UNRAVELING THE PROTEOME CHANGES UNDERLYING INSECTICIDES RESISTANCE IN THE DENGUE VECTOR *Aedes aegypti* USING QUANTITATIVE PROTEOMICS ANALYSES

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by

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In the name of Allah, the most Gracious, the most Merciful. All praise and thanks be to Allah. Peace and blessings of Allah be upon the Prophet of humanity, Muhammad.

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LIST OF SYMBOLS

α	Alpha
β	Beta
Δ	Delta
Ɛ	Epsilon
°C	Degree Celsius
µg/µl	Microgram/microlitre
g	Gram
g	Centrifugal force
-	Hyphen or minus
×	Multiplication sign
=	Equal sign
<	Less than
>	Greater than
≤	Less than or equal to
≥	Greater than or equal to
±	Plus, minus sign
%	Percent sign
®	Registered sign

LIST OF ABBREVIATION

1DE SDS-PAGE	1-dimensional Sodium-dodecyl sulphate gel electrophoresis
2DE SDS-PAGE	2-dimensional Sodium-dodecyl sulphate gel electrophoresis
AChE	Acetylcholinesterase
ACN	Acetonitrile
Ae. aegypti	*Aedes aegypti*
Ambic	Ammonium bicarbonate
ANOVA	Analysis of variance
APS	Ammonium persulfate
BSA	Bovine Serum Albumin
CBB	Coomassie brilliant blue
CDC	Centres for disease control
CID	Collision-induced dissociation
Con	Concentration
Cx	Carbamate
CYP450	Cytochrome P450
ddH_2O	Distilled deionized water
DDT	Dichlorodiphenyltrichloroethane
DEPs	Differential expressed proteins
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ESI	Electrospray ionisation
FA	Formic acid
FDR	False discovery rate
GO	Gene ontology
GST	Glutathione S-transferases
HCD	High-energy collision-induced

HCL	Hydrochloric acid
HPLC	High-performance liquid chromatography
IAA	Iodoacetamide
IEF	Isoelectric focusing
iTRAQ	Isobaric tags for relative and absolute quantification
Kdr	Knockdown-resistant gene
LC	Liquid chromatography
LC50	Lethal concentration
LC–ESI–MS/MS	Liquid chromatography Electrospray ionisation tandem Mass spectrometry
LFQ	Label-free quantification
m/z	Mass to charge ratio
MALDI	Matrix-assisted laser desorption ionisation
Mg	Milligram
ml	Millilitre
mM	Millimolar
MudPIT	Multidimensional protein identification technology
ng	Nanogram
OMSSA	The Open Mass Spectrometry Search Algorithm
OP	Organophosphate
PCA	Principal component analysis
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulfonyl fluoride
PPI	Protein-protein interaction
Q-IT	Quadrupole ion trap
Q-TOF	Quadrupole/time-of-flight
RAId	Robust Accurate Identification
Rep	Replicate
RNA	Ribonucleic acid
RR	Resistance ratio

RT	Room temperature
SDS-PAGE	Sodium Dodecyl sulphate polyacrylamide gel electrophoresis
SD	Standard deviation
TCA	Trichloroacetic acid
TFA	Trifluoracetic acid
TOF–TOF	Time-of-flight/time-of-flight
TQ	Triple quadrupole
USM	Universiti Sains Malaysia
VCRU	Vector Control Research Unit
VGSC	Voltage-gated sodium channel
WHO	World Health Organisation

LIST OF APPENDICES

MERUNGKAI PERUBAHAN PROTEOM MENDASARI KERINTANGAN INSEKTISID DALAM VEKTOR DENGGI *Aedes aegypti* MENGGUNAKAN ANALISIS PROTEOMIK KUANTITATIF

ABSTRAK

Aedes aegypti merupakan vektor signifikan kepada pelbagai penyakit flavivirus di kawasan tropika dan subtropika. Racun serangga sintetik merupakan kaedah utama dalam pengawalan vektor. Walaubagaimanapun, pengggunaan pyrethroid secara meluas telah menyebabkan kerintangan dalam *Ae. aegypti*. Oleh hal yang demikian, kajian ini dijalankan bertujuan untuk menghuraikan profil ekspresi protein *Ae. aegypti* yang rintang terhadap temephos dan permethrin menggunakan kaedah proteomik kuantitatif. Kajian ini menilai status kerintangan *Ae. aegypti* dari kawasan denggi di kawasan khas dan juga bukan kawasan khas di Pulau Pinang terhadap 0.75% permethrin dan 31.25 mg/l temephos menggunakan protokol bioasai piawai Pertubuhan Kesihatan Sedunia (WHO). Ekstrak protein daripada nyamuk telah dianalisis menggunakan LC–ESI–MS/MS untuk identifikasi dan pengkuantitian protein menggunakan kaedah proteomik kuantitatif tanpa label (LFQ). Kajian ini menggunakan perisian statistik Perseus 1.6.14.0 untuk menganalisis pembezaan pengekspresan protein menerusi ANOVA dan ujian t. Protein terpilih daripada ujian t adalah protein yang menunjukkan perubahan ≥2.0-kali ganda (FC) dan mempunyai ≥2 peptida unik dan protein ini dianalisis untuk pengekspresan gen menggunakan qPCR. Kajian ini juga menggunakan perisian STRING untuk analisis pengayaan fungsi ontologi dan interaksi protein-protein (PPI). Keputusan bioasai menunjukkan sebanyak 28% dan 53% kematian nyamuk dari kawasan khas dan bukan kawasan khas apabila didedahkan kepada permethrin. Kerintangan larva *Ae. aegypti* menunjukkan

