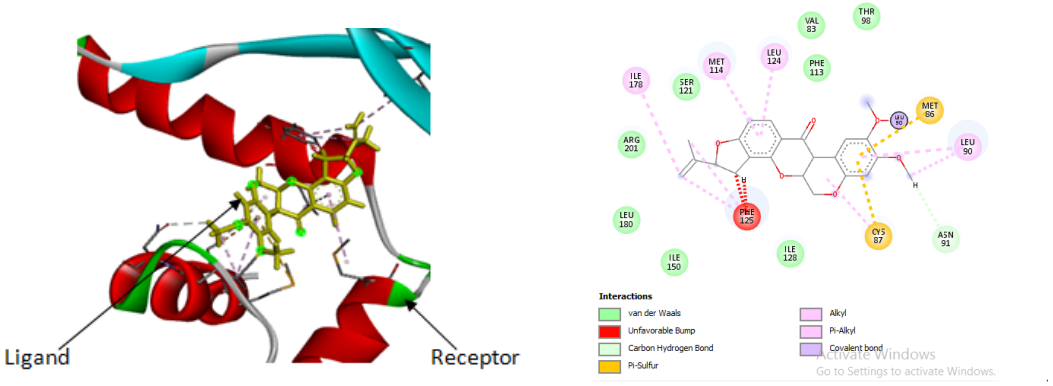

FIGURE 4.23: ISWosp5 family of *Wolbachia*

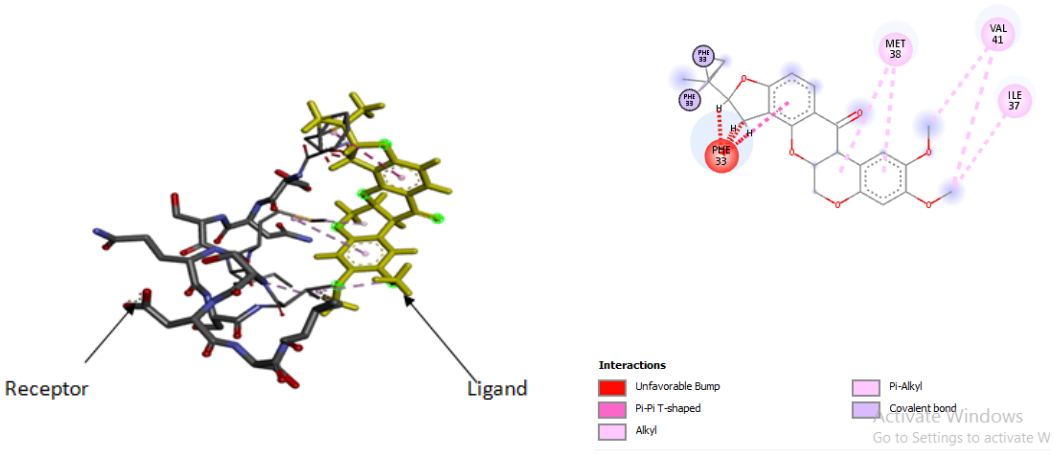

FIGURE 4.24: ISWpi18 family of *Wolbachia*

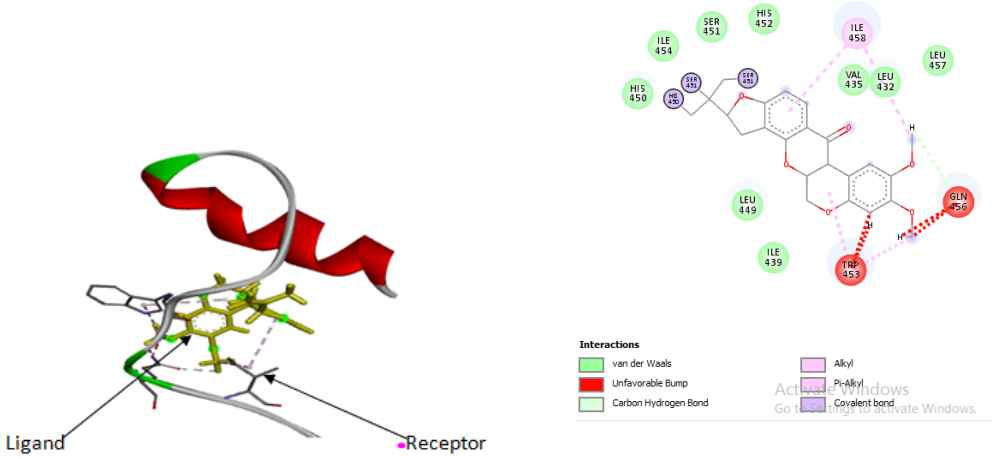

FIGURE 4.25: IS4 family of *E.coli*

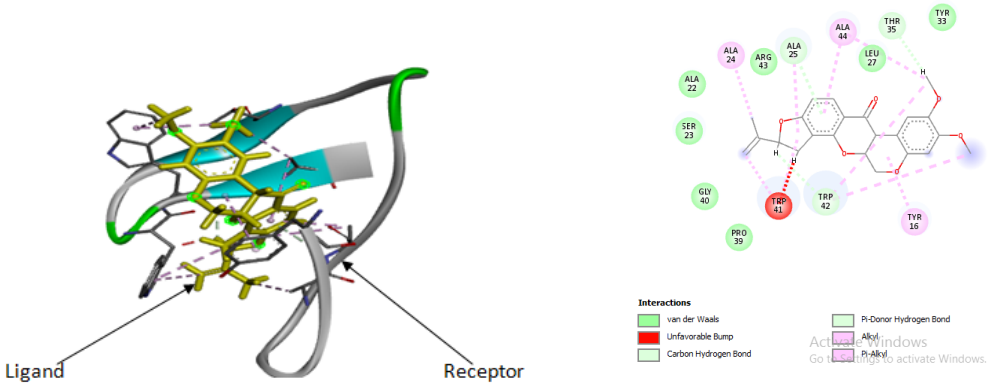

FIGURE 4.26: ISCro3 family of *E.coli*

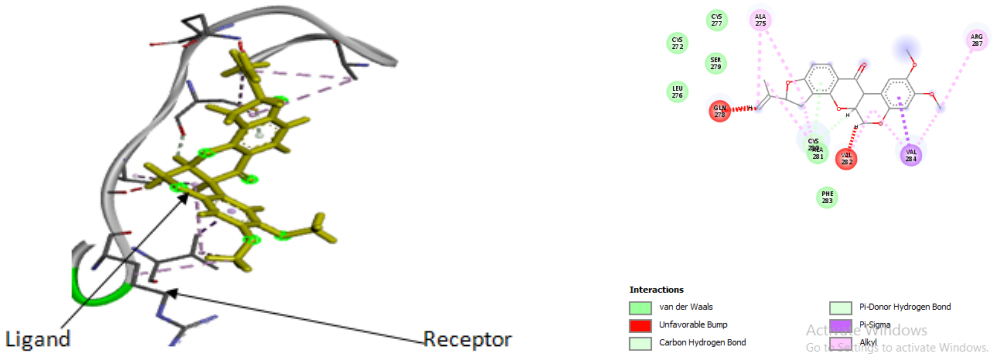

FIGURE 4.27: ISPcc3 family of *E.coli*

4.8.2.4 ISAS1 Family

ISAS1 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*.

Docking was performed for ISAS1 insertion sequence against Derris. Only *Escherichia coli* containing different families like ISEc5 and ISEc26 shows unfavorable bumps for this insertion sequence.

The binding affinities of these sequences were –201.02 and -124.78. The results were shown in figure.

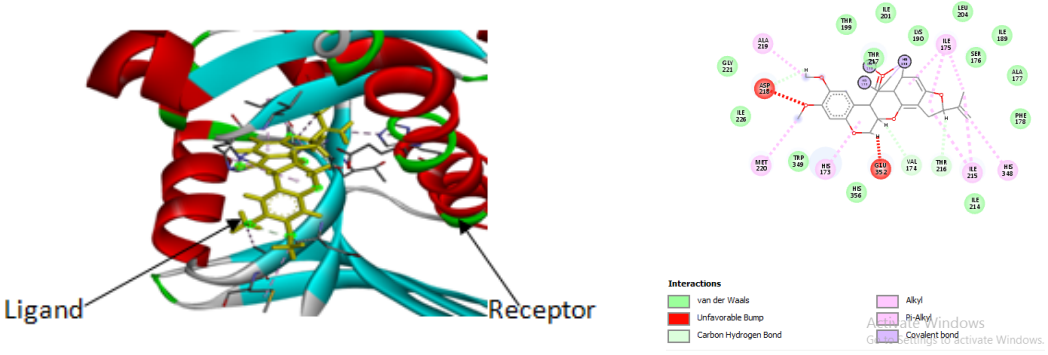

FIGURE 4.28: ISEc5 family of *E.coli*

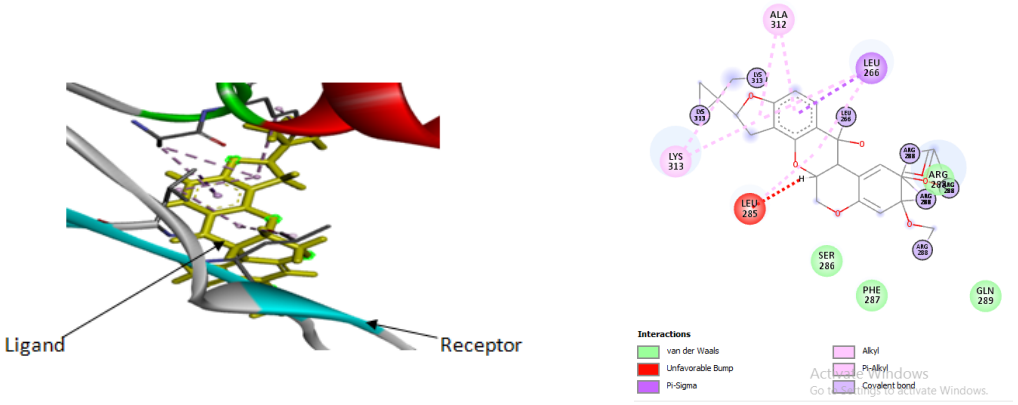

FIGURE 4.29: ISec26 family of *E.coli*

4.8.2.5 IS481 Family

IS481 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*.

Docking was performed for IS481 insertion sequence against Derris. Only *Wolbachia* with certain families such as ISFW3 and ISWpi4 and *Escherichia coli* containing different families such as ISErsp1 and ISSod13 shows unfavorable bumps for this insertion sequence. The binding affinities of the sequences of *Wolbachia* were -137.71 and –212.85. The binding affinities of *E.coli* were -211.92 and -244.29 respectively. The results were shown in figure.

