INVESTIGATING INSECTICIDE RESISTANCE MECHANISMS IN DENGUE VECTORS POPULATION; *Aedes aegypti* AND *Aedes albopictus* IN PENANG ISLAND

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LIST OF ABBREVIATIONS

Ach	Acetylcholine
AchE	Acetylcholinesterase
Ace-1	Acetylcholinesterase 1
BP	Balik Pulau
BPNE	Balik Pulau Non-exposed
BPR	Balik Pulau Resistance
BPS	Balik Pulau Susceptible
Bti	*Bacillus thuringiensis israelensis*
CB	Carbamate
cDNA	complementary DNA
CHIKV	chikungunya virus
CYP450	cytochrome P450
DHF	Dengue haemorrhagic fever
DNTP	Deoxynucleotide Triphosphate
DTNB	5.5'-Dithio-Bis (2- NItrobenzoic Acid)
EST	Esterase
F0	Fillial 0
F1	Fillial 1
GABA	Gamma-aminobutyric acid

gDNA	genomic DNA
GST	glutathione-S-transferase
IGR	insect growth regulator
ITS	internal transcribe spacers
JE	Japanese encephalitis
JG	Jelutong
JGNE	Jelutong Non-exposed
JGR	Jelutong Resistance
JGS	Jelutong Susceptible
kdr	knockdown resistance
KdT	Knockdown Time
MFO	mixed function oxidase
MOH	Ministry of Health
OD	optical density
OP	Organophosphate
OR	odd ratio
P.	*Poecilia*
PCR	Polymerase chain reaction
PY	pyrethroid
qPCR	real-time polymerase chain reaction

Rdl	resistance to Dieldrin
RFLP	restriction fragment length polymorphisms
RNA	ribonucleic acid
RR	resistance ratio
SA	Sungai Ara
SANE	Sungai Ara non-exposed
SAR	Sungai Ara resistance
SAS	Sungai Ara susceptible
TMBZ	3,3’5,5’-Tetramethylbenzidine
USM	Universiti Sains Malaysia
ULV	ultra low volume
VCRU	Vector Control Research Unit
VGSC	Voltage-gated sodium channel
WHO	World Health Organization
ZIKV	Zika Virus

MENYIASAT MEKANISME KERINTANGAN RACUN SERANGGA DALAM POPULASI VEKTOR DENGGI; *Aedes aegypti* DAN *Aedes albopictus* DI PULAU PINANG

ABSTRAK

Di Malaysia, vektor penyakit arbovirus terutamanya *Aedes aegypti* dan *Ae. albopictus* telah lama dikenali sebagai vektor yang boleh menyebarkan virus denggi. Pada masa ini, kawalan kimia dianggap sebagai cara yang paling berkesan untuk mengawal vektor denggi di dalam kawasan wabak denggi. Walau bagaimanapun, pergantungan melampau terhadap kawalan kimia telah menyebabkan perkembangan terhadap kerintangan racun serangga di dalam vektor denggi akibat kehadiran mekanisme kerintangan racun serangga. Kajian ini mengetengahkan status kerentanan vektor denggi yang terdedah kepada racun serangga permethrin dan malathion melalui ujian bioasai nyamuk dewasa WHO. Mekanisme kerintangan racun serangga yang terlibat dalam vektor denggi dengan menumpukan kepada mekanisme tapak sasaran melalui penjenisan gen voltage gated sodium channel (VGSC) dan gen Acetylcholinesterase (Ace-1) dikenal pasti melalui asai tindak balas berantai polymerase (PCR). Kehadiran mekanisme kerintangan metabolik di dalam sampel vektor denggi dari kawasan Sungai Ara (SA), Jelutong (JG), dan Balik Pulau (BP) di Pulau Pinang telah dikenal pasti melalui ujian biokimia dan PCR kuantitatif (qPCR). Kajian kerentanan racun serangga menunjukkan bahawa sampel *Ae. aegypti* dewasa dari kawasan SA, JG, dan BP adalah rintang terhadap permethrin 0.25% dan 0.75%, manakala *Ae. aegypti* dari semua kawasan pensampelan didapati rentan kepada malathion 5%. Mutasi F1534C dan S989P didapati pada sampel *Ae. aegypti* yang diuji

dari semua kawasan pensampelan yang dipilih tetapi tiada korelasi diperhatikan antara fenotip dan genotip rintang di setiap individu *Ae. aegypti*. Dalam kajian ini, kehadiran kedua-dua kerintangan tapak sasaran dan metabolik dapat dilihat pada sampel *Ae. aegypti* dari JG. Sampel *Ae. aegypti* dari JG dikesan mempunyai gabungan mutasi F1534C, peningkatan dalam enzim oksidasi fungsi campuran (MFO) dan peningkatan regulasi gen CYP9J32. Ujian bioasai pada sampel *Ae. albopictus* dari semua kawasan penyampelan yang terdedah kepada permethrin 0.75% menunjukkan status kerentanan yang tinggi. Berbeza dengan *Ae. aegypti*, *Ae. albopictus* dari SA dan JG didapati rintang terhadap malathion 5%. Walau bagaimanapun, tiada mutasi dikesan pada gen tapak sasaran di dalam sampel yang diuji dari semua kawasan. Peningkatan dalam peratus AchE selepas perencatan oleh propoxur dan kehadiran CYP6N3 dalam sampel *Ae. albopictus* yang tidak terdedah mungkin menyumbang kepada kerintangan *Ae. albopictus* terhadap OP. Kajian ini mededahkan bahawa status kerentenan vektor denggi terhadap racun serangga permethrin dan malathion tidak semata-mata bergantung kepada mekanisme kerintangan gen tapak sasaran, tetapi mekanisme rintangan jenis lain seperti mekanisme rintangan detoksifikasi metabolik juga boleh memainkan peranan dalam menyebabkan rintangan permethrin dan malathion dalam sampel vektor denggi dari SA, JG dan BP. Oleh itu, pengurusan kerintangan racun serangga perlu dilakukan untuk mengelakkan keadaan yang lebih teruk di dalam kawasan wabak denggi akibat perkembangan kerintangan racun serangga terhadap racun yang digunakan di dalam program kawalan vektor.