peningkatan tahap kerintangan terhadap temephos di kawasan khas dan juga bukan kawasan khas dengan 80% dan 91% kematian. Analisis proteomik kuantitatif tanpa label (LFQ) merekodkan 501 dan 557 (nilai-q <0.05) pembezaan protein terekspresi (DEP) di dalam nyamuk dewasa dan larva *Ae. Aegypti*. Ujian t menunjukkan 114 protein pengawalaturan yang meningkat dan 74 protein pengawalaturan yang menurun dalam nyamuk dewasa yang rintang berbanding strain makmal yang telah didedahkan kepada permethrin. Manakala, terdapat 13 protein pengawalaturan yang meningkat dan 105 protein pengawalaturan yang menurun dalam larva rintang temephos berbanding strain makmal yang telah didedahkan kepada temephos. Kemudian, empat protein DEP yang signifikan daripada setiap nyamuk dewasa dan larva *Ae. aegypti* telah dipilih untuk pengesahsahihan menggunakan pengekspresan gen. Ujian t menunjukkan pengawalaturan menaik bagi natrium/potassium-bersandar ATPase β2 dalam strain rintang permethrin, domain mengandungi protein H15, 60S protein ribosom, dan protein PB dalam strain yang rintang terhadap temephos. Pengawalaturan menurun bagi troponin i, enolase fosfatase E1, glukosida 2β dalam strain rintang permethrin dan rantai tubulin β dalam strain rintang temephos juga telah diperhatikan. Seterusnya, pengesahsahihan qPCR menunjukkan persamaan corak pengekspresan protein dalam kelapan-lapan protein DEP. Pengayaan fungsi ontologi DEP yang signifikan ialah proses metabolik dadah, proses metabolik molekul kecil, dan aktiviti hidrolase bertindak terhadap ikatan ester dan keaktifan bermangkin. PPI daripada DEP menunjukkan nilai p pada $<1.0 \times 10^{-16}$ dalam *Ae*. *aegypti* yang rintang terhadap permethrin dan temephos. Pengayaan laluan DEP yang signifikan telah diperkayakan melibatkan beberapa laluan antaranya laluan metabolik, pemfosforilan oksidatif, metabolisme karbon, biosintesis asid amino, glikolisis, dan kitaran sitrat. Kesimpulannya, hasil kajian ini telah menunjukkan bahawa DEP serta menonjolkan

protein pengawalaturan yang menaik dan menurun adalah berhubung kait dengan kerintangan racun serangga dalam *Ae. aegypti*. DEP yang telah disahkan memerlukan kajian lanjutan sebagai penanda protein berpotensi bagi memantau dan menjangka kerintangan terhadap racun serangga dalam *Ae. aegypti.*

UNRAVELING THEPROTEOME CHANGES UNDERLYING INSECTICIDES RESISTANCE IN THE DENGUE VECTOR *Aedes aegypti* USING QUANTITATIVE PROTEOMICS ANALYSES

ABSTRACT

Aedes aegypti is a significant vector for many tropical and subtropical flavivirus diseases. Synthetic insecticides are the primary vector control method. However, the widespread use of pyrethroid is causing resistance in *Ae. aegypti*. Hence, this study was aimed to elucidate permethrin and temephos resistant protein expression profiles in *Ae. aegypti* using quantitative proteomics. The study evaluated the susceptibility status of *Ae. aegypti* from dengue hotspot and non-hotspot areas of Penang Island against 0.75% permethrin and 31.25 mg/l temephos using the world health organisation (WHO) standard bioassay protocols. Protein extracts from the mosquitoes were analysed using LC–ESI–MS/MS for protein identification and quantification via label-free quantitative proteomics (LFQ). The study used Perseus 1.6.14.0 statistical software to perform differential protein expression (DEP) analysis via ANOVA and student's T-test. The t-test selected proteins that showed ≥2.0-fold change (FC) and ≥2 unique peptides were used for gene expression via qPCR. The study also used STRING software for functional ontology enrichment and protein-protein interaction (PPI) analyses. Bioassay results showed 28% and 53% mortalities in mosquitoes exposed to permethrin from the hotspot and non-hotspot areas. The susceptibility of *Ae. aegypti* larvae revealed high resistance to temephos in hotspot and non-hotspot areas with 80% and 91% mortalities. The LFQ analysis revealed 501 and 557 (q-value <0.05) DEPs in adults and larvae *Ae. aegypti*. The t-test showed 114 up-regulated and 74 down-regulated adult proteins in resistant versus laboratory strain

exposed to permethrin. Meanwhile, there were 13 up-regulated and 105 down-regulated larvae proteins in resistant versus laboratory strain exposed to temephos. Then, four significant DEPs each from adults and larvae *Ae. aegypti* were chosen for validation by gene expression. The t-test revealed the up-regulation of sodium/potassium-dependent ATPase β2 in permethrin resistant strain, H15 domain-containing protein, 60S ribosomal protein and PB protein in temephos resistant strain. The down-regulation of troponin i, enolase phosphatase E1, glucosidase 2β in permethrin resistant strain and tubulin β chain in temephos resistant strain were also observed. Furthermore, the qPCR validation revealed similar expression patterns in the above eight DEPs. The significant functional ontology enrichment of the DEPs indicated drug-metabolic process, small molecule metabolic process, hydrolase activity acting on ester bonds and catalytic activity. The PPI of DEPs showed a p-value at $<1.0 \times 10^{-16}$ in permethrin and temephos resistant *Ae. aegypti*. Significantly enriched pathways in DEPs revealed metabolic pathways, oxidative phosphorylation, carbon metabolism, biosynthesis of amino acids, glycolysis, and citrate cycle. In conclusion, this study has revealed DEPs and highlighted up-regulated and down-regulated proteins associated with insecticide resistance in *Ae. aegypti*. The validated DEPs merit further investigation as a potential protein marker to monitor and predict insecticide resistance in field *Ae. aegypti*.

CHAPTER 1

INTRODUCTION

1.1 Background

Insecticide resistance in *Aedes aegypti* (Linnaeus) contributes to the dengue burden in Malaysia and other tropical countries. Apart from dengue, *Ae. aegypti* is also a vector for transmitting chikungunya, yellow fever, and zika viral diseases. A chemical-based control method is the primary control method used for dengue vectors in Malaysia. Some insecticides include permethrin, deltamethrin, malathion used against adult mosquitoes and temephos used against the larval stage. Nonetheless, the widespread use of various synthetic insecticides has triggered insecticide resistance against the chemical-based control methods deployed to fight vector-borne diseases (Selvi et al., 2010). In many parts of Malaysia, insecticide resistance has been reported in mosquitoes (Chen et al., 2005b, 2005a; Othman et al., 2008; Rong et al., 2012; Wan-Norafikah et al., 2010). Ishak et al., (2015) detected pyrethroid-resistant *Ae. aegypti* and *Ae. albopictus* (Skuse), based on their resistant ratios and mortality percentages in Penang and Kuala Lumpur.