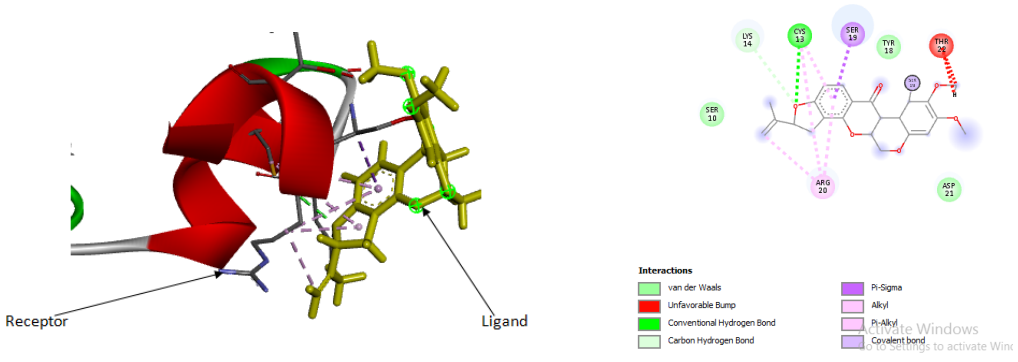

FIGURE 4.30: ISFW3 family of *Wolbachia*

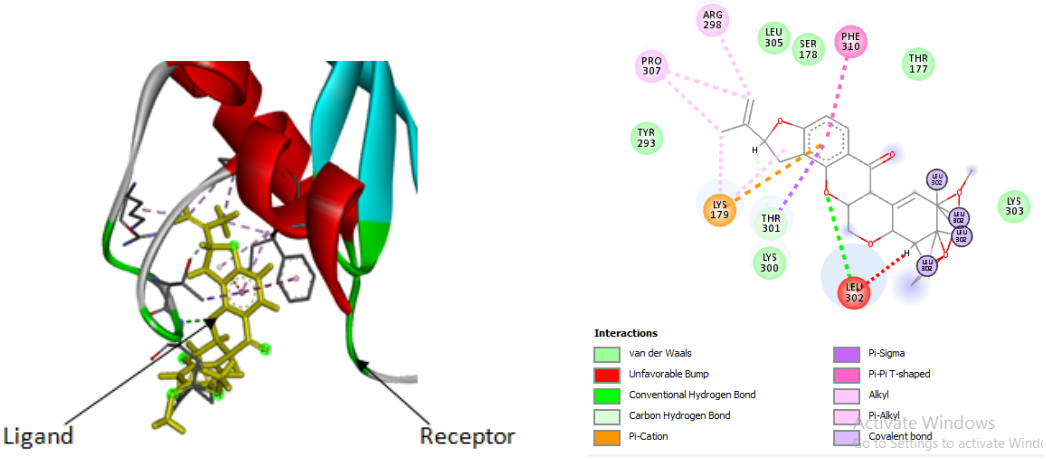

FIGURE 4.31: ISWpi4 family of *Wolbachia*

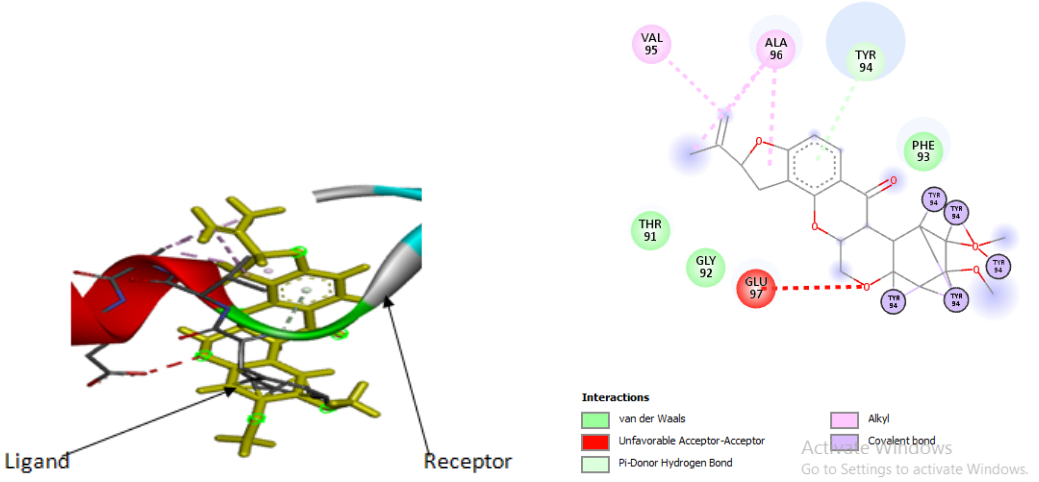

FIGURE 4.32: ISErsp1 family of *E.coli*

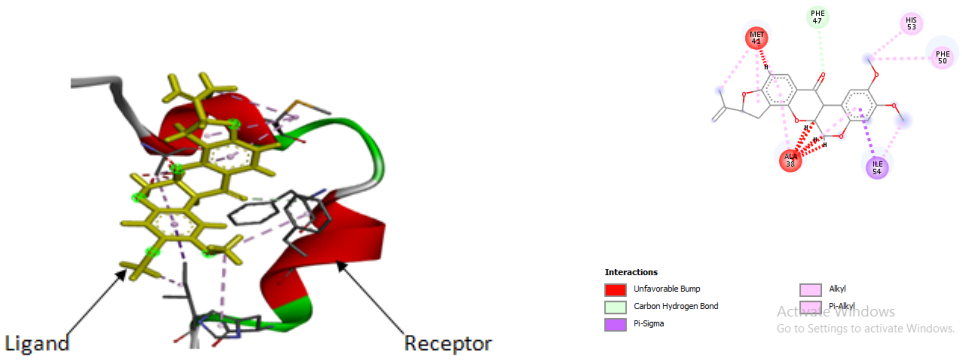

FIGURE 4.33: ISSod13 family of *E.coli*

4.8.2.6 IS30 Family

IS30 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*.

Docking was performed for IS30 insertion sequence against Derris. Only *Escherichia coli* containing different families such as IS3OH show unfavorable bumps for this insertion sequence. The binding affinity of this sequence was -130.90. The results were shown in figure.

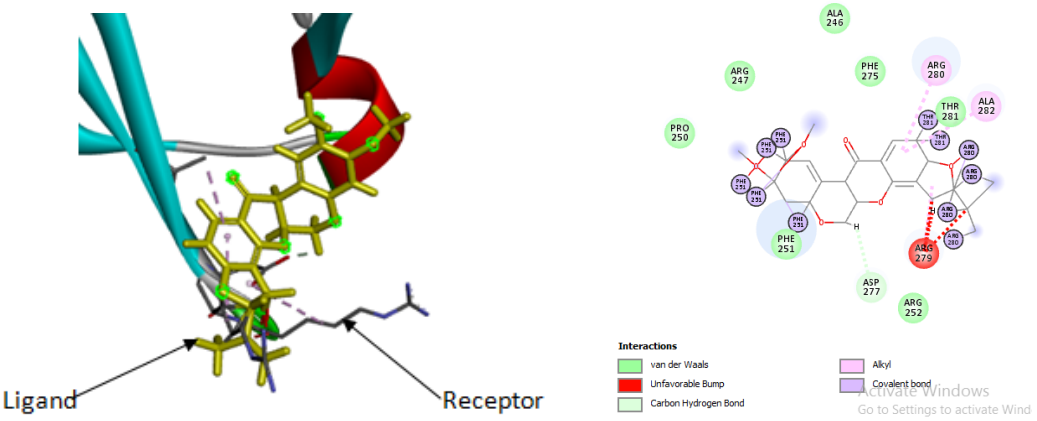

FIGURE 4.34: IS3OH family *E.coli*

4.8.2.7 IS5 Family

IS5 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*. Docking was performed for IS5 insertion sequence against Derris. Only *Escherichia coli* containing different families such as ISPa41, ISpmo2, IS5, IS5D, ISD1sp1, ISEc68, ISPa26, ISPa52, ISPpu21, ISVch5 and ISVch9 show unfavorable bumps for this insertion sequence. The binding affinities of these sequences were -146.45, -146.45, -142.81, -197.49, -217.86, -197.49, -146.45, -145.46, -146.45, -203.03 and -160.28 respectively. The results were shown in figure.