INVESTIGATING INSECTICIDE RESISTANCE MECHANISMS IN DENGUE VECTORS POPULATION; *Aedes aegypti* AND *Aedes albopictus* IN PENANG ISLAND

ABSTRACT

In Malaysia, the vector of arboviral diseases especially *Aedes aegypti* and *Ae. albopictus* has long been known as a vector that could transmit dengue viruses. Currently, chemical control is considered as the most efficient way to control the dengue vectors in the dengue outbreak areas. However, the heavy reliance on chemical control has resulted in the development of insecticide resistance in the dengue vectors due to the presence of insecticide resistance mechanism. This study highlights the susceptibility status of both dengue vectors exposed to permethrin and malathion insecticides via WHO adult mosquito bioassay test. The mechanisms involved in the insecticide resistance of dengue vectors by focusing on target-site resistance mechanism via genotyping the voltage gated sodium channel gene (VGSC), and Acetylcholinesterase gene (Ace-1) have been identified via polymerase-chain reaction (PCR) assays. The presence of metabolic resistance mechanisms has been investigated through biochemical enzyme assays and quantitative PCR (qPCR) in the samples of dengue vectors collected from Sungai Ara (SA), Jelutong (JG) and Balik Pulau (BP) areas of Penang Island. The insecticide susceptibility study showed that the adult *Ae. aegypti* samples from SA, JG and BP were resistant towards permethrin 0.25% and 0.75%, while the *Ae. aegypti* from all three sampling areas were susceptible towards malathion 5%. The F1534C and S989P mutation were found in the tested *Ae. aegypti* samples from all selected sampling areas but no correlation was observed between

resistant phenotype and genotype in *Ae. aegypti* individuals. In this study, the presence of both target-site and metabolic resistance mechanisms could be observed in the *Ae. aegypti* samples from JG. The samples of *Ae. aegypti* from JG were detected to have F1534C mutation, elevation in the mixed function oxidase enzyme (MFO) and the upregulation of CYP9J32 gene. The bioassay test on *Ae. albopictus* samples from all three sampling areas which was exposed to permethrin 0.75% showed a high susceptibility status. In contrast to *Ae. aegypti*, the *Ae. albopictus* from SA and JG was found to be resistance towards malathion 5%. However, no mutation in target-site gene were detected in the tested samples of *Ae. albopictus* from all areas. The elevated level in the percentage of AChE after propoxur inhibition and the presence of CYP6N3 in the non-exposed samples of the *Ae. albopictus* might contribute to the developing resistance of *Ae. albopictus* towards OP. Based on this research, it reveals that the susceptibility status of dengue vectors towards permethrin and malathion insecticides does not solely dependent on target-site gene resistance mechanism, however other type of resistance mechanisms such as metabolic detoxification resistance mechanism might also play role in causing the permethrin and malathion resistance in dengue vectors samples from SA, JG and BP. Therefore, a proper insecticide resistance management must be done to avoid a worse situation in the dengue outbreak areas due to the development of insecticide resistance towards the insecticide used in the vector control programmes.

CHAPTER 1

GENERAL INTRODUCTION

Dengue is the most important arthropod-borne virus disease infecting humans worldwide namely in tropical, subtropical and temperate areas (Murray et al., 2013). Previously, dengue fever is primarily known as an urban disease in tropical and subtropical areas, however, nowadays it has spread into rural areas (Vong et al., 2010). The dengue virus is carried by mosquitoes particularly from the species of *Aedes aegypti* and *Ae. albopictus*. The main vector for dengue transmission is *Ae. aegypti*, meanwhile the Asian tiger mosquito, *Ae. albopictus* is the secondary vector that could transmit the dengue virus. *Ae. aegypti* or known as yellow fever mosquito originates from sub-Saharan Africa whereby their immature stages lived in tree-holes (Powell & Tabachnick, 2013). The oviposition sites of *Ae. aegypti* has also been recorded in several types of natural habitats such as rock hole, leaf axils and tree holes (Chadee et al., 1998). This also shows the *Ae. aegypti* has a wide adaptability by being able to lay their eggs in both natural and man-made container such as in plastic containers and coconut shells or husks in rural areas (Basari et al., 2016; Chareonviriyaphap et al., 2003). Meanwhile, the secondary vector of dengue virus, *Aedes albopictus* originated from Southeast Asia, and spread to the islands of the Western Pacific and the Indian Ocean (Delatte et al., 2009).

The dengue virus transmitted by these two dengue vectors belong to the genus flavivirus in the family *Flaviviridae* which has four strains known as DENV-1, DENV-2, DENV-3 and DENV-4 (Guzman et al., 2010). The recent findings on DENV-5 virus which is only found in Sarawak and only found circulating in the sylvatic cycle particularly amongst the non-human primates is believed to be limited

to the forest canopies of Southeast Asia (Mustafa et al., 2015). A person who gets infected with any four of the dengue virus will have a lifelong immunity to that specific serotype (Murray et al., 2013). However, the infections with one specific serotype would not results in the cross-protective immunity against other serotypes (Gubler & Clark, 1995). Secondary infection by another serotype of dengue virus would cause a severe form of dengue diseases such as dengue haemorrhagic fever due to immune enhancement (Peeling et al., 2010). Other than dengue virus, the recent Zika outbreak in Latin and South America indicates that the *Ae. aegypti* are also capable in transmitting the Zika virus (Sikka et al., 2016).

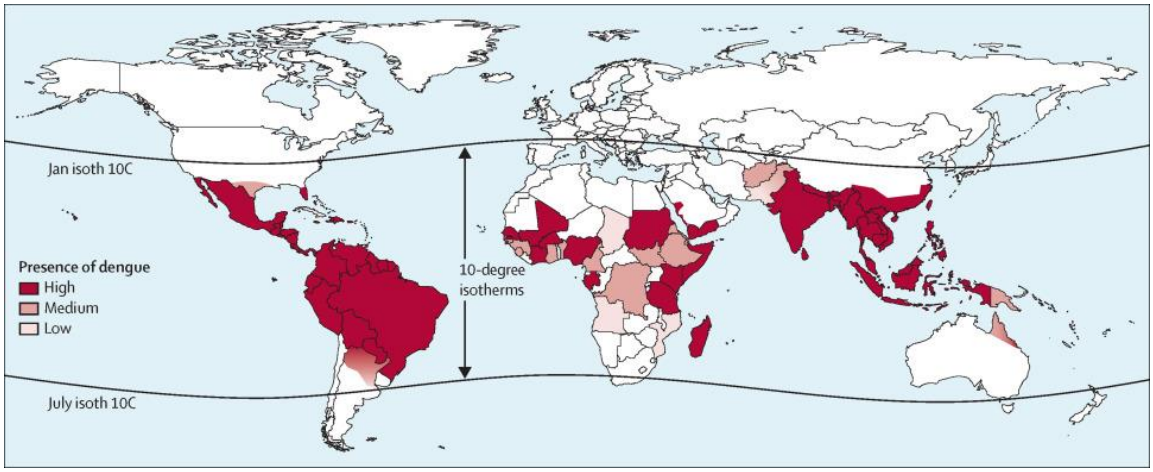

Figure 1.1: Global Map of Dengue Fever, 2014 (Guzman & Harris, 2015).