Mutations and modifications in the knockdown-resistant gene (*kdr*) and the voltage-gated sodium channel (VGSC) prompt pyrethroid insensitivity in mosquitoes. This mutation in the VGSC diminishes the knock-down effect of the insecticide (Dusfour et al., 2015). In Malaysia, F1534C and V10206G *kdr* gene sites confer resistance against pyrethroids (Ishak et al., 2015). Certain detoxifying enzymes naturally degrade a toxic compound into a non-toxic form before removing it from the insect body (Dusfour et al., 2015). The feature of this mechanism depends on the overproduction of detoxifying enzymes. Hence, the gene expression studies using

microarray on the detoxifying enzymes conducted in French territories in South America and the Caribbean have shown the metabolic insecticide-resistant mechanism in *Ae. aegypti*. The microarray analysis demonstrated the involvement of Cytochrome P450 (CYP450) genes in conferring resistance (Dusfour et al., 2015). Furthermore, other genes involved in pyrethroids-resistance are carboxylesterases and glutathione S-transferases (GST) (Bariami et al., 2012; Marcombe et al., 2009; Saavedra-Rodriguez et al., 2012; Strode et al., 2008). Dusfour et al., (2015) reported cytochrome gene variants CYP6BB2, CYP6M11(Q16WQ7), CYP6N12(Q16WR9), CYP9J9(Q174T1), CYP9J10, and carboxylesterase (CCE) gene variant CCE3 found overexpressed in *Ae. aegypti* deltamethrin-resistant strain identified in French Guiana, Guadeloupe, and New Caledonia. Furthermore, 3 and 2 gene variants of the carboxylesterases and glutathione S-transferases families were also overexpressed. Ishak et al., (2016) confirmed the overexpression of cytochrome CYP450 and CYP6P12 genes exerts pyrethroid resistance in Malaysian *kdr*-free *Ae. albopictus* populations.

This study utilised LC–ESI–MS/MS for protein identification and quantification, known as tandem mass spectrometry (MS). Furthermore, this study used the label-free quantitative proteomics (LFQ) approach to quantify differential expressed proteins (DEPs) in the insecticide-resistant *Ae. aegypti* versus laboratory strain. Differential protein expression (DEP) analysis determines the comparative abundance of identical proteins in two or more samples representing different treatment groups (Gibb & Strimmer, 2015).

This study utilised the electrospray ionisation (ESI) system, coupled to a quadrupole-orbitrap MS system for a parallel accumulation of a serial fragmentation

acquisition via collision-induced and high energy collision-induced dissociation. This model of tandem MS enhanced the speed and sensitivity by increasing overall mass-ion intensity and resolution detection for better proteome coverage and quantification (Cui et al., 2020). The advantage of the orbitrap analyser is due to its high-resolution power. The high-resolution has given an edge to the orbitrap in analysing proteins characterised with high molecular weight (Haag, 2016). The advantage of using the LFQ approach is that it does not require tedious sample preparation, while the labelled approach requires additional pre-treatments and expensive labelling reagents. Thus, the LFQ approach is less likely than labelled techniques to cause errors in sample preparations. This approach can be applied to nearly all numbers and types of samples and enable comparison across many experimental conditions (Wang et al., 2015), allowing greater study design flexibility. In contrast, isotopic labelling approaches are limited to the number of samples or experiments that may be directly evaluated (Distler et al., 2016).

1.2 Problem statement and rationale of the study

Many mosquito genes encode detoxifying enzymes making insecticides harmless. Metabolic detoxification of insecticides is part of the acquired resistant mechanism controlled by some oxidizing enzymes. These enzymes include cytochrome P450 (CYP450), glutathione S-transferase (GST) and carboxylesterases (CCE). Identifying the specific genes exerting resistance is a challenge. As the primary functional biological molecules, proteins are the architects that function in many different physiological processes. Therefore, comparative proteomics analysis brings a practical, robust method for gaining insight into insecticide-resistant related proteins at the proteome level. Still, few studies have been made to apply proteomics to

elucidate insecticide resistance protein profiles in *Ae. aegypti* in Malaysia. Therefore, this study aimed to identify DEPs in insecticide-resistant *Ae. aegypti*, to reveal biomarkers for detection of insecticide resistance in *Ae. aegypti*. There is no single study about DEPs in insecticide-resistant *Ae. aegypti* in Penang Island, Malaysia, to date.

1.3 Objectives of the studies

General objective:

To elucidate permethrin and temephos resistant protein expression profiles in the *Ae. aegypti* using quantitative proteomics and the DEPs predictive functions.

The specific objectives are:

1. To determine the *Ae. aegypti* insecticides susceptibility in dengue hotspot and non-hotspot areas of Penang Island.
2. To compare the extraction methods to observe the quantity and quality of protein for mass spectrometry analysis.
3. To identify differentially expressed proteins (DEP) of the insecticide-resistant *Ae. aegypti* and validate selected DEPs by gene expression analysis.
4. To analyse the predictive protein-protein interaction (PPI), functional gene ontology (GO) enrichment and pathways of the DEPs in insecticide-resistant *Ae. aegypti*.

The flow chart of the study is presented in Figure 1.1.