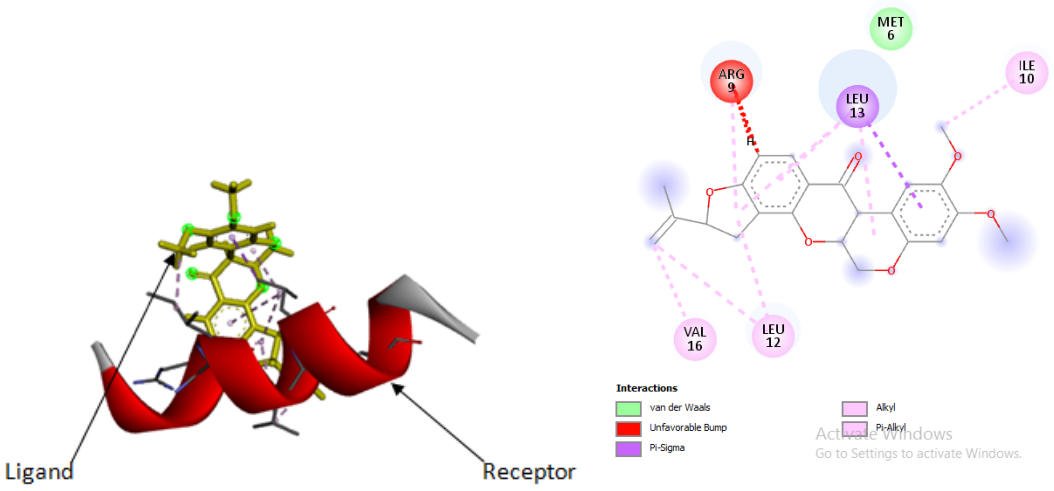

FIGURE 4.35: ISPa41 family of *E.coli*

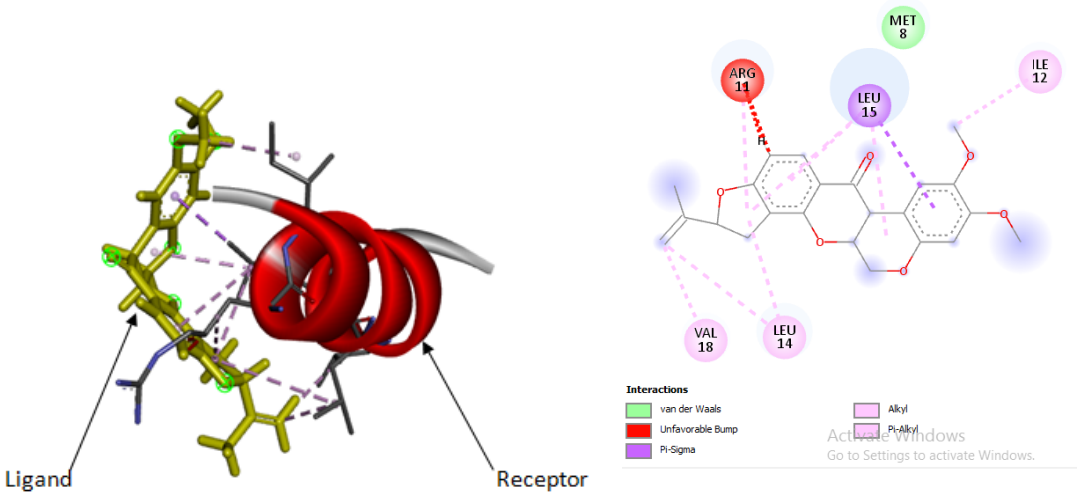

FIGURE 4.36: ISpmo2 family of *E.coli*

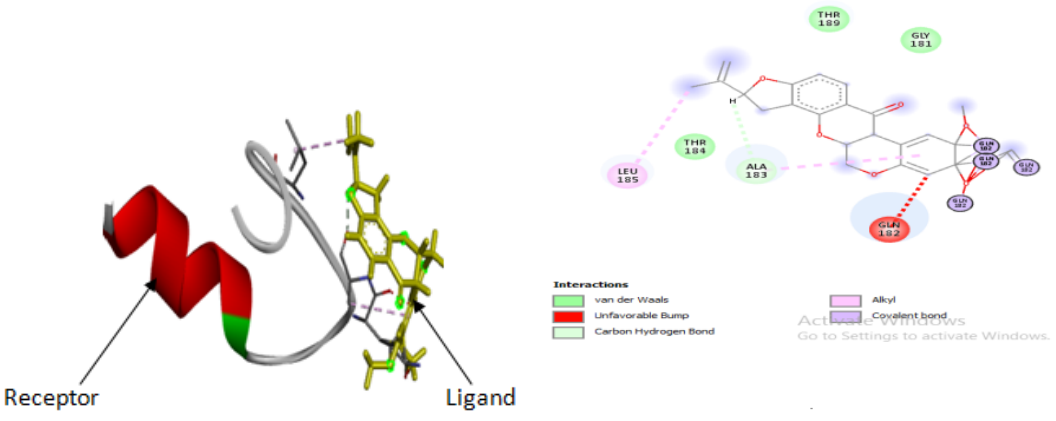

FIGURE 4.37: IS5 family of *E.coli*

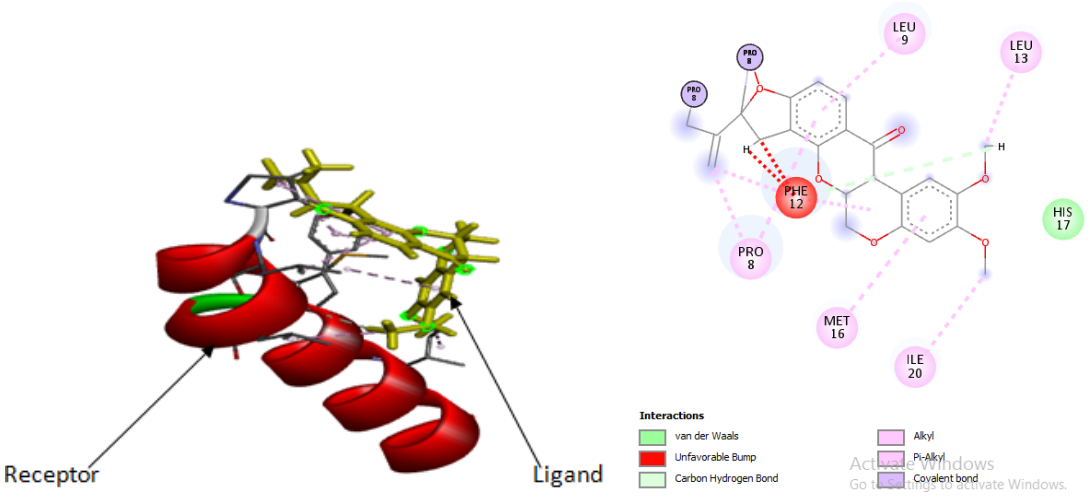

FIGURE 4.38: ISD1sp1 family of *E.coli*

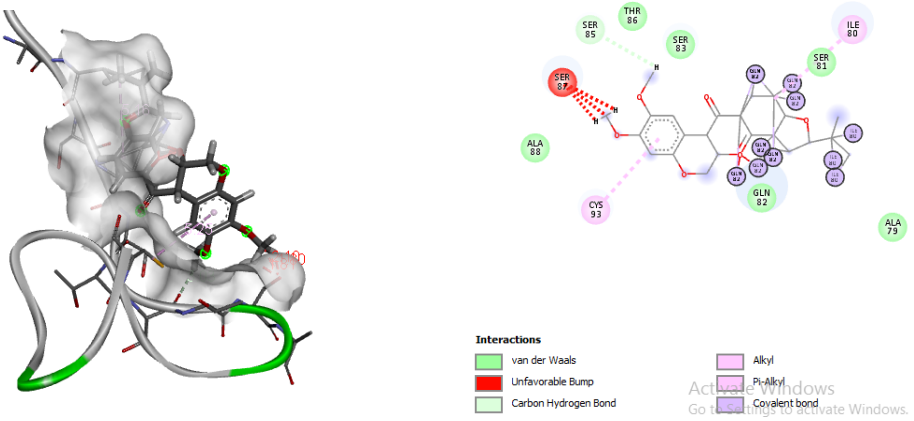

FIGURE 4.39: ISEc68 family of *E.coli*

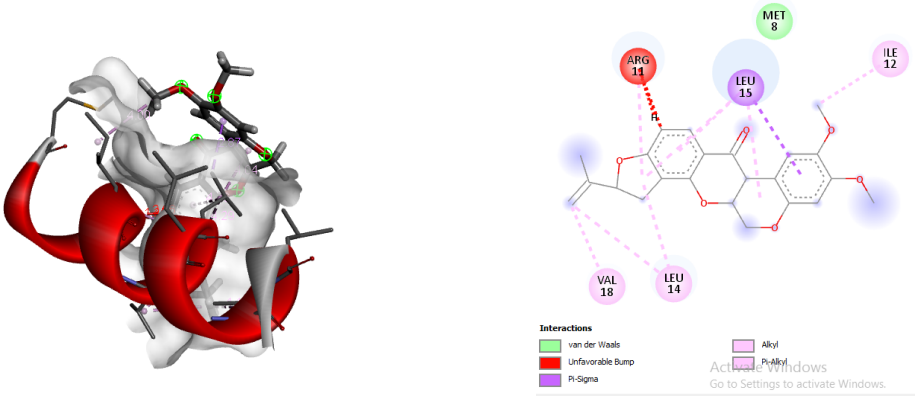

FIGURE 4.40: ISPa26 family of *E.coli*

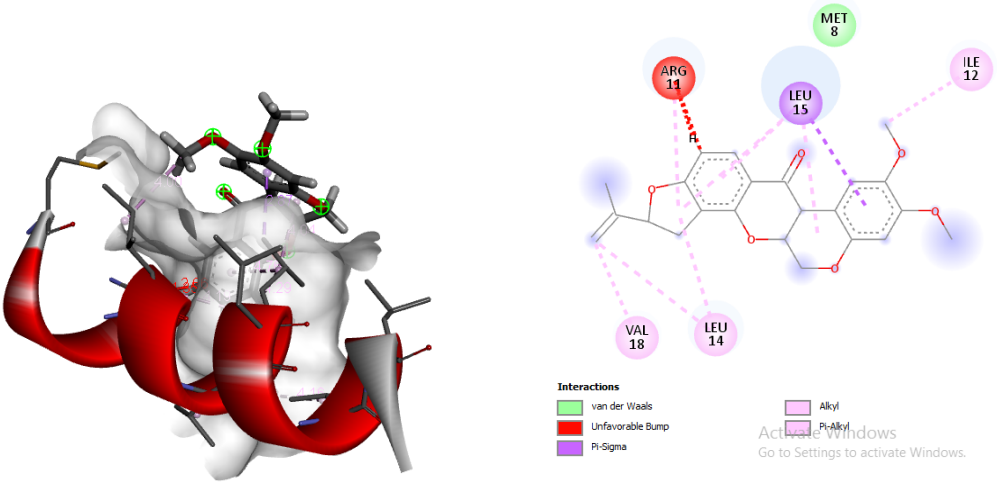

FIGURE 4.41: ISPa52 family of *E.coli*

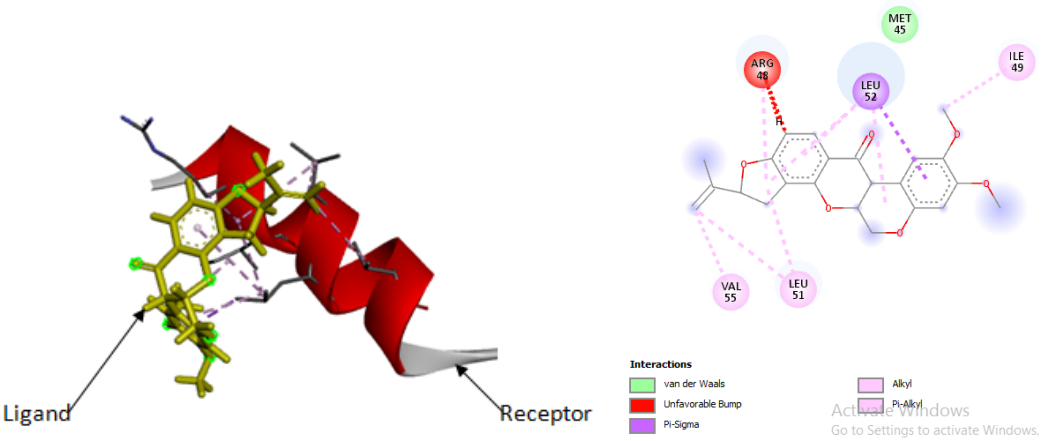

FIGURE 4.42: ISPpu21 family of *E.coli*

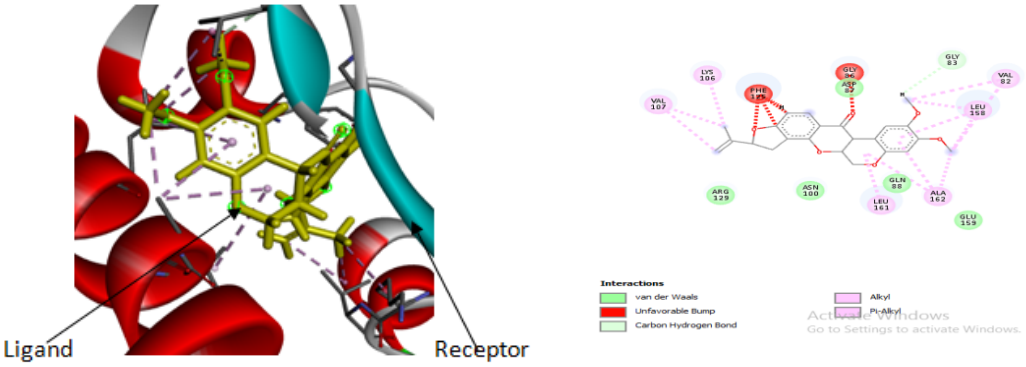

FIGURE 4.43: ISVch5 family of *E.coli*

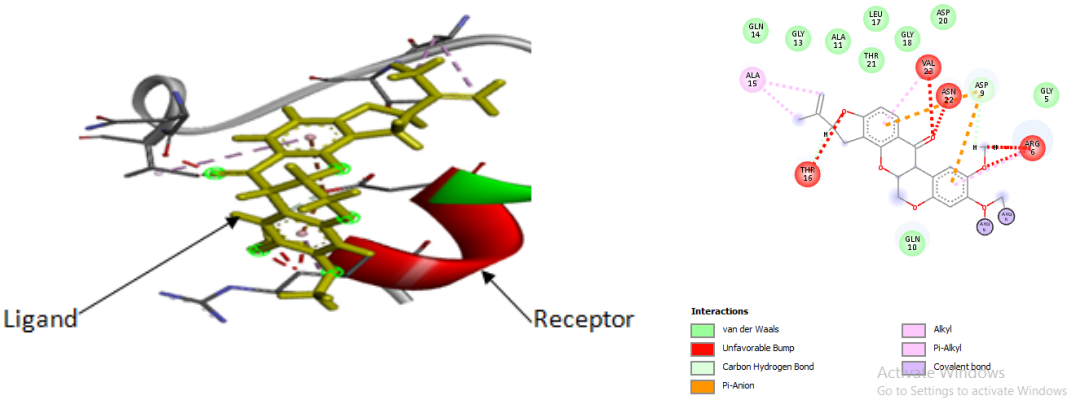

FIGURE 4.44: ISVch9 family of *E.coli*

4.8.2.8 IS630 Family

IS630 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*.

Docking was performed for IS630 insertion sequence against Derris. Only *Escherichia coli* containing different family MITEEc1 show unfavorable bumps for this insertion sequence. The binding affinity of this sequence was -220.98.

The results were shown in figure.

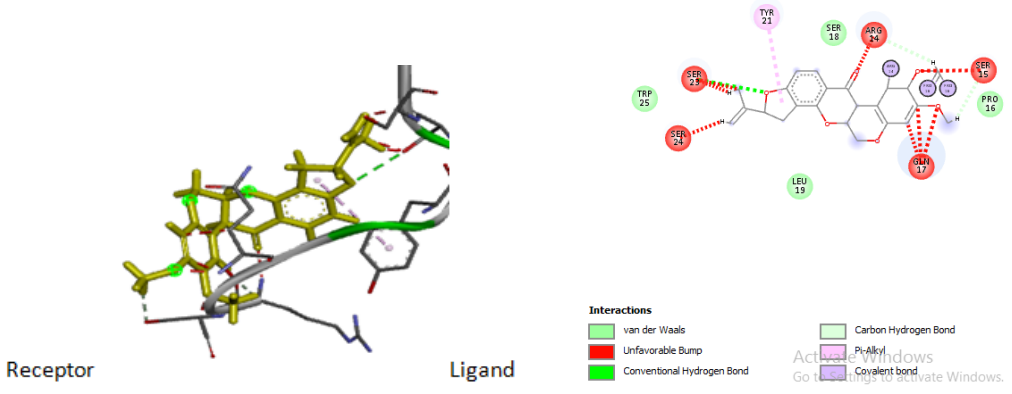

FIGURE 4.45: MITEEc1 family of *E.coli*

4.8.2.9 IS110 Family

IS110 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*.

Docking was performed for IS110 insertion sequence against Derris. Only *Wolbachia* with ISWen2, ISWpi12 and ISWpi13 family and *Escherichia coli* containing family IS621 shows unfavorable bumps for this insertion sequence. The binding affinity of sequences of *Wolbachia* was -168.32, -163.54 and -220.41. The binding affinity of *E.coli* family was -248.39.

The results were shown in figure.

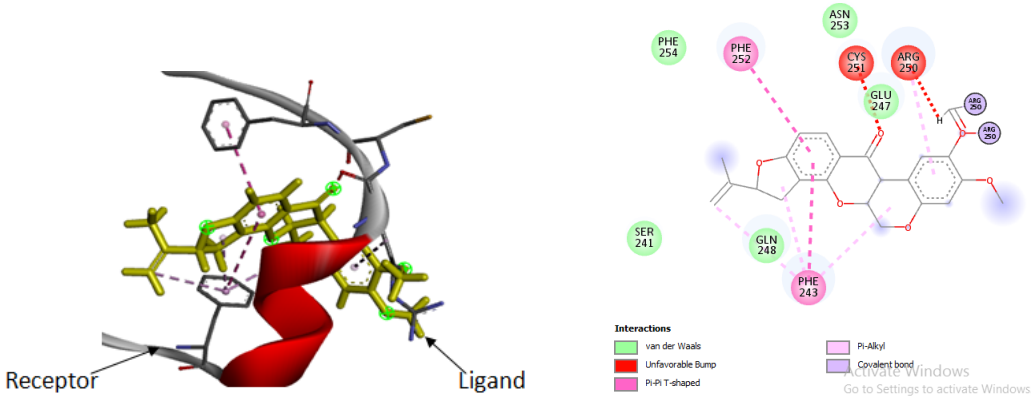

FIGURE 4.46: ISWen2 family of *Wolbachia*

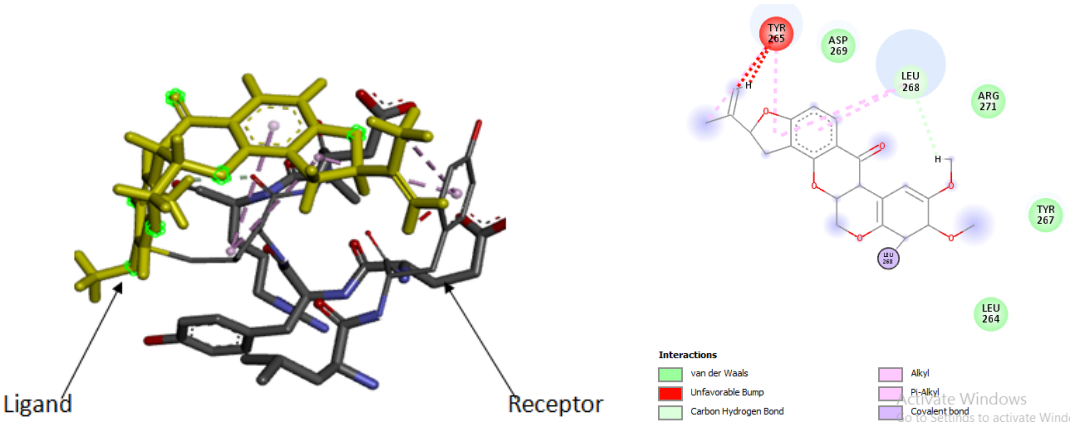

FIGURE 4.47: ISWpi12 family of *Wolbachia*

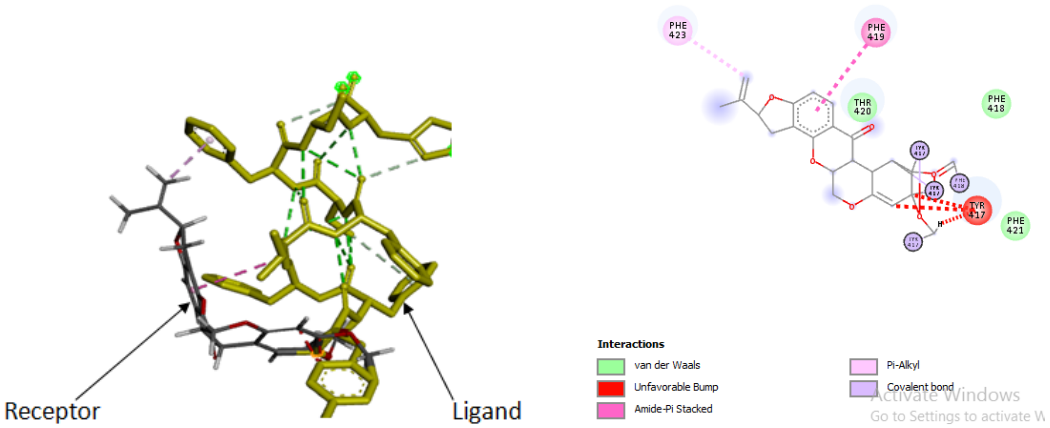

FIGURE 4.48: ISWpi13 family of *Wolbachia*

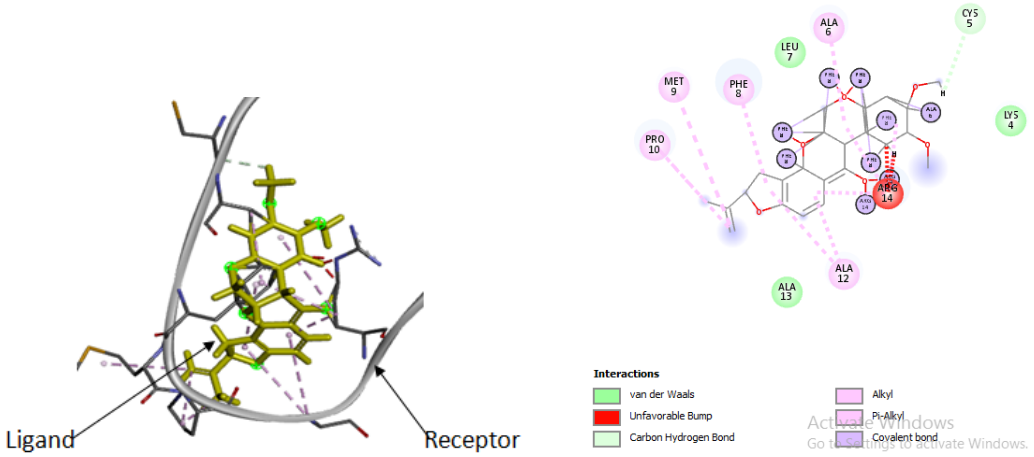

FIGURE 4.49: IS621 family of *E.coli*

4.8.2.10 IS256 Family

IS256 was the common family among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*.