The global expansion of international trade and travel has increased the distribution of *Ae. aegypti* and *Ae. albopictus* (Kraemer et al., 2015). *Aedes aegypti* is widely distributed in tropical and subtropical regions of Asia, Africa, Southern United States, Northern Australia and the Indian Ocean Islands. Meanwhile, *Ae. albopictus* which originated from Southeast Asia is now present in United States, Northern Australia and some countries in Africa and Europe (Medlock et al., 2012). However, the countries located in tropical and subtropical regions such as in the South-East Asia and Western Pacific Regions are severely affected by dengue fever disease

(Thisyakorn & Thisyakorn, 2015; WHO, 2009). Historically, the severe form of dengue cases such as dengue haemorrhagic fever (DHF) is first recognized in a few Asian countries such as Phillipines and Thailand starting from the year of 1950s (WHO, 2017a).

In Malaysia, the dengue cases were first reported in the year of 1901-1902 from Penang Island (Skae, 1902). The dengue incidence has continued to increase since 2001 until 2014 due to the uncontrollable factors such as environmental conditions, human behavior, rapid urbanization and also international travel (Mudin, 2015). Recent evidence also shows that insecticide resistance in Malaysian dengue vectors might play a role in the increasing cases of dengue fever (Hasan et al., 2016). In the mid of 2017, the dengue cases in Malaysia showed a reduction with 43,807 cases compared to the mid of 2016 with 52,185 cases (WHO, 2017b).

In order to control the *Aedes* mosquito from transmitting the dengue virus, several vector control strategies such as environmental management by modification of building design such as firstly by modifying the house-design that would eliminate the potential breeding habitat e.g eliminate roof gutters and installing mosquito screen on windows and doors (Zainon et al., 2016; Montgomery & Ritchie, 2002). Furthermore, the cracks and crevices that would allow the mosquito to enter the house and at the same time laying the eggs have to be sealed (McCall et al., 2009). Secondly, environmental sanitation by improving solid waste management, and biological and as well as chemical control of *Aedes* has to be strictly implemented so that an effective vector control management could be achieved (McCall et al., 2009; Kumarasamy, 2006). Currently in Malaysia, several vector control strategies have been taken into place such as for larval control; sources reduction, usage of Abate larvicides, house

inspections, enforcement of Destruction of Disease-bearing Insect Act 1975 and environmental management (Ong, 2016). Meanwhile for adult control, fogging activities such as thermal fogging by using Organophosphate in fuel oil or ultra-low volume (ULV) fogging were carried out once a dengue case occurs and the health authority is notified as it could rapidly reduce adult *Aedes* mosquitoes in the dengue outbreak areas (Ruhil Amal et al., 2011; Lam, 1994). However, the frequent usage of the same classes of chemical insecticides in the fogging activities and larvicides has allowed or increase the chances of insecticide resistance to develop in the targeted dengue vectors.

Generally, insecticide resistance is caused by the insensitivity of an insect population to an insecticide (Insecticide Resistance Action Committee, n.d). This is reflected in the repeated failure of vector control programme when the same classes of chemical insecticides are being used in the control programme. Insecticide resistance in dengue vectors primarily in *Aedes aegypti* is widespread due to the over-reliance of chemical control used by the public health sectors or the authority (Chanda et al., 2015). Insecticide rotations, mixtures and mosaics have to be implemented to reduce the insecticides resistance problem in the dengue vectors (Hemingway & Ranson, 2000).

In Malaysia, a few cases of insecticide resistance in dengue vectors has been reported. In the major city such as Kuala Lumpur, resistance profiles show that *Ae. aegypti* and *Ae. albopictus* has developed resistance to pyrethroid (permethrin and deltamethrin), while in a recent study reported that the *Ae. albopictus* population from a few selected hotspot dengue areas in Kuala Lumpur have a full susceptibility when exposed to permethrin 0.75% (Ishak et al., 2015; Wan-Norafikah et al., 2013).

Furthermore, both of *Ae. aegypti* and *Ae. albopictus* populations from Penang, Kuala Lumpur and Johor Bharu also shows resistance when exposed to carbamate (bendiocarb), meanwhile both *Ae. aegypti* and *Ae. albopictus* populations from Kuala Lumpur shows resistance when exposed to organophosphate (Malathion) (Ishak et al., 2015). The development of insecticide resistance in dengue vector is a serious threat to an effective and sustainable vector control programs (WHO, 2014). Insecticides resistance mechanisms such as the alteration in the target-site of the gene and the increase in the detoxification rate of metabolic enzymes are the major insecticide resistance mechanisms involved in the insecticides resistance of dengue vectors (Ranson et al., 2010). Thus, an effective vector control program is important to reduce the number of dengue vectors and at the same time to reduce the amount of human-vector contact. This study is conducted to identify the underlying resistance mechanism of dengue vectors in Penang Island, Malaysia.

1.1 Research Objectives

1) To assess the susceptibility status of *Aedes aegypti* and *Ae. albopictus* in Penang Island against permethrin and malathion insecticides used by the Ministry of Health.
2) To identify the main target-site mechanisms associated with the insecticide resistance in *Aedes aegypti* and *Ae. albopictus*.
3) To investigate the metabolic resistant mechanisms involved in *Aedes aegypti* and *Ae. albopictus*.

CHAPTER 2

LITERATURE REVIEW

2.1 Mosquito classification

Generally there are more than 3400 species of mosquitoes worldwide (Khatik et al., 2014). Mosquito is an arthropod from the order Diptera, suborder Nematocera and family *Culicidae* (Reinert, 2009). It can be divided into three subfamilies known as *Anophelinae*, *Culicinae* and *Toxorhynchitinae* (Jeffery et al., 2012). The *Anophelinae* comprises of 430 species with 30 to 40 species are known to be vectors of malarial parasites (Aina et al., 2009). The *Culicinae* comprises of 3046 species of mosquitoes. The most important species of mosquitoes that can acts as a vector belonging to the genera *Anopheles, Culex, Aedes, Ochlerotatus, Psorophora, Haemagogus* and *Sabethes* (Kalaivani et al., 2012). The *Anopheles* is a vector for malaria and filariasis, *Culex* is a vector for filariasis and Japanese Encephalities (JE), meanwhile both of *Aedes aegypti* and *Aedes albopictus* from the Subfamily *Culicinae* are the highlighted species in this research as these two species are the main vector for dengue virus as well as yellow fever and chikungunya (Kovendan et al., 2012). In 2016, Zika virus which is known to be transmitted by *Ae. aegypti* has become epidemic in 18 countries including several regions in America (Ai & Zhang., 2016).