1.4 Experimental design flow chart

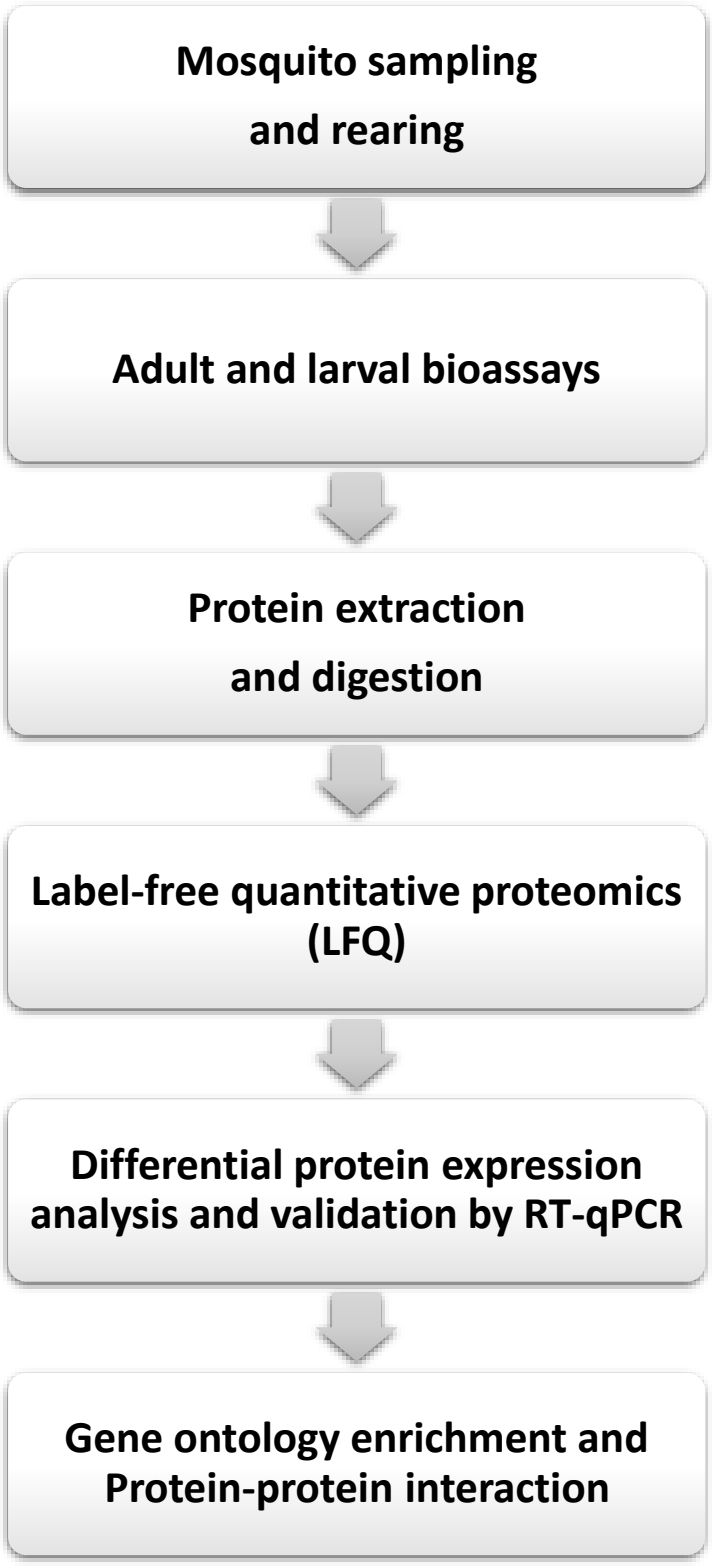


Figure 1.1: Flow-chart of this study.

CHAPTER 2

LITERATURE REVIEW

2.1 The Genus *Aedes*

Mosquitoes are classified in the class *Insecta*, order *Diptera*, and sub-order of *Nematocera*. The family *Culicidae* contains mosquitoes in over 100 genera and subgenera in the *Nematocera*. There are over 3600 species; the most significant subfamilies include *Anophelinae*, *Culicinae*, and *Toxorhynchintinae*. *Culicinae* remains the primary vector of arboviruses and filariasis. *Culex*, *Aedes,* and *Monsonia* are the important genera in this subfamily (Das et al., 2019).

Culicinae has over 2500 species, and *Aedes* is the main genus in the *Aedini* tribe with 1240 established species. Carolus Linnaeus changed the traditional taxonomy classification of *Aedini* by distinguishing a few genera and many subgenera. *Aedini* was a natural group, and some members showed affinities with all other higher-level taxa of the subfamily *Culicinae* (Figure 2.1). These species are challenging to identify and differ extensively at the genus level due to corresponding suites of related morphological traits. The typical features of the *Aedini* tribe in female mosquitoes are a pointed abdomen and toothed ungues, also known as tarsal claws. Before the 20th century, the traditional classification of *Aedini* consists of nine genera and 50 subgenera. *Aedes* was the most prominent genus and was divided into 41 subgenera comprising about 1000 species. *Stegomyia Aedes* is a medically important species. They are vectors for flaviviruses such as yellow fever, dengue, chikungunya, Zika, and filariasis (Das et al., 2019; Harbach, 2007; Reinert, 2000).