Docking was performed for IS256 insertion sequence against Derris. Only *Wolbachia* with ISWpi15 family show unfavorable bumps for this insertion sequence. The binding affinity of this family of *Wolbachia* is-296.56.

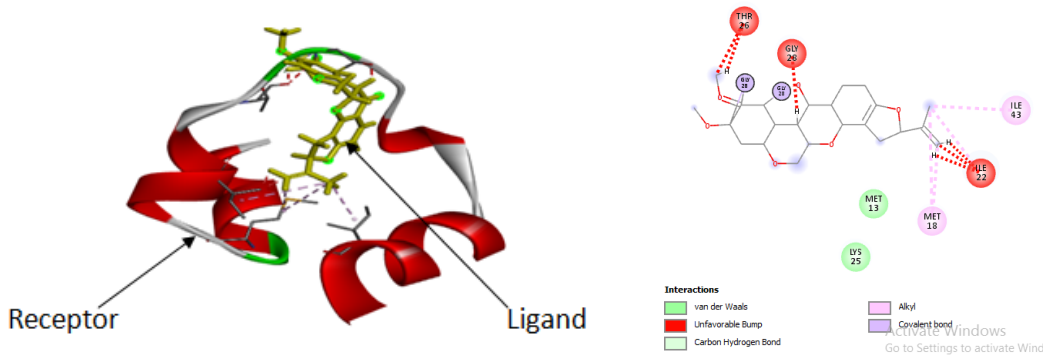

FIGURE 4.50: ISWpi15 family of *Wolbachia*

ISNCY, IS1182, IS21, IS95, IS1634 were common families among the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei*. The docking of these sequences was performed against Derris. No unfavorable bumps were shown in these families respectively. Hence the results prove that there was no insecticide resistance among these families.

Similarly, ISL3 family from the selected mosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and common selected microbes such as *Escherichia coli*, *Plasmodium falciparum*, *P.vivax* and *P.berghei* as a result of docking against Derris show no unfavorable bumps. IS1380 from the same selected mosquitoes and some common microbes such as *Wolbachia*, *Escherichia coli*, *Plasmodium falciparum* and *P.berghei* as a result of docking show no unfavorable bumps. IS607 family from same mosquitoes and some common microbes such as *Wolbachia*, *Plasmodium falciparum* and *P.vivax* as a result of docking against Derris insecticide show no unfavorable bumps.

4.8.2.11 Tn3 Family

Tn3 was the common family among the selectedmosquitoes i.e. *Aedes aegypti*, *Culex pipiens*, *Anopheles gambiae*, *Aedes albopictus* and it was also common

among the selected microbes such as *Escherichia coli*, *Plasmodium falciparum* and *P.vivax*. Docking was performed for Tn3 insertion sequence against Derris. Only *Escherichia coli*having family ISSBa14 shows unfavorable bumps for this insertion sequence. The binding affinity ofthis*E.coli* family was -263.91. The results were shown in figure:

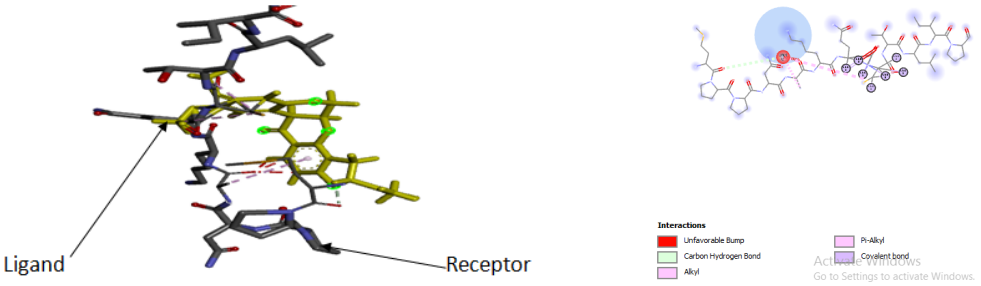

FIGURE 4.51: ISSba14 family of *E.coli*

4.8.3 Second Group

4.8.3.1 ISkra4 Family

ISkra4 was the common family among the threeselectedmosquitoesi.e.*Aedes aegypti*, *Anopheles gambiae*, *Aedes albopictus* and it was also common among the selected microbes such as *Escherichia coli*, *Plasmodium vivax* and *Wolbachia*. Docking was performed for ISkra4 insertion sequence against Derris. No microbe show unfavorable bumps for this insertion sequence.

4.8.4 Third Group

4.8.4.1 IS6 Family

IS6 was the common family among the three selectedmosquitoesi.e.*Aedes aegypti*, *Culex pipiens*, *Aedes albopictus* and it was also common among two selected microbes which were *Plasmodium berghei* and *Wolbachia*. Docking was performed

for IS6 insertion sequence against Derris. No microbe show unfavorable bumps for this insertion sequence.

4.8.5 Fourth Group

4.8.5.1 IS66Family

IS66 was the common family among the three selected mosquitoes i.e. *Aedes albopictus* and *Anopheles gambiae* and it was also common among four selected microbes which were *Escherichia coli*,*Plasmodium berghei*, *Plasmodium vivax* and *Wolbachia*. Docking was performed for IS66 insertion sequence against Derris. Only *Wolbachia* ISWen3 family shows unfavorable bumps for this insertion sequence. The binding affinity of this family was -294.16. The results were shown in figure:

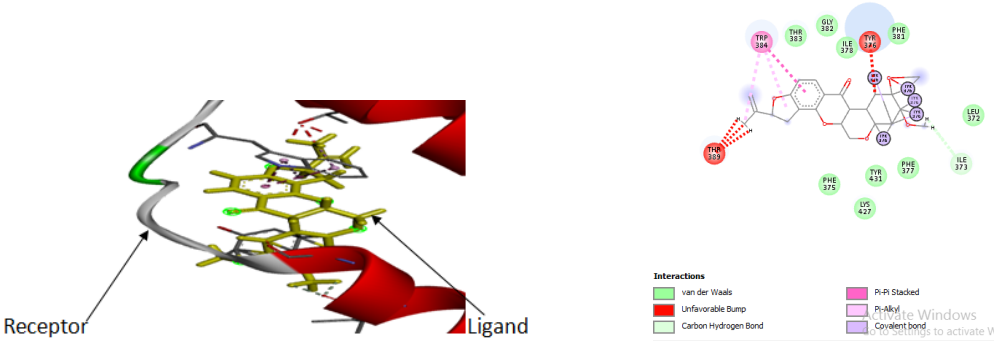

FIGURE 4.52: ISWen3 family of *Wolbachia*

4.8.6 Fifth Group

4.8.6.1 ISLre2 Family

ISLre2 was the common family among the two selectedmosquitoesi.e.*Aedes aegypti* and *Culex pipiens* and it was also common among three selected microbes which were *Plasmodium berghei*, *Plasmodium falciparum* and *Wolbachia*. Docking was performed for ISLre2 insertion sequence against Derris. Only *Plasmodium vivax*

ISCbe4 family shows unfavorable bumps for this insertion sequence. The binding affinity of this family was -228.72.

The results were shown in figure:

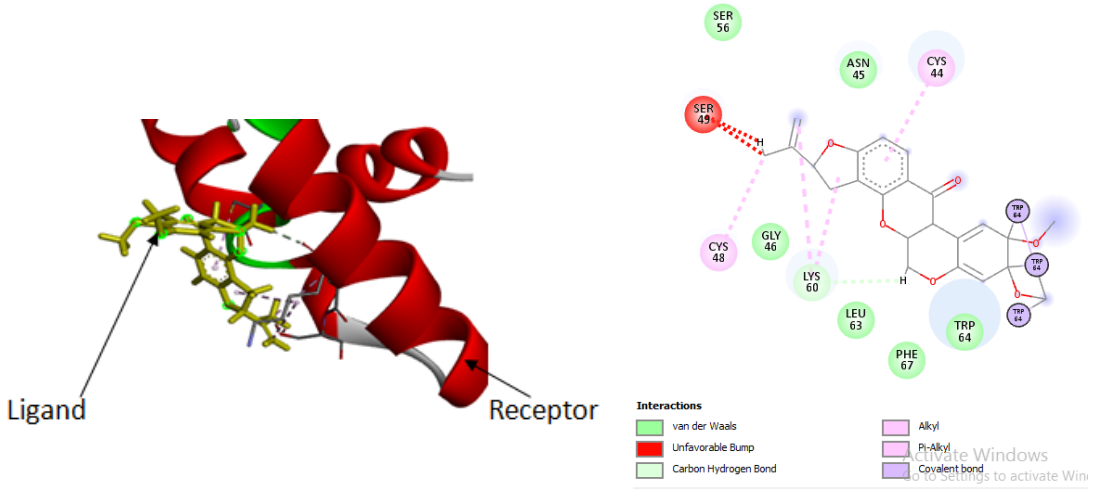

FIGURE 4.53: ISLre2 family of *Plasmodium vivax*

IS982 family having the same two common mosquitoes and same microbes like ISLre2 family as a result of docking against derris shows no unfavorable bumps.

IS701 was another member from the same group that was just found in *Plasmodium falciparum* also show no unfavorable bump against derris.

4.8.7 Sixth Group

4.8.7.1 ISH3Family

ISH3 was the common family among the two selected mosquitoes i.e. *Aedes albopictus* and *Aedes aegypti* and it was also common among four selected microbes which were *Plasmodium berghei*, *Plasmodium falciparum*.

Docking was performed for ISH3 insertion sequence against Derris. No microbe show unfavorable bumps for this insertion sequence.

4.8.8 Seventh Group

4.8.8.1 IS1 Family

IS1 was the common family among the only one selected mosquitoi.e.*Anopheles gambiae* and it was also common among five selected microbes which were *Plasmodium berghei*, *Plasmodium vivax*, *Plasmodium falciparum*, *Wolbachia* and *Escherichia coli*. Docking was performed for IS1 insertion sequence against Derris. Only *Escherichia coli* having families IXIX1 and IXIX4 show unfavorable bumps for this insertion sequence. The binding affinity of these families of *E.coli* was -283.87 and -283.87 respectively. The results were shown in figure:

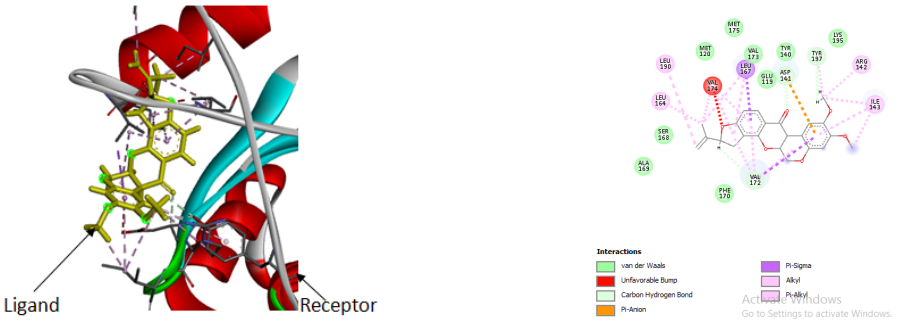

FIGURE 4.54: IXIX1 family of *E.coli*

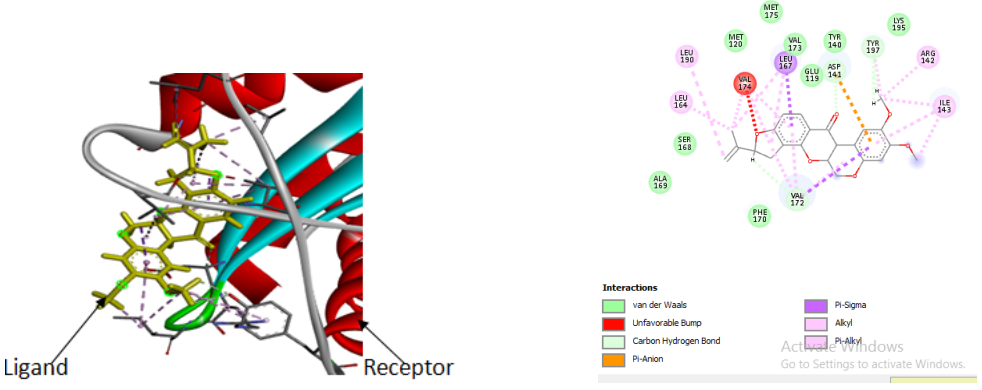

FIGURE 4.55: IXIX4 family of *E.coli*

IS91 was another family from the same group shown in *Escherichia coli* and *Plasmodium vivax* as a result of docking against Derris show no unfavorable bumps.