2.2 Morphology of *Aedes* mosquitoes

The adult stage of *Ae. aegypti* and *Ae. albopictus* is easy to recognize especially by observing their morphological characteristics under the stereo microscope with the help of identification keys. The general distinct morphology that distinguishes between the adult of *Ae. aegypti* and *Ae. albopictus* is their

scales on the scutum (Figure 2.1). The *Ae. aegypti* regardless of male or female has a pair of white lyre-shaped scales on their scutum. Their scutum also has a pair of longitudinal white line scales in between of the white lyre-shaped scales (Andrew & Bar, 2013). The *Ae. aegypti* also has two separated patches of white scales on the mesepimeron. The clypeus of *Ae. aegypti* has white scales, whereas the anterior surface of their midfemur has a longitudinal white stripe. Meanwhile, the adult of *Ae. albopictus* regardless of male or female has a median-longitudinal white scale on their scutum. The white scale patches on mesepimeron of *Ae. albopictus* are not separated. The *Ae. albopictus* also has a clypeus without white scales, and the anterior surface of their midfemur has no longitudinal stripe (Rueda, 2004).

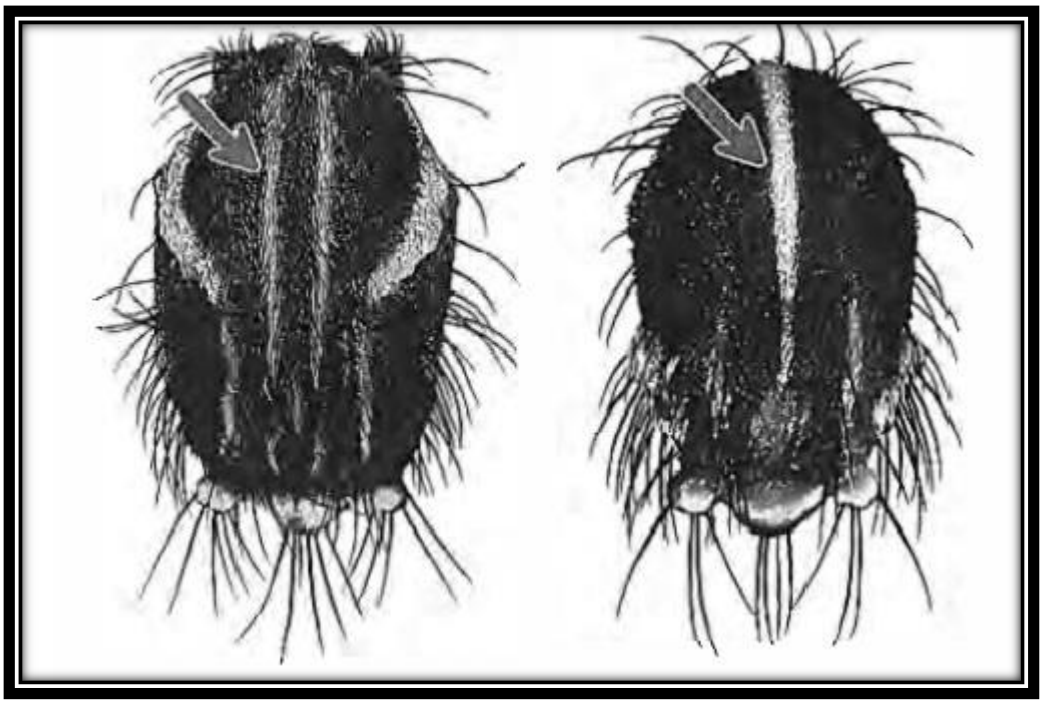

Figure 2.1: The white scales on the scutum of *Aedes aegypti* and *Aedes albopictus* (Rueda, 2004).

2.3 Distribution of *Aedes*

2.3.1 *Aedes aegypti* Linnaeus

The *Ae. aegypti* (Linnaeus) (*Stegomyia aegypti*) mosquito originated from Africa where their original habitat is from the forested area. However, the species has developed an adaptation to live in the peridomestic environment of the urban and suburban area (WHO, 2011). Most females of *Ae. aegypti* spends their adult life time in or around the houses. The females of *Ae. aegypti* prefers to rest in bedrooms which is not the same for male *Ae. aegypti* (Perich et al., 2000). Both male and female *Ae. aegypti* prefers to rest indoor. Normally, the *Ae. aegypti* which is also known as endophilic mosquito tends to rest indoors and at distance of more than 6m from the street (Perich et al., 2000). This behaviour will reduce the efficacy of space spray such as thermal fogging due to fewer insecticide droplets may reach the *Ae. aegypti* resting place (Mount, 1998 & Lofgren, 1972).

2.3.2 *Aedes albopictus* Skuse

Ae. albopictus (Skuse) (*Stegomyia albopicta*) which belongs to the *Scutellaris* group of the subgenus *Stegomyia* is also well known as a secondary vector for dengue fever and dengue haemorrhaegic fever (WHO, 2011). *Aedes albopictus* is known to have less adaptation to urban environment and is less anthropophilic compared to *Ae. aegypti* (Rezza, 2012). It originates from the South-East Asia, islands of the Western Pacific and the Indian Ocean (WHO, 2011). It has spread from the South-East Asia to Europe, the Middle East, Africas and Americas over the past 30 years (Kaufmann et al., 2013). Their habitat can be found within urban and suburban areas, peri rural as well as forested area. It breeds in containers containing water such as tyres, vases and tin can (Knudsen, 1995). The *Ae. albopictus* was more likely to breed and transmit

dengue virus outside the building and it could normally be found in semi urban and rural areas (Ali et al., 2003). The eggs of *Ae. albopictus* could withstand the dry season and cold temperature during the winter in temperate region by becoming dormant (Hanson & Craig, 1995).