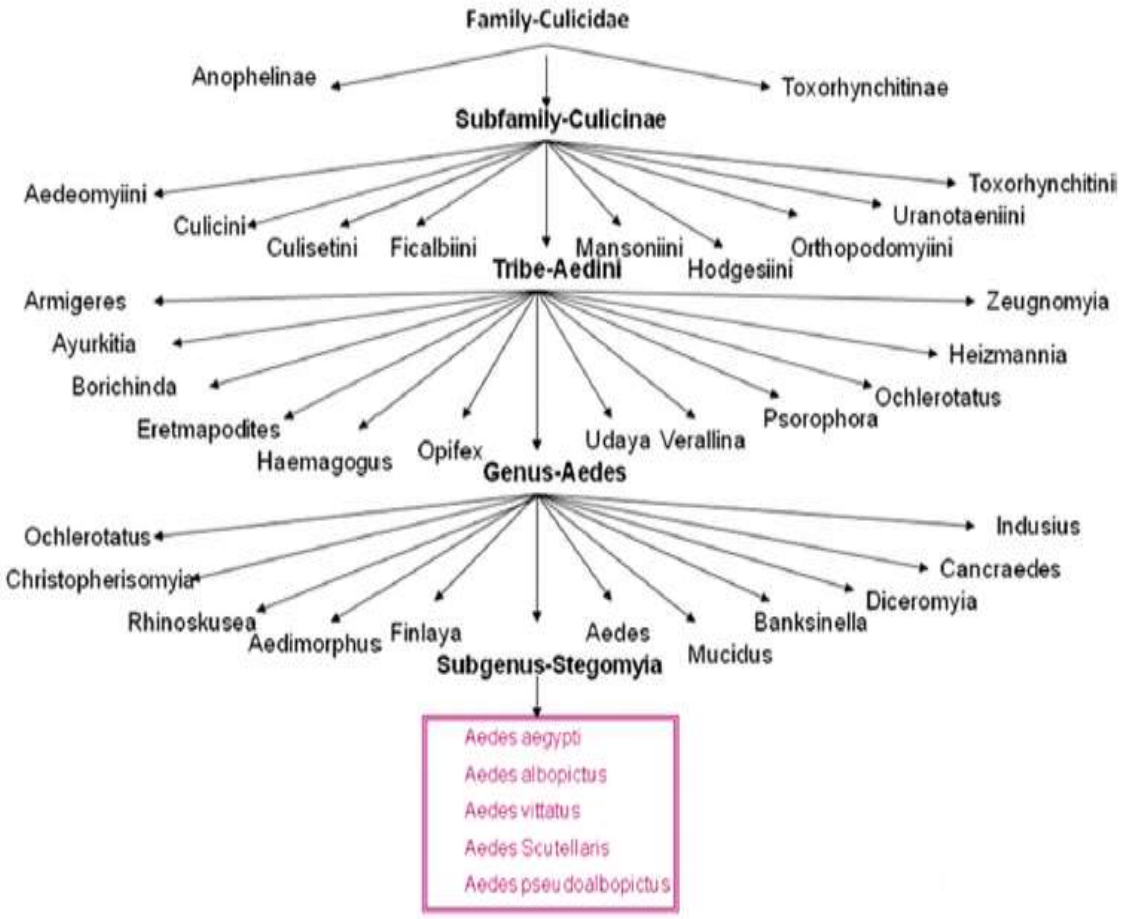


Figure 2.1: Classification of *Aedes* mosquitoes up to the genus level displaying some essential species of medical important subgenus *Stegomyia* (Das et al., 2019).

2.2 Biology of *Ae. aegypti*

Aedes aegypti (Linnaeus) mosquitoes have a characteristic small to medium size of about 4 to 5 millimetres. Morphologically adult *Ae. aegypti* looks like the Asian tiger mosquitoes *Aedes albopictus* (Skuse) with a slight distinction in size and thorax pattern (Muktar et al., 2016). On the dorsal thorax surface, adult *Ae. aegypti* has a white/silver scale that creates a violin or lyre shape. In contrast, adult *Ae. albopictus* possess a longitudinal white/silver stripe on the thorax surface (Figure 2.2).

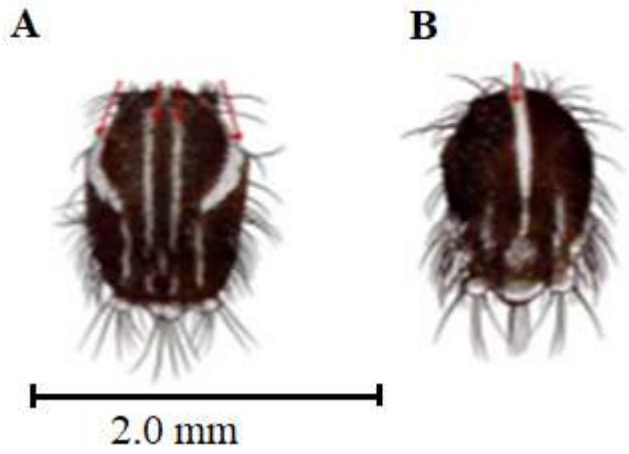


Figure 2.2: Morphological differences in *Ae. aegypti* and *Ae*. *albopictus* thorax. (A) *Ae. aegypti* with lyre shape white scales on the thorax versus (B) *Ae. albopictus* with the longitudinal stripe on the thorax surface (RUEDA, 2004).

The *Ae. aegypti* abdomen has a dark brown pigment with black contrast and a white scale. The body size of female *Ae. aegypti* are larger than males and differentiated by the small palps tinted with silver or white scales and a lyre on the upper surface of the thorax (Figure 2.3). The male has the characteristic plumose antennae, while the female has scattered tiny hairs. The male mouthpart is adopted for nectar-feeding, while the female mouthpart is for blood-feeding (Figure 2.3) (Cutwa & O'Meara, 2006; Muktar et al., 2016).

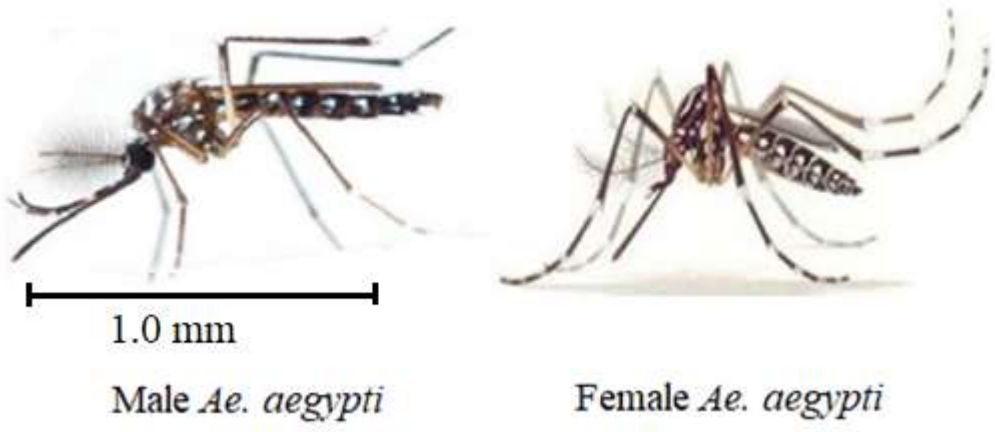


Figure 2.3: *Ae*. *aegypti* male and female mosquitoes (Fahad et al., 2018). The male *Ae. aegypti* has plumose antennae and the female has scattered tiny hairs on the antennae.

Aedes mosquitoes are attracted to human residences and lay eggs all over the waters near houses. The accumulation of plastics and tyre cavities storing water provide excellent breeding sites for *Aedes* mosquitoes. Thus, their population densities increased mainly in urban settings (Vontas et al., 2012). *Ae. aegypti* and *Ae. albopictus* are extensively distributed all over the tropics and subtropics regions of the world. In their geographical distribution, *Ae. aegypti* mainly breeds around developing urban areas with little vegetation, while *Ae. albopictus* prefers areas with heavier vegetation and is generally called exophilic than *Ae. aegypti* (Ishak et al., 2015).

2.3 Distribution and taxonomy of *Ae. aegypti*

Ae. aegypti originated in Africa, currently in tropical and subtropical areas globally. *Ae. aegypti* has a cosmo-tropical distribution (Figure 2.4). During the summer months, *Ae. aegypti* moved to more temperate regions (Zettel & Kaufman, 2013).