4.8.9 Molecular Docking of Microbes with Derris

The selected microbes were docked with Derris to check the binding affinity, interaction residues and unfavorable bumps produced in several insertion sequences. The following tables show docking results:

TABLE 4.18: Docking results of *Plasmodium berghei* against Derris

Sr no.	**Protein**	**Binding Affinty**	**Interactions Residues/ Hydrogen Bond**	**Unfavourable Bump**
1	ISCpe2	-238.67	ILE-21 PHE-17 GLU-23	TYR-20

Docking results of *Plasmodium berghei* were shown in table. The binding affinity of protein and ligand was -238.67 which shows that there was less binding affinity between insecticides and protein.

The appearance of unfavorable bumps show weak interactions.

TABLE 4.19: Docking results of *Plasmodium vivax* against Derris

Sr No.	**Protein**	**Binding Affinty**	**Interactions Residues/ Hydrogen Bond**	**Unfavourable Bump**
1	ISLre2	-228.72	CYS-44 CYS-48 LYS-60	SER-49

Docking results of *Plasmodium vivax* were shown in table. The binding affinity of protein and ligand was -228.72which shows that there was less binding affinity between insecticides and protein.

The appearance of unfavorable bumps i.e. SER-49 show weak interactions between ligand and protein.

TABLE 4.20: Docking results of *Wolbachia* spp. against Derris

Sr No.	**Protein**	**Binding Affinty**	**Interactions Residues/ Hydrogen Bond**	**Unfavourable Bump**
1	ISFw3	-137.71	LYS-14	THR-22
			CYS-13	
			SER-19	
			ARG-20	
2	ISWen1	-118.9	Met-261	ASP-264
			ARG-245	PHE-262
			ARG-282	ARG-244
			LEU-276	ASN-286
			LYS-285	
3	ISWen2	-168..32	PHE-252	CYS-251
			PHE-243	
			ARG-250	
4	ISWen3	-294.16	TRP-384	TYR-376
			ILE-373	THR-389
5	ISWosp8	-274.89	TYR-134	SER-133
			GLN-112	
6	ISWosp9	-131	SER-227	GLN-230
			SER-229	GLN-235
			SER-231	
7	ISWpi4	-212.85	ARG-298	LEU-302
			PHE-310	
			PRO-307	
			LYS-179	
			THR-301	
8	ISWpi12	-163.54	LEU-268	TYR-265
9	ISWosp5	-217.47	MET-86	PHE-125
			MET-114	

			LEU-90	
			LEU-124	
			ILE-78	
			ASN-91	
			CYS-87	
10	ISWpi13	-220.41	PHE-423	TYR-417
			PHE-419	
11	ISWpi15	-296.56	MET-18	THR-26
			ILE-43	GLY-28
				ILE-22
12	ISWpi18	-162.1	MET-38	PHE-33
			VAL-41	
			ILE-37	

Docking results of *Wolbachia* were shown in table. The binding affinity of proteins and ligands were extreme negative valueswhich show that there was less binding affinity between insecticides and protein. The appearance of unfavorable bumps in red circles show weak interactions. The docking results of *E.coli* against Derris is given in Table 1 (ref to An Appendix)

Docking results of *Escherichia coli* were shown in table. The binding affinity of proteins and ligands were extreme negative valueswhich show that there was less binding affinity between insecticides and protein. The appearance of unfavorable bumps in red circles show weak interactions.

Chapter 5

Conclusion and Future Prospects

Mosquitoes as biological vectors, contribute a lot in onset of various epidemics. This project was executedas an effort to explore the gut microbiota of mosquitoes and resistance genes these microbota harbour, as these resistance genes play a significant role in insecticide resistance. For this purpose, systematic review through metanalysis was conducted, the objective of which was to prioritise the most frequently reported mosquito species as well as the gut microbial species. Literature mining helped us in gathering information about the insecticide resistance genes and the pathways that are involved in insecticide resistance mechanim. Seven generas from the three related species are found to be involved directly in insecticide resistance. The first step was to check the microbial interactions among the different mosquitoes. The meta-analysis techniques was performed which showed that there is a same level of variations in microbes among different mosquitoes. The microbial diversity was also visualized inside the gut of mosquitoes and they are found to consists of bacteria, viruses and parasites respectively. The phylogenetic analysis was performed to checked the closely related and differently related microbial species. Phylogenetic results showed that there is complex variety of parasites inside mosquitoes gut that are basically involved in disease transmission. Orthologs were found using the BLASTn to identify the same genes in other organisms. Once the microbial species were identified, pangenome analysis was conducted to fine common genes (core genome) of all the selected species. As

the microbiota varies between viruses to bacteria to parasites, the number of core genes was very low. Pangenome analysis showed that only few mosquitoes and microbes have the common genes/insertion sequences. After this last major step was performed to check the metabolic pathways in which insertion sequences are involved in insecticide resistance. For this purpose docking was performed using the online Patch-dock software and results are visualized using the Discovery studio. Poor docking results and presence of bumps showed that there is a weak interactions between the proteins and insecticides respectively.

In future the prioritized insertion sequences and their inhibitors could be validated in lab. The control stretegies for mosquitoe borne epidemics could be more effective if they are designed targeting the mechanism behind insecticide resistance. And thus by targeting these insertion sequences better drugs could be designed that are able to control the mosquitoes growth and mosquito born diseases like dengue and malaria etc.

Bibliography

[1] M. A. Tolle, "Mosquito-borne Diseases," Curr. Probl. Pediatr. Adolesc. Health Care, vol. 39, no. 4, pp. 97–140, Apr. 2009.

[2] R. C. Kessler, G. Andrews, D. Mroczek, B. Ustun, and H.-U. Wittchen, "The World Health Organization Composite International Diagnostic Interview short-form (CIDI-SF)," Int. J. Methods Psychiatr. Res., vol. 7, no. 4, pp. 171–185, Nov. 1998.

[3] K. Bashar, M. S. Rahman, I. J. Nodi, and A. J. Howlader, "Species composition and habitat characterization of mosquito (Diptera: Culicidae) larvae in semi-urban areas of Dhaka, Bangladesh," Pathog. Glob. Health, vol. 110, no. 2, pp. 48–61, 2016..

[4] M. Ashfaq, P. D. N. Hebert, J. H. Mirza, A. M. Khan, Y. Zafar, and M. S. Mirza, "Analyzing mosquito (Diptera: Culicidae) diversity in Pakistan by DNA barcoding," PLoS One, vol. 9, no. 5, p. e97268, May 2014.

[5] S. Ronald and G.-C. Doris, "(2), julio-diciembre 2013, Maestre."

[6] A. Montoya, H. Rodríguez, P. Posos-Ponce, E. Pimienta-Barrios, and V. Antonio Aceves-Nùñez, "Selectivity of Amblyseius largoensis (Muma) with phytosanitarys products used in the protected production of pepper (Capsicum annuum L.)."

[7] P. De and A. Aegypti, "Determinación de los grados de resistencia al insecticida temefos en," 2011.

[8] M. W. Skidmore and S. Z. Cohen, "Pesticides Report 38. Pesticide fate in tropical soils (Technical Report) The Importance of Chemistry in Maintaining a Secure Food Supply View project nitrogen loading to surface water at a golf course View project."

[9] G. W. Ware, "Effects of pesticides on nontarget organisms.," Residue reviews, vol. 76. Springer, New York, NY, pp. 173–201, 1980.

[10] M. Ashfaq, P. D. N. Hebert, J. H. Mirza, A. M. Khan, Y. Zafar, and M. S. Mirza, "Analyzing mosquito (Diptera: Culicidae) diversity in Pakistan by DNA barcoding," PLoS One, vol. 9, no. 5, May 2014.

[11] C. B. Ocampo, M. J. Salazar-Terreros, N. J. Mina, J. McAllister, and W. Brogdon, "Insecticide resistance status of Aedes aegypti in 10 localities in Colombia," Acta Trop., vol. 118, no. 1, pp. 37–44, Apr. 2011.

[12] L. S. Varón, B. C. Córdoba, and H. L. Brochero, "Susceptibilidad de Aedes aegypti a DDT, deltametrina y lambdacialotrina en Colombia," Rev. Panam. Salud Publica/Pan Am. J. Public Heal., vol. 27, no. 1, pp. 66–73, Jan. 2010.

[13] "(No Title)." [Online]. Available: https://www.redalyc.org/ pdf/ 843 / 84329 151014.pdf. [Accessed: 27-Feb-2021].

[14] Y. Granada, A. Mejia-Jaramillo, C. Strode, and O. Triana-Chavez, "A Point Mutation V419L in the Sodium Channel Gene from Natural Populations of Aedes aegypti Is Involved in Resistance to λ-Cyhalothrin in Colombia," Insects, vol. 9, no. 1, p. 23, Feb. 2018.

[15] R. Maestre-Serrano, D. Gomez-Camargo, G. Ponce-Garcia, and A. E. Flores, "Susceptibility to insecticides and resistance mechanisms in Aedes aegypti from the Colombian Caribbean Region," Pestic. Biochem. Physiol., vol. 116, pp. 63–73, Nov. 2014.

[16] A. Aponte, R. P. Penilla, A. D. Rodríguez, and C. B. Ocampo, "Mechanisms of pyrethroid resistance in Aedes (Stegomyia) aegypti from Colombia," Acta Trop., vol. 191, pp. 146–154, Mar. 2019.

[17] L. G. de Almeida, L. A. B. de Moraes, J. R. Trigo, C. Omoto, and F. L. Cônsoli, "The gut microbiota of insecticide-resistant insects houses insecticide-degrading bacteria: A potential source for biotechnological exploitation," PLoS One, vol. 12, no. 3, p. e0174754, Mar. 2017.

[18] N. Dada, M. Sheth, K. Liebman, J. Pinto, and A. Lenhart, "Whole metagenome sequencing reveals links between mosquito microbiota and insecticide resistance in malaria vectors," Sci. Rep., vol. 8, no. 1, pp. 1–13, Dec. 2018.

[19] "Sharma,A.; Bhatt,P.; Khati,P.; Gangola, S.;Kumar, G.Microbial. - Google Scholar." [Online]. Available: https://scholar. google. com/ scholar ?hl=en& assdt= 0,5& q= Sharma, A BBhatt, P. BKhati,P.B Gangola, S.% 3BKumar, G.Microbial Degradation of Pesticides for Environmental Cleanup. [Accessed: 27-Feb-2021].

[20] W. Li, D. Jin, C. Shi, and F. Li, "Midgut bacteria in deltamethrin-resistant, deltamethrin-susceptible, and field-caught populations of Plutella xylostella, and phenomics of the predominant midgut bacterium Enterococcus mundtii," Sci. Rep., vol. 7, no. 1, pp. 1–13, Dec. 2017.

[21] J. E. Pietri and D. Liang, "The Links Between Insect Symbionts and Insecticide Resistance: Causal Relationships and Physiological Tradeoffs," Ann. Entomol. Soc. Am., vol. 111, no. 3, pp. 92–97, Apr. 2018.

[22] H. Itoh et al., "Infection dynamics of insecticide-degrading symbionts from soil to insects in response to insecticide spraying," ISME J., vol. 12, no. 3, pp. 909–920, Mar. 2018.

[23] D. W. Kim and C. J. Cha, "Antibiotic resistome from the One-Health perspective: understanding and controlling antimicrobial resistance transmission," Exp. Mol. Med., vol. 53, no. 3, pp. 301–309, 2021.

[24] C. M. Thomas and K. M. Nielsen, "Antibiotic biosynthesis investigatuion View project," nature.com, vol. 3, no. 9, pp. 711–721, Sep. 2005.

[25] M. Service and M. Service, "Introduction to mosquitoes (Culicidae)," Med. Entomol. Students, pp. 1–33, 2012.

[26] N. Burkett-cadena, "Morphology of Adult and Larval Mosquitoes," Morphol. Adult Larval Mosquitoes, pp. 1–14, 2013.

[27] W. Mohrig, "Die Culiciden Deutschlands," Anzeiger für Schädlingskd. und Pflanzenschutz, vol. 42, no. 8, pp. 126–127, Aug. 1969.

[28] I. C. Yadav and N. L. Devi, "Pesticides Classification and Its Impact on Human and Environment," no. February, 2017.

[29] J. Kosova, "Longevity Studies of Sindbis Virus Infected Aedes Albopictus," All Vol., Jan. 2003.

[30] H. Y. M. Yousif, "Susceptibility of Anopheles arabiensis Patton and Culex quinquefasciatus Say Larvae to Ixora (Ixora coccinea L) Leaves and Flowers Extracts," May 2013.

[31] Centers for Disease Control and Prevention, "Life Cycle": Aedes aegypti," Centers Dis. Control Prev., p. 2, 2012.

[32] K. Lebl et al., "Mosquitoes (Diptera: Culicidae) and their relevance as disease vectors in the city of Vienna, Austria," Parasitol. Res., vol. 114, no. 2, pp. 707–713, Feb. 2015.

[33] Centers for Disease Control and Prevention, "Life Cycle: Aedes aegypti," Centers Dis. Control Prev., p. 2, 2012.

[34] L. Khatoon, F. N. Baliraine, M. Bonizzoni, S. A. Malik, and G. Yan, "Genetic structure of Plasmodium vivax and Plasmodium falciparum in the Bannu district of Pakistan," Malar. J., vol. 9, no. 1, 2010.

[35] P. G.-M. P. and other Haemosporidia. and undefined 1966, "Malaria parasites and other Haemosporidia.," cabdirect.org.

[36] N. Becker et al., "Morphology of Mosquitoes," Mosquitoes Their Control, pp. 63–87, 2010.

[37] W. Shabbir, J. Pilz, and A. Naeem, "A spatial-temporal study for the spread of dengue depending on climate factors in," pp. 1–10, 2020.