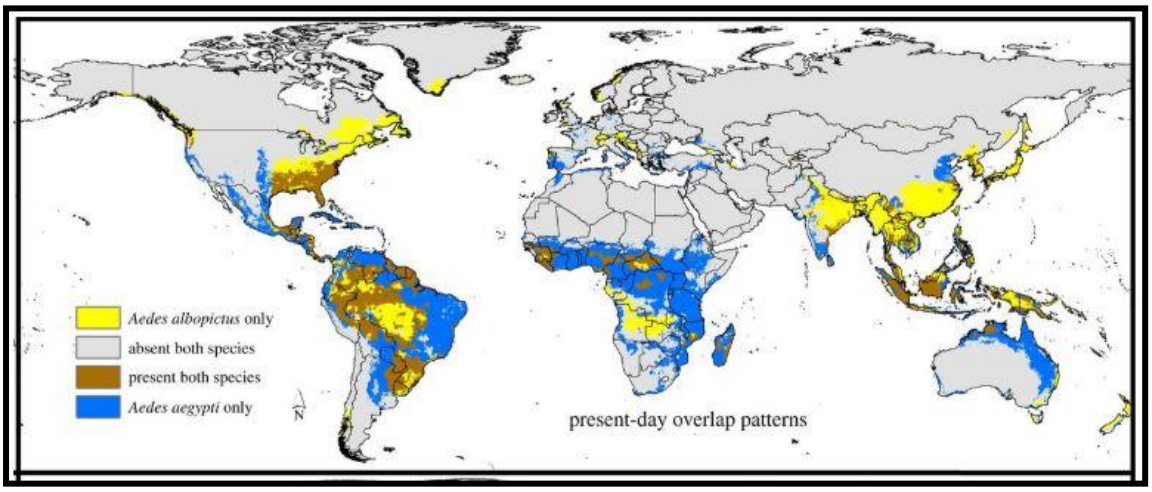

Figure 2.2: Global distribution of *Aedes aegypti* and *Aedes albopictus* (Campbell et al., 2015).

2.4 Medical Importance of *Aedes*

2.4.1 Vectors of dengue fever

Dengue is found in tropical and subtropical regions worldwide (WHO, 2011). The primary dengue vector is *Ae. aegypti*, meanwhile the Asian tiger mosquito, *Ae. albopictus* is known as the secondary vector of dengue viruses. The dengue viruses which comprises of 4 virus serotypes known as DENV-1, DENV-2, DENV-3 and DENV-4 of the genus flavivirus and family *flaviviridae* are spread by both of *Ae. albopictus* and *Ae. aegypti* (WHO, 2011). Patient who is infected with one of the virus serotypes will get a lifelong immunity to that serotype. However, if the secondary infection occurs, the patient will obtain a severe form of dengue such as dengue

haemorrhagic fever or dengue shock syndrome. Patient who suffers from the dengue haemorrhagic fever will have symptoms such as fever, abdominal pain, vomiting, bleeding and breathing difficulty (Teixeira & Barreto., 2009).

2.4.2 Vectors of chikungunya

Chikungunya virus (CHIKV) is the virus that is responsible for the chikungunya fever. The CHIKV virus is from the genus of *Alphavirus* and family *Togaviridae*. The first recorded outbreak of chikungunya virus was in Tanzania, Africa in the year of 1952. In Africa, the virus is believed to be circulating in the sylvatic cycle of *Aedes* spp. mosquitoes among a group of reservoir hosts such as African green monkey (*Chlorocebus sabeus*), patas monkeys (*Erythrocebus patas*), and Guinea baboon (*Papio papio*) (Diallo et al., 2012). In Asia, Chikungunya virus is transmitted by *Ae. aegypti* and *Ae. albopictus* and the virus is often transmitted to the humans residing in urban areas (Thiboutot et al., 2010). The person who are infected with the chikungunya virus often have symptoms such as fever, headache, severe joint pain, rashes and lymphadenitis (Chua, 2010). In Malaysia, the first infection of CHIKV is recorded in the year of 1998-1999 in Port Klang which affected more than 51 people followed by the outbreak in Perak in the year of 2006 (Apandi et al., 2009). The outbreak of the chikungunya virus involving more than 10000 cases with one death has occurred between April 2008 until March 2010 in Malaysia (Sam et al., 2010).

2.4.3 Vectors of Zika

Zika virus (ZIKV) is a virus from the family of *flaviviridae*. It was first discovered in 1947 in the Zika Forest in Uganda. It is believed to have a sylvatic cycle as it was isolated from both a sentinel rhesus monkey and *Aedes africanus* mosquitoes (Howard, 2016). In Malaysia, the ZIKV is isolated from the *Ae. aegypti* mosquitoes (Hayes, 2009). Recently, the ZIKV infection has been associated with microcephaly and other neurological disorders. In the outbreak of ZIKV in French Polynesia in the year of 2013-2014, the neurological disorder such as Guillain-Barré syndrome has been linked with the Zika virus infection (Kindhauser et al., 2016).

2.5 Vector control and management

Preventing the transmission of the dengue virus from spreading across the region depends entirely on the control of the mosquito vectors to reduce the human-vector contact. A few control measures could be taken to prevent the dengue virus from continuously spreading. The steps are physical control, biological control and chemical control.

2.5.1 Physical Control

Physical control such as by having a good environmental management is the key factor that leads to the major reduction of dengue vectors. This is because the dengue vector; *Ae. albopictus* and *Ae. aegypti* prefers to breed in the water container such as clogged drain, leftover tin can, abandoned tyres or plastic bottles containing water. Source reduction by clean-up campaigns, regular container cleaning and emptying, installation of water supply systems, solid waste management and urban planning are important elements in the environmental management that must be taken seriously by the responsible authorities and also the public (Guzman et al., 2010).

Furthermore, the community engagement activities through active involvement of the community in vector control interventions via having routine dengue control measures would help to rapidly reduce the dengue vectors (Lin et al., 2016).

Furthermore, physical control of immature stages of *Aedes* by using the monomolecular film (polydimethylsiloxane) has proven to be effective in controlling immature stages of mosquitoes. The ability of the Aquatain AMF to spread throughout the water surface by forming a thin film on the surface and reduce the surface tension will eventually suffocate the larvae and drown them to the bottom of the container (Mbare et al., 2014).

2.5.2 Biological Control

Biological control is the use of natural enemies to reduce the number of pest organism (Holmes et al., 2016). In the field, larvivorous fish are used to control the immature stages of mosquito populations. The larvivorous fish used in mosquito control should be able to withstand drought, able to live in either deep or shallow water, having small body size and also able to live in water containers such as water tanks without contaminating the water (Chandra et al., 2008). The most effective larvivorous fish for the control of mosquitoes immature stage are from the family of *Poeciliidae*, *Cyprinidae*, *Cyprinodontidae* and *Chichlidae* (WHO, 2003b). In Malaysia, the effectiveness of *Poecilia reticulata* from the family *Poeciliidae* as the biocontrol agent for the dengue vectors have been studied. The results from the studies showed that the female *Poecilia reticulata* has the highest rate of predatory on *Ae. aegypti* with the highest density in daily feeding rate of 121.3 larvae per day while 105.6 larvae per day for *Ae. albopictus* (Saleeza et al., 2014). Furthermore, the *P. reticulata* or commonly known as the South African guppy has a well-developed

tolerance with water of high temperature, organically polluted water and it can also live in domestic water storage containers (Saleeza et al., 2014). Besides the larvivorous fish, the larvae of *Toxorhynchites* spp. can also be used as the natural predator for immature stages of *Ae. aegypti* and *Ae. albopictus*. The *Toxorhynchites splendens* and *Toxorhynchites amboinensis* have similar breeding habitat preference with the *Ae. aegypti* and *Ae. albopictus* which is the artificial container (Steffan & Evenhuis, 1981). Thus, it could effectively be used as the natural biocontrol agent as they have similar breeding habitat.