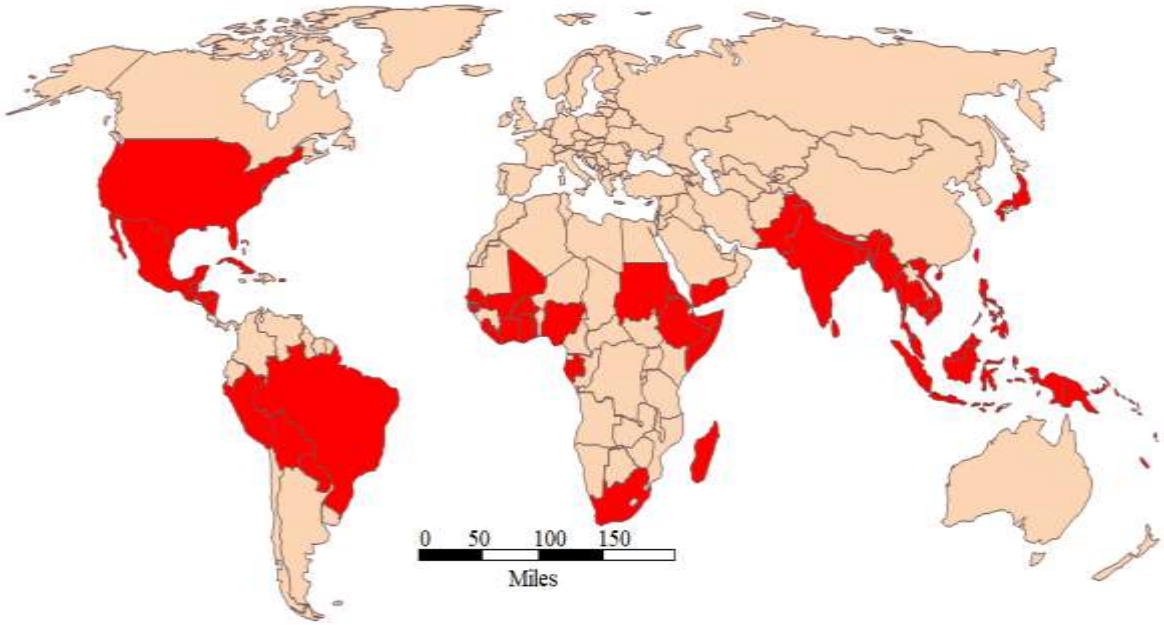


Figure 2.4: Global distribution of *Ae*. *aegypti*. Countries shown in red colour are hotspots for *Ae. aegypti* mosquitoes (Zettel & Kaufman, 2013).

Taxonomically *Ae. aegypti* was classified in the kingdom of Animalia, subkingdom Bilateria, phylum Arthropoda, class Insecta, order Diptera, family Culicidae, genus *Aedes*, and specie *aegypti* (Das et al., 2019; Muktar et al., 2016).

2.4 Habitation and feeding patterns of *Ae. aegypti*

Ae. aegypti mosquitoes usually live below 1000 meters high between the latitude of 35ºN and 35ºS. In general, mosquitoes are very good at adapting to climate change. *Ae. aegypti* live in both natural and artificial environments, either terrestrial or aquatic. Their successful adaptation to urban domestic habitats has resulted in several human-made containers often related to human habitation, such as flower pots, water tanks, and tires (Dom et al., 2013; Tsuda et al., 2006). The global distribution of *Ae. aegypti* is directly proportional to climatic conditions. Lower temperatures of less than 10 ºC limit larval development and adult survival (Hopp & Foley, 2001; Marinho et al.,

2016). The optimal temperatures, from 22 ºC to 32 ºC, are needed for development, longevity, and productiveness (Beserra et al., 2009).

Female *Ae. aegypti* are strong sneaky biters. They exclusively feed on human blood. They can also feed on other hosts such as bovine, canine, equine, and other mammals, accounting for less than 1% of the blood meal. *Ae. aegypti* females feed readily and routinely under favourable conditions. It also can sustain long-term existence on meals other than blood (Ponlawat & Harrington, 2005), such as flower nectars and fruit juices (Barredo & Degennaro, 2020). *Aedes* mosquitoes bite their host in search of a blood meal throughout the day, indoors or outdoors, in contrast to *anopheline* species, where they only bite in search of blood meal from dusk to dawn (Zettel & Kaufman, 2013).

2.5 Reproduction and life cycle of *Ae. aegypti*

Ae. aegypti mosquito is a holometabolous insect. It passes through a successful metamorphosis from an egg, to larvae, and pupae, to the adult stage. *Aedes* mosquitoes lay one egg at a time on a wet surface. The eggs hatch into larvae in 48 hours when submerged in water. The life cycle from an egg to an adult mosquito requires not more than 8 to 10 days, depending on optimum growth conditions. The lifecycle of *Ae. aegypti* has aquatic and terrestrial phases (Reinert, 2000). Larvae hatch from the eggs once the water increases and sinks the eggs entirely. The larvae grow and develop through four instar larval stages. Metamorphosis into pupae takes place after the fourth instar larvae. Finally, a fully developed mosquito emerges and breaks out over the pupal skin and flies (Figure 2.5) (Clements, 2000).