[38] R. U. Rahman et al., "Insecticide resistance and underlying targets - site and metabolic mechanisms in Aedes aegypti and Aedes albopictus from Lahore , Pakistan," Sci. Rep., pp. 1–15, 2021.

[39] D. J. Perkins, T. Were, G. C. Davenport, P. Kempaiah, J. B. Hittner, and J. M. Ong'echa, "Severe Malarial Anemia: Innate Immunity and Pathogenesis," Int. J. Biol. Sci., vol. 7, no. 9, pp. 1427–1442, Nov. 2011.

[40] B. Franke-Fayard, J. Fonager, A. Braks, S. M. Khan, and C. J. Janse, "Sequestration and Tissue Accumulation of Human Malaria Parasites: Can We Learn Anything from Rodent Models of Malaria?," PLoS Pathog., vol. 6, no. 9, p. e1001032, Sep. 2010.

[41] N. J. White, "Plasmodium knowlesi: The Fifth Human Malaria Parasite," January) Editor. Comment. Clin. Infect. Dis., vol. 46, pp. 172–175, 2008.

[42] "Genome sequence of the intracellular bacterium Wolbachia," PLoS Biology, vol. 2, no. 3. Public Library of Science, 2004.

[43] The malERA Consultative Group on Vector Control, "A research agenda for malaria eradication: Vector control," PLoS Medicine, vol. 8, no. 1. 2011.

[44] A. Alperi, A. J. Martínez-Murcia, W. C. Ko, A. Monera, M. J. Saavedra, and M. J. Figueras, "Aeromonas taiwanensis sp. nov. and Aeromonas sanarellii sp. nov., clinical species from Taiwan," International Journal of Systematic and Evolutionary Microbiology, vol. 60, no. 9. Microbiology Society, pp. 2048–2055, 01-Sep-2010.

[45] O. Tenaillon and D. Skurnik, "The Population Genetics of Comensal Phylogeny and molecular pathogenesis of ExPEC View project CRISPR-cas9-recombineering mediated deep mutational scanning of essential genes View project," Nat. Rev. Microbiol., vol. 8, no. 3, pp. 207–217, Mar. 2010.

[46] G. Wei, Y. Lai, G. Wang, H. Chen, F. Li, and S. Wang, "Insect pathogenic fungus interacts with the gut microbiota to accelerate mosquito mortality," Proc. Natl. Acad. Sci. U. S. A., vol. 114, no. 23, pp. 5994–5999, 2017.

[47] M. S.-C. opinion in insect science and undefined 2018, "Composition and functional roles of the gut microbiota in mosquitoes," Elsevier.

[48] V. Pidiyar, K. Jangid, ... M. P.-T. A. journal of, and undefined 2004, "Studies on cultured and uncultured microbiota of wild Culex quinquefasciatus mosquito midgut based on 16s ribosomal RNA gene analysis," academia.edu.

[49] D. Duguma, M. Hall, C. Smartt, J. N.- Msphere, and undefined 2017, "Effects of organic amendments on microbiota associated with the Culex nigripalpus mosquito vector of the Saint Louis Encephalitis and West Nile viruses," Am Soc Microbiol, vol. 2, no. 1, Feb. 2017.

[50] K. Zouache, F. Raharimalala, ... V. R.-F. M., and undefined 2011, "Bacterial diversity of field-caught mosquitoes, Aedes albopictus and Aedes aegypti, from different geographic regions of Madagascar," academic.oup.com.

[51] M. David, L. Santos, ... A. V.-M. do I., and undefined 2016, "Effects of environment, dietary regime and ageing on the dengue vector microbiota: evidence of a core microbiota throughout Aedes aegypti lifespan," SciELO Bras.

[52] P. A. Lynch, U. Grimm, M. B. Thomas, and A. F. Read, "Prospective malaria control using entomopathogenic fungi: Comparative evaluation of impact on transmission and selection for resistance," Malar. J., vol. 11, 2012.

[53] E. J. Muturi, J. L. Ramirez, A. P. Rooney, and C. H. Kim, "Comparative analysis of gut microbiota of mosquito communities in central Illinois," PLoS Negl. Trop. Dis., vol. 11, no. 2, pp. 1–18, 2017.

[54] B. Bolling, S. Weaver, R. Tesh, N. V.- Viruses, and undefined 2015, "Insect-specific virus discovery: significance for the arbovirus community," mdpi.com, 2015.

[55] N. Vasilakis, R. T.-C. opinion in virology, and undefined 2015, "Insect-specific viruses and their potential impact on arbovirus transmission," Elsevier.

[56] M. Shi, P. Neville, J. Nicholson, J.-S. Eden, A. Imrie, and E. C. Holmes, "High-Resolution Metatranscriptomics Reveals the Ecological Dynamics of Mosquito-Associated RNA Viruses in Western Australia," J. Virol., vol. 91, no. 17, Sep. 2017.

[57] B. O. Encyclopedia, "Modes of penetration," pp. 1–5, 2021.

[58] N. Liu, Q. Xu, T. Li, L. He, L. Z.-J. of medical entomology, and undefined 2009, "Permethrin Resistance and Target Site Insensitivity in the Mosquito Culex quinquefasciatus in Alabama," academic.oup.com.

[59] O. Mittapalli, X. Bai, P. Mamidala, S. P. Rajarapu, P. Bonello, and D. A. Herms, "Tissue-specific transcriptomics of the exotic invasive insect pest emerald ash borer (agrilus planipennis)," PLoS One, vol. 5, no. 10, 2010.

[60] M. Michel, L. G.-T. Lancet, and undefined 1997, "Methicillin-resistant Staphylococus aureus and vancomycin-resistant enterococci: therapeutic realities and possibilities," Elsevier.

[61] J. Hemingway, N. Hawkes, ... L. M.-I. biochemistry and, and undefined 2004, "The molecular basis of insecticide resistance in mosquitoes," Elsevier.

[62] S. J. Gan et al., "Dengue fever and insecticide resistance in Aedes mosquitoes in Southeast Asia: a review," Parasites and Vectors, vol. 14, no. 1, pp. 1–19, 2021.

[63] D. Weetman et al., "Contemporary evolution of resistance at the major insecticide target site gene Ace-1 by mutation and copy number variation in the malaria mosquito Anopheles gambiae," Mol. Ecol., vol. 24, no. 11, pp. 2656–2672, Jun. 2015.

[64] N. Liu, "Insecticide Resistance in Mosquitoes: Impact, Mechanisms, and Research Directions," http://dx.doi.org/10.1146/annurev-ento-010814-020828, vol. 60, pp. 537–559, Jan. 2015.

[65] T. Nkya, I. Akhouayri, W. Kisinza, J. D.-I. biochemistry and, and undefined 2013, "Impact of environment on mosquito response to pyrethroid insecticides: facts, evidences and prospects," Elsevier.

[66] D. Carrasco, T. Lefèvre, N. Moiroux, C. P.-C. opinion in insect, and undefined 2019, "Behavioural adaptations of mosquito vectors to insecticide control," Elsevier.

[67] J. Grieco, N. Achee, M. Sardelis, K. C.-J. of the A., and undefined 2005, "A Novel High-Throughput Screening System to Evaluate the Behavioral Response of Adult Mosquitoes to Chemicals1," BioOne.

[68] N. L.-A. review of entomology and undefined 2015, "Insecticide resistance in mosquitoes: impact, mechanisms, and research directions," annualreviews.org, vol. 60, pp. 537–559, Jan. 2015.

[69] C. Saisawang and J. Wongsantichon, "Insect glutathione transferases," Taylor Fr., vol. 43, no. 2, pp. 253–265, May 2011.

[70] J. S.-I. biochemistry and molecular biology and undefined 1999, "Cytochromes P450 and insecticide resistance," Elsevier.

[71] F. D. Rinkevich, Y. Du, and K. Dong, "Diversity and convergence of sodium channel mutations involved in resistance to pyrethroids," Pestic. Biochem. Physiol., vol. 106, no. 3, pp. 93–100, Jul. 2013.

[72] K. Dong et al., "Molecular biology of insect sodium channels and pyrethroid resistance," Insect Biochemistry and Molecular Biology, vol. 50, no. 1. Elsevier Ltd, pp. 1–17, 01-Jul-2014.

[73] M. Wooding, Y. Naudé, E. Rohwer, and M. Bouwer, "Controlling mosquitoes with semiochemicals: A review," Parasites and Vectors, vol. 13, no. 1, pp. 1–20, 2020.

[74] C. M. Jones et al., "Footprints of positive selection associated with a mutation (N1575Y) in the voltage-gated sodium channel of Anopheles gambiae," Proc. Natl. Acad. Sci. U. S. A., vol. 109, no. 17, pp. 6614–6619, Apr. 2012.

[75] L. J. Wanga, "One hundred years of general relativity-A critical view," Physics Essays, vol. 28, no. 4. Physics Essays Publication, pp. 421–442, 01-Dec-2015.

[76] M. Weill et al., "The unique mutation in ace-1 giving high insecticide resistance is easily detectable in mosquito vectors," Insect Mol. Biol., vol. 13, no. 1, pp. 1–7, Feb. 2004.

[77] J. Hemingway, N. J. Hawkes, L. McCarroll, and H. Ranson, "The molecular basis of insecticide resistance in mosquitoes," in Insect Biochemistry and Molecular Biology, 2004, vol. 34, no. 7, pp. 653–665.

[78] M. J. Donnelly, A. T. Isaacs, and D. Weetman, "Identification, Validation, and Application of Molecular Diagnostics for Insecticide Resistance in Malaria Vectors," Trends in Parasitology, vol. 32, no. 3. Elsevier Ltd, pp. 197–206, 01-Mar-2016.

[79] C. Yunta, K. Hemmings, ... B. S.-P. biochemistry, and undefined 2019, "Cross-resistance profiles of malaria mosquito P450s associated with pyrethroid resistance against WHO insecticides," Elsevier.

[80] P. Pignatelli, V. A. Ingham, V. Balabanidou, J. Vontas, G. Lycett, and H. Ranson, "The Anopheles gambiae ATP-binding cassette transporter family: phylogenetic analysis and tissue localization provide clues on function and role in insecticide resistance," Insect Mol. Biol., vol. 27, no. 1, pp. 110–122, Feb. 2018.

[81] P. Tawidian, V. Rhodes, K. M. and molecular biology, and undefined 2019, "Mosquito-fungus interactions and antifungal immunity," Elsevier.

[82] B. D. Menze et al., "Multiple insecticide resistance in the malaria vector Anopheles funestus from Northern Cameroon is mediated by metabolic resistance alongside potential target site insensitivity mutations," PLoS One, vol. 11, no. 10, Oct. 2016.

[83] S. A. S. Diakité et al., "A comprehensive analysis of drug resistance molecular markers and Plasmodium falciparum genetic diversity in two malaria endemic sites in Mali," Malar. J., vol. 18, no. 1, Nov. 2019.

[84] E. Peterson and P. Kaur, "Antibiotic resistance mechanisms in bacteria: Relationships between resistance determinants of antibiotic producers, environmental bacteria, and clinical pathogens," Front. Microbiol., vol. 9, no. NOV, pp. 1–21, 2018.

[85] J. P. Toutant, "Insect acetylcholinesterase: Catalytic properties, tissue distribution and molecular forms," Progress in Neurobiology, vol. 32, no. 5. Pergamon, pp. 423–446, 01-Jan-1989.

[86] M. Weill, P. Fort, A. Berthomieu, M. P. Dubois, N. Pasteur, and M. Raymond, "A novel acetylcholinesterase gene in mosquitoes codes for the insecticide target and is non–homologous to the ace gene Drosophila," Proc. R. Soc. London. Ser. B Biol. Sci., vol. 269, no. 1504, pp. 2007–2016, Oct. 2002.

[87] A. Mutero, M. Pralavorio, J. M. Bride, and D. Fournier, "Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase," Proc. Natl. Acad. Sci. U. S. A., vol. 91, no. 13, pp. 5922–5926, Jun. 1994.

[88] F. Faucon et al., "Identifying genomic changes associated with insecticide resistance in the dengue mosquito Aedes aegypti by deep targeted sequencing," Genome Res., vol. 25, no. 9, pp. 1347–1359, 2015.

[89] R. N'Guessan et al., "Resistance to carbosulfan in Anopheles gambiae from Ivory Coast, based on reduced sensitivity of acetylcholinesterase," Med. Vet. Entomol., vol. 17, no. 1, pp. 19–25, Mar. 2003.

[90] Ochman, Howard, Lawrence, Jeffrey G., and Groisman, Eduardo A., "Lateral gene transfer and the nature of bacterial innovation," Nature, vol. 405, no. 6784, pp. 299–304, 2000.

[91] T. WATANABE, "Infective heredity of multiple drug resistance in bacteria.," Bacteriol. Rev., vol. 27, no. 1, pp. 87–115, 1963.

[92] N. Willetts and B. Wilkins, "Processing of plasmid DNA during bacterial conjugation," Microbiological Reviews, vol. 48, no. 1. American Society for Microbiology (ASM), pp. 24–41, 1984.

[93] G. Stotzky and H. Babich, "Survival of, and Genetic Transfer by, Genetically Engineered Bacteria in Natural Environments," Adv. Appl. Microbiol., vol. 31, no. C, pp. 93–138, Jan. 1986.

[94] I. Sultan, S. Rahman, A. T. Jan, M. T. Siddiqui, A. H. Mondal, and Q. M. R. Haq, "Antibiotics, resistome and resistance mechanisms: A bacterial perspective," Front. Microbiol., vol. 9, no. SEP, 2018.

[95] N. S. PATIL, K. S. LOLE, and D. N. DEOBAGKAR, "Adaptive larval thermotolerance and induced cross-tolerance to propoxur insecticide in mosquitoes Anopheles stephensi and Aedes aegypti," Med. Vet. Entomol., vol. 10, no. 3, pp. 277–282, Jul. 1996.