Microbial larvicides such as the *Bacillus thuringiensis* H-14 and *Bacillus sphaericus* are widely used in vector control programmes to eliminate the dengue vectors in their immature stages. The *Bacillus thuringiensis var israelensis* (Bti) produced protoxins crystals that could cause mortality of the targeted organisms that ingested the Bti (Araújo et al., 2013). Microbial larvicides could be an alternative in controlling the immature stages of dengue vectors if the mosquito larvae have developed resistance towards the commonly used larvicides such as temephos (Yap et al., 1994). A study conducted in Malaysia by using *Bti* (VectoBac WG) showed a significant reduction in the ovitrap index of both of *Ae. aegypti* and *Ae. albopictus* in the treated sites compared to the untreated sites (Tan et al., 2012).

Furthemore, *Wolbachia* is another biological based method that could be used in the vector control programme. *Wolbachia* is a gram-negative intracellular bacterium that could naturally be found in *Ae. albopictus* (Joanne et al., 2014). *Wolbachia* is known for its ability to alter the host reproductivity through cytoplasmic incompatibility (Afizah et al., 2015). A study conducted in Australia showed that the *Wolbachia*-infected mosquitoes is able to block the dengue virus from replicating thus enable it to be a good biological control technique (Frentiu et al., 2014). In Malaysia,

the laboratory *Wolbachia*-infected *Ae. aegypti* had been released in March 2017 in Keramat area which is situated in Klang Valley and followed by Shah Alam to curb the dengue virus transmission problems (Azil et al., 2018).

2.5.3 Chemical control

Larviciding and adulticiding mosquitoes by using chemical is another option to control the vectors of dengue. Larvicides are the product used to kill mosquitoes at their immature stages while adulticide is used to kill the adult mosquito. Chemical larvicides such as temephos from the group of organophosphates with the trade name of Abate 1-SG™ and Abate 500 EC™ are used by the public health practitioners to control the dengue vectors. Abate 1-SG™ is in granular form, whereas Abate 500EC™ is an emulsifiable concentrate formulation (Lee et al., 2015). The temephos is an organophosphorus compound that is safe, effective and easy to use as larvicides. It also has a low mammalian toxicity, thus, would not be harmful to human if we consumed the water containing Abate (Edward & Sogbesan, 2007). In Malaysia, the Abate 1-SG™ in granular forms are easily accessible to public (Lam, 1994). The public is encouraged to put the Abate 1-SG™ into large water storage located in their housing compound. Meanwhile, the Abate 500EC™ can be used as both adulticide and larvicide as it usually used as a space-sprayed insecticide (Lee et al., 2015). As a biolarvicide, the insect growth regulator, (IGR) such as pyriproxyfen is also effective in controlling the immature stages of mosquito. The pyriproxyfen will delay the rate of larval development and at the same time will inhibit the pupal emergence. Previous study by using *Ae. aegypti* showed that the pyriproxyfen shows 100% mortality when tested against larvae of *Ae. aegypti* except for pupal stage with only 1.5-7.8% mortality (Wang et al., 2013).

The adulticide from the class of pyrethroid and organophosphate are normally used in the space spray treatment during fogging activities (Table 2.1). In Malaysia, the thermal or cold fogging via Ultra Low Volume (ULV) machine is carried out at dengue outbreak areas by using insecticides from the class of organophosphate and pyrethroid with the active ingredients such as malathion, pirimiphos-methyl and s-bioallethrin (Ong, 2016; Omar et al., 2011).

Table 2.1: Insecticides for space spray treatment (Ong, 2016).

Product name	Active ingredient(s)
Malathion	malathion
Sumithion	fenitrothion
Bayetex	fenthion
Resigen	s-bioallethrin + permethrin + piperonyl butoxide
Pesguard	d-tetramethrin + cyphenothrin
Gokilat	cyphenothrin
Actellic	primiphos-methyl
Abate	temephos

2.6 Insecticide Classification and Mode of Action

Insecticides could be classified into different classes with different modes of action. Generally, there are several classes of insecticides. In Malaysia, the insecticides mainly used by Public Health operators are usually from the class of organophosphate and pyrethroid.

2.6.1 Organophosphate

Organophosphate (OP) is a class of insecticide widely used around the world in both agriculture and public health sector. OP is an ester or thiols derived from phosphoric acid (Figure 3) (Balali-Mood & Saber, 2012). OP is highly toxic to vertebrate animals and it is also usually used as a poison to kill targeted insect pest (Costa et al., 2008). OP inhibits AChE activity and causes the accumulation of ACh at the synaptic cleft resulting in over stimulation of the ACh receptors (Costa et al., 2008).

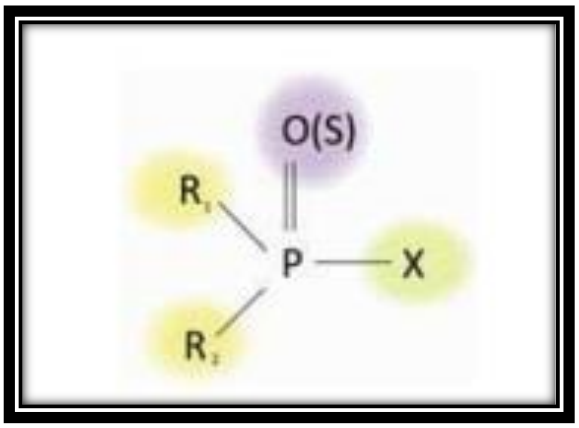

Figure 2.3: General structure formula of organophosphate (Eleršek & Filipič, 2011).