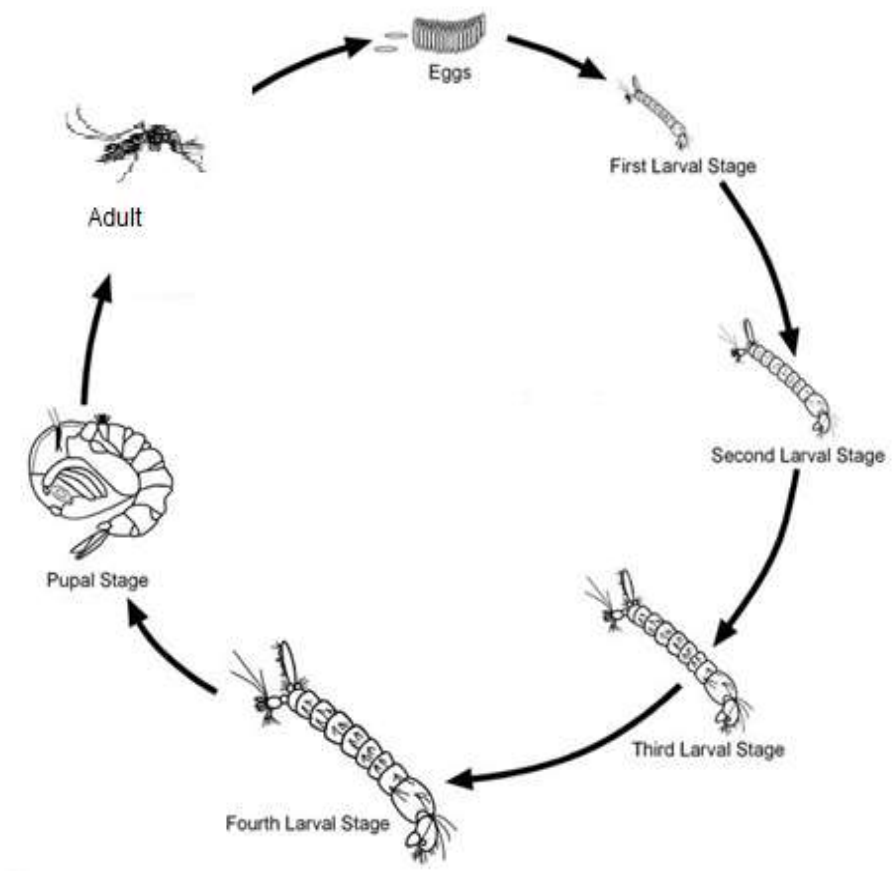


Figure 2.5: *Ae*. *aegypti* life cycle displays aquatic and terrestrial habitats (Muktar et al., 2016).

After the male and female *Ae. aegypti* have mated, and the female takes a blood meal from a suitable human host. Adult female *Ae. aegypti* develop eggs for 2 to 3 days and lay them on moist surfaces. Female *Ae. aegypti* require a blood meal to synthesize the protein yolk stored in the eggs for the developing embryo. The pre-requisite blood meal to produce eggs cause mosquitoes to be an effective disease vector. The mosquito requires a minimum of a single bite from a host for each egg batch they develop. After female adult *Ae. aegypti* has a blood meal; it can produce around 100 to 200 eggs per batch, depending on the quantity of blood meal. An adult female can make a maximum of five eggs batch. Based on optimum environmental conditions, *Ae. aegypti* life expectancy may differ from two weeks to a month (Bhatt et al., 2013; Muktar et al., 2016; Rutledge, 2008).

Ae. aegypti larvae are legless with a well-formed head. They do not appear like a maggot. The larvae are often referred to as wrigglers or wigglers. Once disturbed, the larvae wiggle in the water and breathe oxygen via a siphon posteriorly located and held above the water. At the same time, the remainder of the body dangles vertically. Morphologically *Aedes* larvae are differentiated from other genera by the appearance of a short siphon. Larval stages feed on organic matter like algae and other microscopic lifeforms. They live in or on any container holding water. The larval development passes through four different instars, spending a moment on the first, second, and third instars and almost three days in the fourth instar. The larval development is temperature-dependent. The males develop faster than female mosquitoes. They pupated and emerged into adult mosquitoes earlier than the females (Foster & Walker, 2018; Rutledge, 2008).

The *Ae. aegypti* pupae are often referred to as tumblers and spend two days developing from the fourth instar larvae. They do not feed. Adult mosquitoes emerge by enlarging the abdomen through air ingestion, consequently separating open the pupa, and the head appears first (Rutledge, 2008).

2.6 *Ae. aegypti* and flaviviruses

Flaviviridae is a positive-strand RNA virus (Lindenbach & Rice, 2007). The Flaviviridae derived its name from the Latin word "flavus", characteristics of jaundice triggered by infection with the yellow fever virus (Huang et al., 2014). The Flaviviridae family consists of over 70 members divided into four genera: Flavivirus (flavus, "yellow"), Pestivirus (pestis, "plague"), Pegivirus, and Hepacivirus (hepar, hepatos, "liver") (Salas-Benito & Nova-Ocampo, 2015).

The flavivirus genus consists of four categories: mosquito-borne, tick-borne, no known vector, and insect-specific viruses. Mosquitoes and tick-borne flaviviruses are significant human pathogens. They cause encephalitis, fever, and hemorrhagic fever. These flaviviruses include Dengue virus, Japanese Encephalitis Virus, Yellow Fever Virus, Saint Loius Encephalitis Virus, West Nile Virus, Murray Valley Encephalitis, and Tick-Borne Encephalitis (Salas-Benito & Nova-Ocampo, 2015).

Mosquito-borne flaviviruses are primarily transmitted to susceptible humans during mosquito blood meal feed. Flaviviruses contracted from vertebrate hosts multiply in the mosquito midgut within minutes of contact. Thus, the midguts remained the first tissues to be infected. The virus disseminates to neighbouring tissues by the hemolymph after evading the midgut and is transmitted via infected salivary glands and saliva (Salas-Benito & Nova-Ocampo, 2015). The extrinsic incubation period (EIP) varies from 7 to 10 days. The EIP is the initial infection in the mosquito midgut until it transmits the virus (Muktar et al., 2016).

Flaviviruses are single-stranded, positive-sense RNA viruses of about 11kb in length. The genome has a single open reading frame, encoding three structural and seven non-structural (NS) proteins. The structural proteins involve capsid (C), membrane (M), and envelop (E), while the NS are 1, 2A, 2B, 3, 4A, 4B, and 5. They are cleaved co-translationally across the endoplasmic reticulum and post-translationally into the endoplasmic membrane from the polyprotein. The encoding precursor polyprotein region is significant in viral translation and replication. The virus flanked at 5 and 3 ends with non-coding parts (Huang et al., 2014; Lindenbach & Rice, 2007; Salas-Benito & Nova-Ocampo, 2015). The virus is spherical, with a diameter of about 50nm.

2.7 Mosquito control

Since the establishment of mosquitoes' connection with the transmissibility of various pathogens to humans in the late 19th century, eradicating mosquito vectors became the primary control approach against major vector-borne diseases (Niang et al., 2018). In the early 20th century, reducing the target population and environmental management were the main mosquito control strategies, using petroleum oil and larvivorous fish (Mulla, 1994; Niang et al., 2018). Target population reduction utilises predators, parasites, pathogens, competitors, or microbial toxins. The strategy of the target population reduction ensures mosquito population reduction satisfactorily. This control measure, otherwise termed biological control, combines human protection from vector-borne diseases and conservation (Scarnecchia et al., 2004). Environmental management control involves antagonizing vector populations and disease transmission risk. Modification and manipulation of the environment and human habitation or behavioural changes are crucial for the success of environmental management (Scarnecchia et al., 2004).