[96] D. M. Livermore, "beta-Lactamases in laboratory and clinical resistance.," Clin. Microbiol. Rev., vol. 8, no. 4, pp. 557–584, Oct. 1995.

[97] F. Vidal-Aroca, A. Meng, T. Minz, M. G. P. Page, and J. Dreier, "Use of resazurin to detect mefloquine as an efflux-pump inhibitor in Pseudomonas aeruginosa and Escherichia coli," J. Microbiol. Methods, vol. 79, no. 2, pp. 232–237, Nov. 2009.

[98] J. Dreier, "Active Drug Efflux in Bacteria," in Enzyme-Mediated Resistance to Antibiotics, ASM Press, 2014, pp. 235–264.

[99] D. M. Livermore, "Multiple mechanisms of antimicrobial resistance in Pseudomonas aeruginosa: Our worst nightmare?," Clin. Infect. Dis., vol. 34, no. 5, pp. 634–640, Mar. 2002.

[100] L. J. V. Piddock, "Clinically relevant chromosomally encoded multidrug resistance efflux pumps in bacteria," Clinical Microbiology Reviews, vol. 19, no. 2. American Society for Microbiology Journals, pp. 382–402, 01-Apr-2006.

[101] H. P. Schweizer and H. P. Schweizer, "Efflux as a mechanism of resistance to antimicrobials in Pseudomonas aeruginosa and related bacteria: unanswered questions," 2003.

[102] R. Misra and V. N. Bavro, "Assembly and transport mechanism of tripartite drug efflux systems," Biochimica et Biophysica Acta - Proteins and Proteomics, vol. 1794, no. 5. Elsevier, pp. 817–825, 01-May-2009.

[103] J. A. Perry and G. D. Wright, "The antibiotic resistance 'mobilome': Searching for the link between environment and clinic," Frontiers in Microbiology, vol. 4, no. MAY. 2013.

[104] W. C Reygaert, "An overview of the antimicrobial resistance mechanisms of bacteria," AIMS Microbiol., vol. 4, no. 3, pp. 482–501, 2018.

[105] H. P. Schweizer and H. P. Schweizer, "Efflux as a mechanism of resistance to antimicrobials in Pseudomonas aeruginosa and related bacteria: unanswered questions," 2003.

[106] G. G. Zhanel, D. J. Hoban, K. Schurek, and J. A. Karlowsky, "Role of efflux mechanisms on fluoroquinolone resistance in Streptococcus pneumoniae and Pseudomonas aeruginosa," International Journal of Antimicrobial Agents, vol. 24, no. 6. Elsevier B.V., pp. 529–535, 01-Dec-2004.

[107] K. ichi Yoshida et al., "MexAB-OprM specific efflux pump inhibitors in Pseudomonas aeruginosa. Part 6: Exploration of aromatic substituents," Bioorganic Med. Chem., vol. 14, no. 24, pp. 8506–8518, Dec. 2006.

[108] Y. Hirakata et al., "Efflux pump inhibitors reduce the invasiveness of Pseudomonas aeruginosa," Int. J. Antimicrob. Agents, vol. 34, no. 4, pp. 343–346, Oct. 2009.

[109] T. Mima, H. Sekiya, T. Mizushima, T. Kuroda, and T. Tsuchiya, "Gene Cloning and Properties of the RND-Type Multidrug Efflux Pumps MexPQ-OpmE and MexMN-OprM from Pseudomonas aeruginosa," Microbiol. Immunol., vol. 49, no. 11, pp. 999–1002, Nov. 2005.

[110] K. Poole, "Multidrug Efflux Pumps and Antimicrobial Resistance in Pseudomonas aeruginosa and Related Organisms JMMB Symposium," 2001.

[111] S. Murakami, R. Nakashima, E. Yamashita, T. Matsumoto, and A. Yamaguchi, "Crystal structures of a multidrug transporter reveal a functionally rotating mechanism," Nature, vol. 443, no. 7108, pp. 173–179, 2006.

[112] M. K. Higgins, E. Bokma, E. Koronakis, C. Hughes, and V. Koronakis, "Structure of the periplasmic component of a bacterial drug efflux pump," Proc. Natl. Acad. Sci. U. S. A., vol. 101, no. 27, pp. 9994–9999, Jul. 2004.

[113] S. Eda, H. Maseda, and T. Nakae, "An elegant means of self-protection in gram-negative bacteria by recognizing and extruding xenobiotics from the periplasmic space," J. Biol. Chem., vol. 278, no. 4, pp. 2085–2088, Jan. 2003.

[114] S. Jalal, O. Ciofu, N. Høiby, N. Gotoh, and B. Wretlind, "Molecular mechanisms of fluoroquinolone resistance in Pseudomonas aeruginosa isolates from cystic fibrosis patients," Antimicrob. Agents Chemother., vol. 44, no. 3, pp. 710–712, Mar. 2000.

[115] C. J. Papadopoulos, C. F. Carson, B. J. Chang, and T. V. Riley, "Role of the MexAB-OprM efflux pump of Pseudomonas aeruginosa in tolerance to tea tree (Melaleuca alternifolia) oil and its monoterpene components terpinen-4-ol, 1,8-cineole, and α-terpineol," Appl. Environ. Microbiol., vol. 74, no. 6, pp. 1932–1935, Mar. 2008.

[116] J. MASSOULIE and S. BON, "L'acétylcholinestérase: une structure originale pour une fonction vitale," Ann. l'Institut Pasteur. Actual., vol. 4, no. 1, 1993.

[117] A. Mutero, M. Pralavorio, J. M. Bride, and D. Fournier, "Resistance-associated point mutations in insecticide-insensitive acetylcholinesterase," Proc. Natl. Acad. Sci. U. S. A., vol. 91, no. 13, pp. 5922–5926, Jun. 1994.

[118] Y. Chen, J. Au, P. Kazlas, A. Ritenour, H. Gates, and M. McCreary, "Flexible active-matrix electronic ink display," Nature, vol. 423, no. 6936, p. 136, 2003.

[119] H. Alout, L. Djogbénou, C. Berticat, F. Chandre, and M. Weill, "Comparison of Anopheles gambiae and Culex pipiens acetycholinesterase 1 biochemical properties," Comp. Biochem. Physiol. - B Biochem. Mol. Biol., vol. 150, no. 3, pp. 271–277, Jul. 2008.

[120] I. Chopra, P. M. Hawkey, and M. Hinton, "Tetracyclines, molecular and clinical aspects," J. Antimicrob. Chemother., vol. 29, no. 3, pp. 245–277, Mar. 1992.

[121] P. Labbé, H. Alout, L. Djogbénou, N. Pasteur, and M. G. Weill, "Evolution of Resistance to insecticide in disease vectors," in Genetics and Evolution of Infectious Diseases, Elsevier Inc., 2011, pp. 363–409.

[122] H. Ranson, R. N'Guessan, J. Lines, N. Moiroux, Z. Nkuni, and V. Corbel, "Pyrethroid resistance in African anopheline mosquitoes: What are the implications for malaria control?," Trends in Parasitology, vol. 27, no. 2. Elsevier Current Trends, pp. 91–98, 01-Feb-2011.

[123] "Global Pesticide Resistance in Arthropods - Google Books." [Online]. Available: https://books.google.com.pk/books?hl en&lr &id yVaM4T60SqgC&oi fnd&pg PR5&dq Hollingworth RM, Dong K. The biochemical and molecular genetic basis of resistance to pesticides in arthropods. Global pesticide resistance in arthropods. Wallingford: CAB International [Accessed: 01-Jan-2021].

[124] D. M. Soderlund and D. C. Knipple, "The molecular biology of knockdown resistance to pyrethroid insecticides," Insect Biochemistry and Molecular Biology, vol. 33, no. 6. Elsevier Ltd, pp. 563–577, 01-Jun-2003.

[125] D. Martinez-Torres, C. Chevillon, A. Brun-Barale, J. B. Berge, N. Pasteur, and D. Pauron, "Voltage-dependent Na channels in pyrethroid-resistant Culex pipiens L mosquitoes," Pestic. Sci., vol. 55, no. 10, pp. 1012–1020, Oct. 1999.

[126] Q. Xu, H. Wang, L. Zhang, and N. Liu, "Kdr allelic variation in pyrethroid resistant mosquitoes, Culex quinquefasciatus (S.)," Biochem. Biophys. Res. Commun., vol. 345, no. 2, pp. 774–780, Jun. 2006.

[127] D. Martinez-Torres et al., "Molecular characterization of pyrethroid knockdown resistance (kdr) in the major malaria vector Anopheles gambiae s.s.," Insect Mol. Biol., vol. 7, no. 2, pp. 179–184, May 1998.

[128] J. G. Scott, M. H. Yoshimizu, and S. Kasai, "Pyrethroid resistance in Culex pipiens mosquitoes," Pesticide Biochemistry and Physiology, vol. 120. Academic Press Inc., pp. 68–76, 01-May-2015.

[129] A. Tomasz1 and R. Muñoz2, "/3-Lactam Antibiotic Resistance in Gram-Positive Bacterial Pathogens of the Upper Respiratory Tract: A Brief Overview of Mechanisms," Mary Ann Liebert, Inc, 1995.

[130] J. Garcia-Bustos and A. Tomasz, "A biological price of antibiotic resistance: Major changes in the peptidoglycan structure of penicillin-resistant pneumococci," Proc. Natl. Acad. Sci. U. S. A., vol. 87, no. 14, pp. 5415–5419, Jul. 1990.

[131] M. Michel and L. Gutmann, "Methicillin-resistant Staphylococus aureus and vancomycin-resistant enterococci: Therapeutic realities and possibilities," Lancet, vol. 349, no. 9069. Lancet Publishing Group, pp. 1901–1906, 28-Jun-1996.

[132] N. Liu, Q. Xu, F. Zhu, and L. Zhang, "Pyrethroid resistance in mosquitoes," Insect Sci., vol. 13, no. 3, pp. 159–166, 2006.

[133] J. M. Schapiro et al., "The effect of high-dose saquinavir on viral load and CD4 t-cell counts in HIV-infected patients," Ann. Intern. Med., vol. 124, no. 12, pp. 1039–1050, 1996.

[134] H. McIlleron, G. Meintjes, W. J. Burman, and G. Maartens, "Complications of antiretroviral therapy in patients with tuberculosis: Drug interactions, toxicity, and immune reconstitution inflammatory syndrome," in Journal of Infectious Diseases, 2007, vol. 196, no. SUPPL. 1, pp. S63–S75.

[135] T. J. Opperman and S. T. Nguyen, "Recent advances toward a molecular mechanism of efflux pump inhibition," Front. Microbiol., vol. 6, no. MAY, pp. 1–16, 2015.

[136] B. Max and R. Sherer, "Management of the Adverse Effects of Antiretroviral Therapy and Medication Adherence," Clin. Infect. Dis., vol. 30, no. Supplement 2, pp. S96–S116, Jun. 2000.

[137] J. Durant et al., "Drug-resistance genotyping in HIV-1 therapy: The VIRADAPT randomised controlled trial," Lancet, vol. 353, no. 9171, pp. 2195–2199, Jun. 1999.

[138] M. Askoura, W. Mottawea, T. Abujamel, and I. Taher, "Efflux pump inhibitors (EPIs) as new antimicrobial agents against Pseudomonas aeruginosa," Libyan J. Med., vol. 6, no. 1, pp. 1–8, 2011.

[139] E. Puchhammer-Stockl, C. Steininger, E. Geringer, and F. Heinz, "Comparison of virtual phenotype and HIV-SEQ program (Stanford) interpretation for predicting drug resistance of HIV strains," HIV Med., vol. 3, no. 3, pp. 200–206, Jul. 2002.

[140] R. W. Shafer, M. A. Winters, S. Palmer, and T. C. Merigan, "Multiple concurrent reverse transcriptase and protease mutations and multidrug resistance of HIV-1 isolates from heavily treated patients," Ann. Intern. Med., vol. 128, no. 11, pp. 906–911, Jun. 1998.

[141] P. S. Eastman et al., "Genotypic Changes in Human Immunodeficiency Virus Type 1 Associated with Loss of Suppression of Plasma Viral RNA Levels in Subjects Treated with Ritonavir (Norvir) Monotherapy," J. Virol., vol. 72, no. 6, pp. 5154–5164, Jun. 1998.

[142] P. R. Harrigan and H. C. F. Côté, "Clinical Utility of Testing Human Immunodeficiency Virus for Drug Resistance," Clin. Infect. Dis., vol. 30, no. Supplement2, pp. S117–S122, Jun. 2000.

[143] K. Stepniewska and N. J. White, "Pharmacokinetic determinants of the window of selection for antimalarial drug resistance," Antimicrob. Agents Chemother., vol. 52, no. 5, pp. 1589–1596, 2008.

[144] F. J. Giordano et al., "199 6 2," Nat. Med., vol. 2, no. 4, pp. 534–539, 1996.

[145] H. A. Duarte et al., "Implementation of a point mutation assay for HIV drug resistance testing in Kenya," AIDS, vol. 32, no. 16, pp. 2301–2308, Oct. 2018.

[146] J. K. Thomas et al., "Pharmacodynamic evaluation of factors associated with the development of bacterial resistance in acutely ill patients during therapy," Antimicrob. Agents Chemother., vol. 42, no. 3, pp. 521–527, Mar. 1998.

[147] G. D. Wright, "The antibiotic resistome: The nexus of chemical and genetic diversity," Nat. Rev. Microbiol., vol. 5, no. 3, pp. 175–186, Mar. 2007.

[148] V. D'Costa, K. McGrann, D. H.- Science, and undefined 2006, "Sampling the antibiotic resistome," science.sciencemag.org, 2006.

[149] K. Lee et al., "Mobile resistome of human gut and pathogen drives anthropogenic bloom of antibiotic resistance," Microbiome, vol. 8, no. 1, Jan. 2020.