2.6.2 Pyrethroid

Pyrethroid is a synthetic insecticide that was developed to improve the efficacy of pyrethrins. Pyrethrin is an active organic compund derived from the flowers of *Chrysanthemum cinerariaefolium* or *Pyrethrum cinerariaefolium* and this organic compound is found in the Pyrethrum extracts (Ensley, 2012). Pyrethroid has a similar chemical structure with pyrethrins, but pyrethroids are more toxic to insects and more stable in the environment depending on the type of soil and species of microbes that will help with pyrethroid degradation process (Mueller-Beilschmidt, 2009). Pyrethroid is an insecticide recommended by World Health Organisation (WHO) for the treatment of insecticidal materials against mosquitoes of the public health importance due to its strong efficacy while using at a low dose, fast killing effect and relatively low cost of production (Bonnet et al., 2009). Pyrethroid is a non-biomagnify insecticide in which they have low water solubility and strongly absorbed to soil particles (Smith & Stratton, 1986). Pyrethroids are effective against agricultural insect's pest and urban pest (Nauen, 2007). Most pyrethroid is used by public health personnel in Ultra Low Volume (ULV) spray for mosquito control (Esu et al., 2010). Furthermore, pyrethroids are known as neurotoxicants because they act directly on excitable membranes (Perger & Szadkowski, 1994). Pyrethroids also causes prolongation of the sodium current thus causing repetitive activity in the nerve fibres of insect (Vijverberg et al., 1982).

2.6.3 Organochlorine

Organochlorine is a synthetic pesticide which belong to the group of chlorinated compounds (Jayaraj et al., 2016). Organochlorine insecticides such as dichlorodiphenyltrichloroethane (DDT) is highly persistence in environment with the

half-life of 2-15 years (Augustijn-Beckers et al., 1994). Due to its low cost and faster killing effects compared to the other classes of insecticides, DDT has been widely used as a pesticide in the agriculture and vector control programmes to eradicate the insect pest (Davies et al., 2007). The presence of DDT insecticide would normally interfere the peripheral nervous system by causing repetitive firing of the neurons. The interference in the peripheral nervous system would likely result in the death of the insects due to the muscle spasms (Davies et al., 2007). In the year of 1998, the Vector Borne Disease Control Programme in Malaysia has replaced DDT insecticide with pyrethroid to eliminate the malaria vectors (Rohani et al., 2014). The usage of DDT in both of agricultural and vector control has been banned due to its deteriorating effects to the non-targeted organisms and persistence in the environments (Davies et al., 2007).

2.6.4 Carbamate

Carbamate is an ester derived from the organic compound called as carbamic acid (Jayaraj et al., 2016). Carbamate insecticide such as propoxur is known to have similar mode of action with organophosphate (Gupta et al., 2017). The carbamate insecticides such as propoxur and bendiocarb will inhibit the AChE resulting in the accumulation of the ACh neurotransmitter at the nerve synapse (Gupta et al., 2017). Furthermore, the carbamate insecticide has been used as an indoor residual spray (IRS) against malaria (Van den Berg et al., 2012).

2.7 Insecticide Resistance Mechanism

Resistance is defined as the ability of some individuals to tolerate doses of toxicant which would cause fatality to the other individuals in a normal population of the same species (Liu et al., 2006). The insecticide resistance in insects might occur due to the natural selection in the population (Mallet, 1989) (Figure 2.4). There are a few mechanisms that are involved in resistance such as behavioral resistance, metabolic detoxification, target-site insensitivity, and physiological resistance mechanisms (Figure 2.5). However, the two main mechanisms responsible for the insecticides resistance are target site insensitivity and increased metabolic detoxification of insecticides (Kamgang et al., 2017).

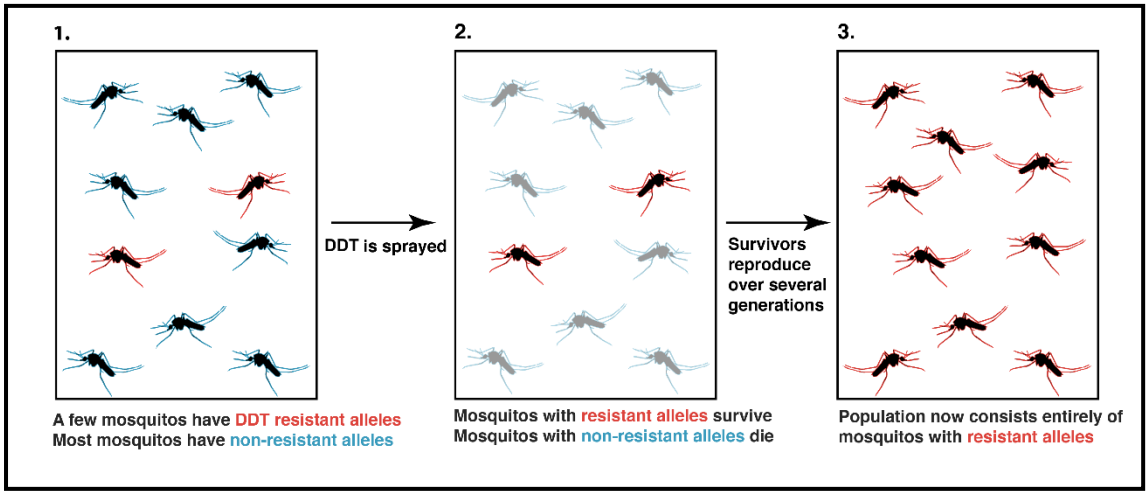

Figure 2.4: Insecticide resistance evolution in dengue vectors (Khan Academy, 2018).

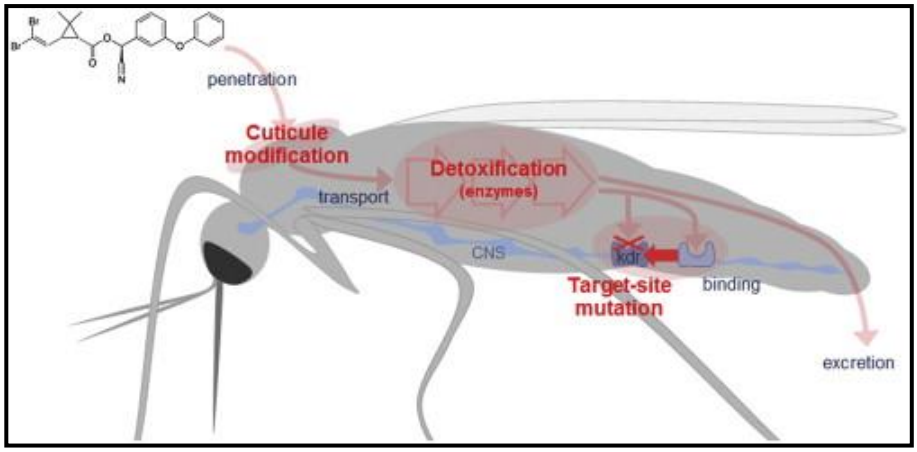

Figure 2.5: Insecticide resistance mechanisms in mosquito (Nkya et al., 2013).

2.7.1 Behavioral resistance

Behavioral resistance is defined as the development of behaviors that reduce an insect's exposure to toxic compounds or that allows an insect to survive in an environment that is harmful and/or fatal to the majority of insects of the same species (Liu et al., 2006). Stimulus-dependent and stimulus-independent are two types of behavioral resistance that normally occur in insects. The stimulus-dependent behavioral resistance includes both irritability and repellency (Liu et al., 2006). The *Ae. aegypti* has been reported to have stimulus-dependent behavioral resistant as contact irritancy was the major behavioral response of *Ae. aegypti* when it is exposed to Deltamethrin (Kongmee et al., 2004). Contact irritancy refers to an insect leaving the insecticides dealt with location solely after making a tarsal contact with the chemical (Chareonviriyaphap et al., 2013). Meanwhile, the stimulus-independent is an innate behaviour that naturally avoids the insecticide due to their natural ability to avoid the chemical exposure (Chareonviriyaphap et al., 2013).

2.7.2 Penetration resistance

The penetration/ cuticular resistance in insects exist by adjusting the thickness of cuticle or digestive track lining to avoid or delay the rate of insecticide's penetration through the insect's cuticle of the cuticular thickness (Ranson et al., 2011). The involvement of the cuticular resistance is proven in the resistant *An. gambiae* of West Africa whereby it has been found for possessing a thicker layer of cuticle when compared to the susceptible strain. Furthermore, it has also been reported to have an upregulated cuticle genes CPAP3-E, CPLCX1, CPLCG3 and CPRs (Yahouédo et al., 2017).

2.7.3 Target-site insensitivity

The target-site insensitivity refers to the ineffective binding of a given dose of insecticides due to the structural modification or point mutation of the target proteins that the insecticides binds to (Liu, 2012). The target site insensitivity usually occurs in voltage gated sodium channel gene (VGSC) conferring knockdown resistance (kdr), synaptic acetylcholinesterase gene (Ace-1) and the gamma-aminobutyric acid (GABA) receptor gene (Hemingway & Ranson, 2000).

2.7.3(a) Voltage gated sodium channel (VGSC) gene

The insensitivity of the sodium-channel is one of the major resistance mechanism involved in pyrethroid resistance and it is referred to as knockdown resistance (kdr) (Soderlund & Knipple, 2003). For *Ae. aegypti* and *Ae. albopictus*, the established target site mutations are found in the sodium channel with the association of pyrethroid and DDT resistance due to the alteration in the sodium channel structure resulting in the insensitivity of the sodium channel to the insecticides binding (Liu, 2015). The kdr mutation has been reported to occur in pyrethroid and DDT resistant

insect populations across the globe. The VGSC gene of insects contain four domains which have six subunits each and connected by loops (Dong et al., 2014) (Figure 2.4). There are several kdr mutations encoding VGSC gene in mosquitoes. In dengue vector such as *Ae. aegypti*, the mutation in domain II of VGSC gene involving S898P, I1011M/V, V1016G/I and in domain III which has the F1534C mutation is responsible for a few reported cases of insecticide resistance (Kawada et al., 2014; Saavedra-Rodriguez et al., 2007). Meanwhile the kdr mutation reported occurring in *Ae. albopictus* is limited. A few studies reported that the kdr mutation encoded for F1534C have been reported in Brazil, Greece, India and Singapore (Rath & Hazra., 2018; Aguirre-Obando et al., 2017; Xu et al., 2016; Kasai et al., 2011). However, none of the mutation is found in the Malaysian population of *Ae. albopictus* (Ishak et al., 2015).

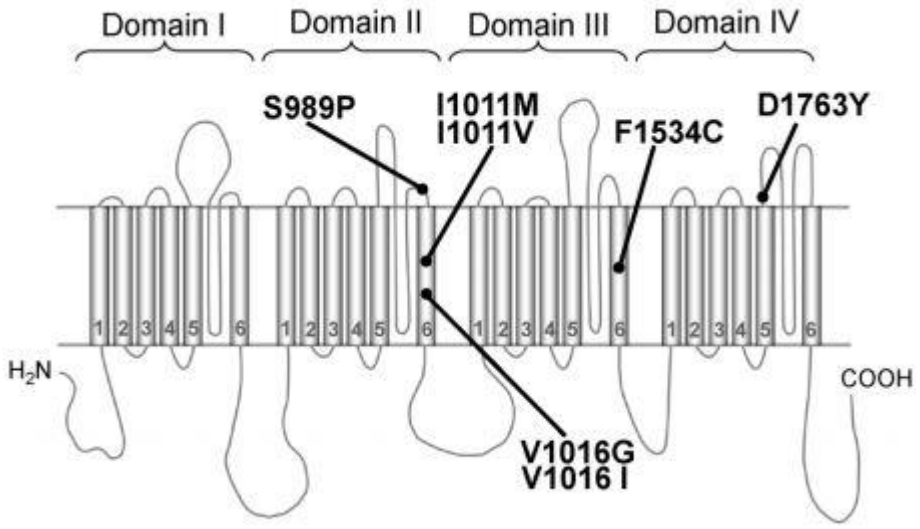

Figure 2.6: The schematic diagram showing the point mutation occurring in the voltage gated sodium channel gene of *Ae. aegypti*. The numbering is based on the amino acid sequence in housefly sodium channel gene (Kasai et al., 2011).

2.7.3(b) Acetylcholinesterase (Ace-1) gene

The target site responsible for the resistance towards organophospahate (OP) and carbamate (CB) is Acetylcholinesterase enzyme encoding Ace-1 gene (Menozzi, 2004). The point mutation alters the structure of Ace-1 gene resulting the insensitivity of the AchE towards OP and CB (Zhao et al., 2014). In mosquito, the most common Ace-1 gene mutation encoding for G119S position has been reported to occur in *Anopheles gambiae*, *An. albimanus*, *An. coluzzii*, and *Culex pipiens* (Weill et al., 2004; Essandoh et al., 2013; Djogbénou et al., 2008 & Liebman et al., 2015). In *Ae. aegypti*, the synonymous mutation in Ace-1 gene encoding G448S and a silent mutation at the position T506T have been reported occurring in *Ae. aegypti* in India and Indonesia (Hasmiwati et al., 2018; Muthusamy & Shivakumar, 2015).

2.7.3(c) GABA receptor

The mutation in the RDL encodes the gamma-aminobutyric acid (GABA) receptor gene is known to be associated with the resistance of insects to Dieldrin insecticides (Wondji et al., 2011). In Africa, the Rdl mutation encoding GABA receptor gene has been reported occurring in *An. funestus* due to having high resistance towards Dieldrin insecticide (Wondji et al., 2011). Furthermore, the Rdl mutation has also been detected in *Ae. albopictus* and *Cx. quinquefasciatus* mosquitoes in La Reunion Island (Tantely et al., 2010).