[150] J. Mahillon and M. Chandler, "Insertion Sequences," Microbiol. Mol. Biol. Rev., vol. 62, no. 3, p. 725, Feb. 1998.

[151] S. He et al., "The IS200/IS605 Family and 'Peel and Paste' Single-strand Transposition Mechanism," 2015.

[152] I. Lysnyansky et al., "Molecular characterization of newly identi¢ed IS3, IS4 and IS30 insertion sequence-like elements in Mycoplasma bovis and their possible roles in genome plasticity."

[153] I. Ujváry, "Pest Control Agents from Natural Products," Hayes' Handb. Pestic. Toxicol., pp. 119–229, 2010.

[154] L. Bennet, "Derris Pest Control Agents from Natural Products Development & Modification of Bioactivity Rotenone Environmental Factors in Neurodegenerative Diseases Complex I Inhibition , Rotenone and Parkinson's Disease Natural Products Structural Diversity-II Secondary Metabolites: Sources , Structures and Chemical Biology Role of Deguelin in Chemoresistance Chemical Ecology and Phytochemistry of Forest Ecosystems," pp. 2014–2016, 2014.

An Appendix

Table 1: Docking results of *Escherichia coli* against Derris

Sr No.	**Protein**	**Binding Affinty**	**Interactions Residues/ Hydrogen Bond**	**Unfavourable Bump**
1	IS3OH	-130.9	ARG-280	ARG-279
			ALA-282	
			ASP-277	
2	ISec5	-201.02	ALA-219	ASP-218
			ILE-175	GLU-352
			ILE-215	
			MET-220	
			HIS-173	
			HIS-348	
3	ISec16	-376.92	ILE-49	TYR-113
			CYS-114	ARG-80
			ALA-89	MET-81
			LEU-90	
			ALA-86	
4	ISErsp1	-211.92	VAL-95	GLU-97
			ALA-96	
			TYR-94	
5	IS Pa41	-146.45	VAL-16	ARG-9
			LEU-12	
			LEU-13	

			ILE-10	
6	ISpmo2	-146.45	LEU-15	ARG-11
			ILE-12	
			VAL-18	
			LEU-14	
7	ISSba14	-263.91	MET-80	ALA-84
			CYS-87	
8	IS2	-183.8	PRO-409	TYR-395
			PRO-400	HIS-401
			TRP-398	
			HIS-394	
			LEU-230	
			ARG-370	
			ASP-371	
9	IS3F	-271.39	GLY-251	GLU-250
			PRO-273	TRP-252
			ARG-357	
10	IS4	-241.02	ILE-458	TRP-453
			LEU-432	GLN-456
11	IS5	-142.81	LEU-185	GLN-182
			ALA-183	
12	IS5D	-197.49	CYS-98	SER-9
			ILE-85	
			SER-86	
			SER-90	
13	IXIX1	-283.87	LEU-164	VAL-174
			LEU-190	
			LEU-167	
			ASP-141	
			VAL-172	
			ILE-143	

			ARG-142	
			TYR-197	
14	IXIX4	-283.87	LEU-190	VAL-174
			LEU-164	
			LEU-167	
			VAL-172	
			ASP-141	
			ARG-142	
			ILE-143	
			TYR-197	
15	IS103	-75.67	LEY-168	ASP-100
			LEU-161	ARG-156
			ARG-171	ARG-157
			ALA-77	
16	IS3OH	-130.9	ARG-217	ILE-227
			ARG-221	
			HIS-224	
17	IS621	-248.39	CYS-5	ARG-14
			ALA-6	
			PHE-8	
			MET-9	
			PRO-10	
			ALA-12	
18	ISCro3	-309.27	ALA-44	TRP-41
			ALA-24	
			ALA-25	
			THR-35	
			TYR-16	
19	ISDIsp1	-217.86	LEU-9	PHE-12
			LEU-13	
			PRO-8	

			MET-16	
			ILE-20	
20	ISEc17	-271.39	PRO-273	GLU-250
			ARG-357	TRP-252
			ILE-355	
			GLY-251	
21	ISEc26	-124.78	ALA-312	LEU-285
			LYS-313	
			LEU-266	
			LEU-285	
			LEU-266	
22	ISEc68	-197.49	SER-85	SER-87
			CYS-93	
			SER-81	
			ILE-80	
23	ISEhe3	-280.25	PHE-38	GLN-41
			PHE-39	
24	ISKpn8	-75.67	LEU-161	ARG-157
			LEU-168	ASP-100
			ARG-171	ARG-156
			ALA-77	
25	ISPa26	-146.45	LEU-14	ARG-11
			LEU-15	
			VAL-18	
			ILE-12	
26	ISPa52	-146.45	LEU-14	ARG-11
			LEU-15	
			VAL-18	
			ILE-12	
27	ISPcc3	-255.02	CYS-281	GLN-278
			ALA-275	VAL-282

			VAL-284	
			ARG-287	
28	ISPeat2	-170.52	ASN-66	HIS-33
			LYS-70	
			GLN-63	
29	ISPpu21	-146.45	ILE-52	ARG-48
			ILE-49	
			LEU-51	
			VAL-55	
30	ISSen6	-109.93	ALA-110	SER-20
			ILE-113	ASP-106
			SER-74	
			ARG-88	
			SER-92	
			ALA-105	
31	ISSFI10	-219.04	LEU-173	GLU-72
			ARG-75	
32	ISSod13	-244.29	PHE-47	MET-41
			HIS-53	ALA-38
			PHE-50	
			ILE-54	
33	ISVch5	-206	GLY-83	PHE-125
			LYS-106	GLY-86
			VAL-107	
			VAL-82	
			LEU-158	
			LEU-161	
			ALA-162	
34	ISVch9	-160.28	ALA-15	VAL-23
			ASP-9	ASN-22
				ARG-6

				THR-16
35	MITEEc1	-220.98	TYR-21	SER-15
				SER-24
				SER-25
				ARG-14
				GLN-17
36	IS150	-27.36	ALA-348	GLN-344
			SER-434	ASP-437
			PHE-360	
			HIS-375	
			LYS-359	
			GLN-438	
			ARG-382	
37	IS609	-75.67	LEU-161	ASP-160
			LEU-168	ARG-156
			ARG-171	ARG-157

Table 2: Orthologs of selected microbes

Type of Microbes	Selected Sequences	Description	Scientific Name	Max Score	Total Score	Query Cover	E Value	% age Identity	Acc. Len	Accession
Escherichia coli	seq CP048439.1	*Escherichia coli* strain NBRC 3301 chromosome, complete genome	*Escherichia coli*	2.232-e+05	2.517-e+05	100%	0.0	100.00%	4656310	CP048439.1
	seq CP027060.1	*Escherichia coli* str. K-12 substr. MG1655 strain K-12 chromosome	*Escherichia coli* str. K-12 substr. MG1655	2.232-e+05	2.449-e+05	100%	0.0	100.00%	4653240	CP027060.1
	seq AP009048.1	*Escherichia coli* str. K-12 substr. W3110 DNA, complete	*Escherichia coli* str. K-12 substr.	2.232-e+05	2.537-e+05	100%	0.0	100.00%	4646332	AP009048.1

		genome	W3110							
	seq CP0-09685.1	*Escherichia coli* str. K-12 substr. MG1655, complete genome	*Escherichia coli* str. K-12 substr. MG1655	2.232-e+05	2.565-e+05	100%	0.0	100.00%	463-6831	CP00-9685.1
	seq CP0-43211.1	*Escherichia coli* O16:H48 strain PG20-180050 chromosome, complete genome	*Escherichia coli* O16:H48	2.232-e+05	2.595-e+05	100%	0.0	100.00%	461-5313	CP04-3211.1
	seq CP0-09644.1	*Escherichia coli* ER2796, complete genome	*Escherichia coli* ER2796	2.232-e+05	2.578-e+05	100%	0.0	99.99%	455-8663	CP00-9644.1
Plasmodium berghei	seq AB5-58173.1	*Plasmodium bergheimito-*	*Plasmodium*	10949	10949	100%	0.0	100.00%	5957	AB55-8173.1

	chondrial DNA, complete genome	*berghei*							
seq M2-9000.1	*Plasmodium yoelii* cytochrome c oxidase subunit 1 (cox1) gene, complete cds; and cytochrome b (cob) gene, complete cds	*Plasmodium yoeliiyoelii*	10333	10333	100%	0.0	97.90%	5956	M290-00.1
seq AB3-79671.1	*Plasmodium chabaudiadami* mitochondrial	*Plasmodium* chabaudia-	9191	9191	99%	0.0	94.49%	5949	AB37-9671.1

	coxIII, coxI, cytb genes for cytochrome c oxidase subunit III, cytochrome c oxidase subunit I, cytochrome b, complete cds, strain: DK	dami							
seq LK0-23131.1	*Plasmodium berghei* ANKA genome assembly, chromosome: MIT	*Plasmodium berghei* ANKA	8641	10939	100%	0.0	99.72%	5957	LK02-3131.1
seq HQ7-12051.1	*Plasmodium atheruri*	*Plasmodium*	8983	9756	98%	0.0	96.63%	5851	HQ71-2051.1

	cytochrome oxidase subunit 3 (cox3) gene, complete cds; cytochrome oxidase subunit 1 (cox1) gene, partial cds; and cytochrome b (cytb) gene, complete cds; mitochondrial	*atheruri*							
seq AB3-54571.1	*Plasmodium ovale* mitochondrial cox3, cox1, cytb genes for	*Plasmodium ovale*	8150	8253	99%	0.0	91.58%	5974	AB35-4571.1

	cytochrome oxidase subunit 3, cytochrome oxidase subunit 1, cytochrome b, complete and partial cds								
seq AB44-4132.1	*Plasmodium fieldi* mitochondrial cox3, cox1, cytb genes for cytochrome oxidase subunit 3, cytochrome oxidase	*Plasmodium fieldi*	8111	8111	100%	0.0	91.16%	5988	AB44-4132.1

	subunit 1, cytochrome b, complete and partial cds, strain: ATCC 30164								
seq AB4-34920.1	*Plasmodium simiovale* mitochondrial cox3, cox1, cytb genes for cytochrome oxidase subunit 3, cytochrome oxidase subunit 1, cytochrome b, complete and partial cds	*Plasmodium* simiovale	8100	8100	100%	0.0	91.12%	5987	AB43-4920.1

seq AB4-44125.1	*Plasmodium cynomolgi* mitochondrial cox3, cox1, cytb genes for cytochrome oxidase subunit 3, cytochrome oxidase subunit 1, cytochrome b, complete and partial cds, strain: Ceylonensis	*Plasmodium* cynomolgi	8093	8093	99%	0.0	91.11%	5983	AB44-4125.1
seq KF6-68407.1	*Plasmodium vivax* isolate 47CDC cytochrome oxidase	*Plasmodium* vivax	8091	8091	100%	0.0	91.09%	5990	KF66-8407.1

		subunit 3 (cox3) gene, complete cds; cytochrome oxidase subunit 1 (cox1) gene, partial cds; and cytochrome b (cytb) gene, complete cds; mitochondrial								
Wolbachia sp.	seq CP01391.1	*Wolbachia* sp.wRi, complete genome	*Wolbachia* sp. wRi	2.093-e+05	6.244-e+05	100%	0.0	100.00%	144-5873	CP001391.1
	seq CP072012.1	*Wolbachia pipientis* strain wCin2USA1 chromosome,	*Wolbachia* pipientis	36616	4.651-e+05	93%	0.0	97.63%	153-8351	CP072012.1

	complete genome								
seq OD0-9258.1	1_Tps_b3v08	Timema poppensis	23852	29508	15%	0.0	98.35%	20126	OD00-9258.1
seq XM_01-2006175.1	PREDICTED: Vollenhoviaemeryi uncharacterized LOC105558474 (LOC105558474), mRNA	Vollenho viaemeryi	3033	7891	4%	0.0	93.04%	7929	XM_0-120061-75.1
seq XM_03-4983981.1	PREDICTED: Maniolahy-perantus aspartyl/ glutamyl-tRNA(Asn /Gln) amidotrans-	Maniolah-yperantus	2093	2093	1%	0.0	95.23%	1335	XM_03-4983-981.1

		ferase subunit B (LOC11-7995968), mRNA								
Plasmodium vivax	seq NC_0-5.1	*Homo sapiens* chromosome 5, GRCh38.p13 Primary Assembly	*Homo sapiens*	71.3	138	0%	9e-07	95.56%	1815-38259	NC_0-00005.10
	seq NC_0-7.14	*Homo sapiens* chromosome 7, GRCh38.p13 Primary Assembly	*Homo sapiens*	65.8	65.8	0%	4e-05	95.12%	1593-45973	NC_0-7.14
	seq NC_0-23.11	*Homo sapiens* chromosome X, GRCh38.p13	*Homo sapiens*	56.5	56.5	0%	0.006	94.44%	1560-40895	NC_0-23.11

		Primary Assembly								
	seq NC_0-17.11	*Homo sapiens* chromosome 17, GRCh38.p13 Primary Assembly	*Homo sapiens*	56.5	56.5	0%	0.028	100.00%	832-57441	NC_0-17.11
	seq NC_0-2.12	*Homo sapiens* chromosome 2, GRCh38.p13 Primary Assembly	*Homo sapiens*	63.9	120	0%	1e-04	93.02%	2421-93529	NC_0-2.12
Plasmodium falciparum	seq AL8-44501.2	*Plasmodium falciparum* 3D7 genome assembly, chromosome: 1	*Plasmodium falciparum* 3D7	3.289-e+05	1.637-e+06	100%	0.0	100.00%	640-851	AL84-4501.2
	seq LT9-									

63414.1	*Plasmodium* sp. *gorilla* clade G1 genome assembly, chromosome: 1	*Plasmodium* sp. *gorilla* clade G1	40915	1.765-e+05	61%	0.0	96.45%	519-140	LT96-3414.1