The discovery of dichlorodiphenyltrichloroethane (DDT) in the 1940s ushered in the era of chemical controls (Niang et al., 2018). DDT was referred to as a miracle insecticide because of its impressive success in eradicating vector populations and agricultural pests (Scarnecchia et al., 2004). DDT remains lethal to mosquitoes a year after its indoor residual spraying because of its high persistent nature. The consequences of disproportionate and indiscriminate DDT use led to its banning in the United States in 1972, ending nearly 30 years of its usage (CDC, 2009). DDT is toxic to several aquatic animals, vertebrates, invertebrates, and other valuable insects

(Beckvar & Lotufo, 2011; Cottam & Higgins, 1946). DDT is also a probable human carcinogen (CDC, 2009; Iarc, 2015).

2.7.1 Classification of insecticides

Four public health insecticides are mainly used to fight mosquitoes and insect vector-borne diseases. These include pyrethroids, organophosphates, carbamates, and chlorinated hydrocarbons or organochlorines (Paine & Brooke, 2016).

The development of insecticides went through generations. The first generations are highly toxic stomach poisons with organic and botanical compounds, such as arsenicals, mercury, lead, and hydrogen cyanide. The latter include nicotine sulphate, rotenone, pyrethrum, and chlordecone. In the second generation, insecticides are synthetic water-soluble toxic compounds referred to as contact insecticides. These include chlorinated hydrocarbons like carbamates, DDT, organophosphates, and pyrethroids. The third-generation insecticides were juvenile hormone analogues and insect growth regulators that were insect-specific to control resistance evolution. Finally, the fourth generation of insecticides is a derivative of entomopathogenic microorganisms (Scarnecchia et al., 2004; Ware & Whitacre, 2006).

2.7.1(a) Pyrethroids

Pyrethroids are a class of insecticides synthetically extracted in the laboratory. Synthetic pyrethrins are cyclopropane carboxylic acid and cyclopentenone alcohol esters. They are primarily present in the flowers of *chrysanthemum* plants. Structurally, pyrethroids are similar between groups (Figure 2.6). The primary acid/alcohol configurations are retained throughout the classes (Burns & Pastoor, 2018). Pyrethroids are constituted with six esters in the *chrysanthemum* head's nonpolar extract solvent to produce the pyrethrum extract. Pyrethrum is the most active plant-

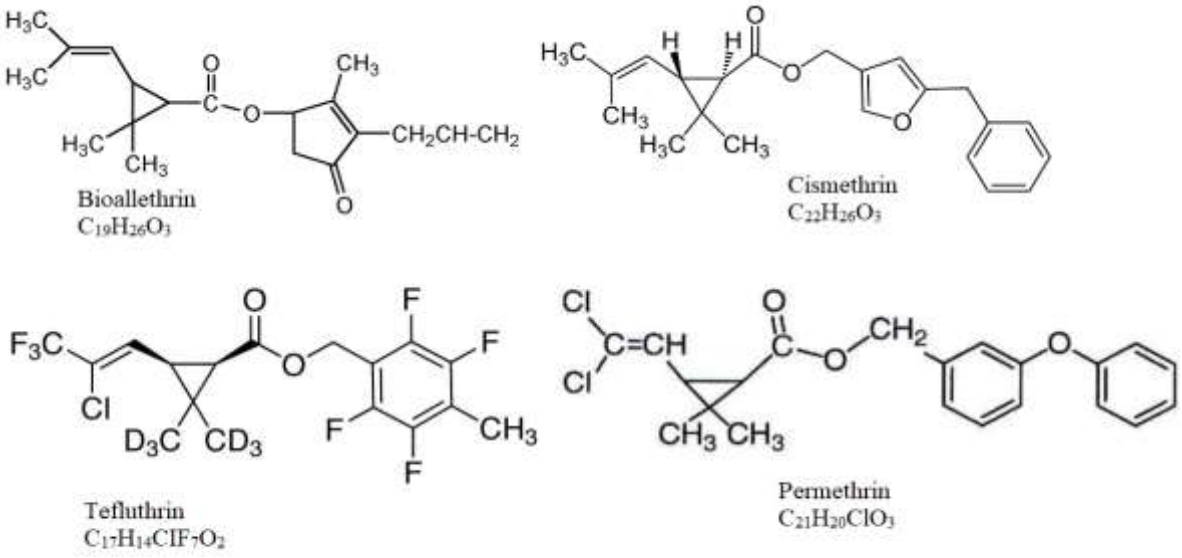
based insecticide with decisive insecticidal actions and low mammalian toxicity, considered harmless for humans and higher animals. However, these esters are easily hydrolysed and are light sensitive. The resulting decomposition hindered its acceptability as an agricultural pesticide. Eventually, a series of structural manipulations were performed, and the biological efficacy was assessed and swapped with synthetic forms. In 1949 the first synthetic pyrethroid insecticide was introduced (Chrustek et al., 2018; Clark & Symington, 2011).

Three main modifications were performed to enhance pyrethroid stability against insect vectors and lower acute mammalian toxicity. First, the change to the methyl group of the photo-stable dimethyl vinyl group with a halogen group is to achieve a photo-stable dichlorovinyl group. Then the replacement of 3-phenoxybenzyl alcohol with chrysanthemates alcohol. These two modifications were combined to produce stable field permethrin. Finally, the nitrile group and the methyl carbon of the 3-phenoxylbenzyl alcohol make cyno-deltamethrin and cypermethrin. The modification in 2-2 dimethylcyclo propane carboxylic acid with α-isopropyl phenylacetic acid present in the pyrethrin produces fenvalerate. The central ester moiety modification through ether linkages resulted in a non-ester pyrethroid called etofenprox (Casida, 2010; Clark & Symington, 2011).

Pyrethroids are classified into two groups, Type I and Type II based on their biological responses. The electrophysiological response in the nerve tissues using Type I results in characteristics of repetitive discharges in the axons, while Type II principally blocks the nerve conductivity at the synapse (Khambay & Jewess, 2005). Type II poisoning effects are slow-acting compared to type I (Khambay & Jewess, 2005). Pyrethroids nomenclature was based on chemical structure, toxicity level, insect poisoning, and nerve activity. Type I pyrethroids are molecules with a

comprehensive structural diversity deficient in α-cyno-3-phenoxybenzyl moieties like pyrethrin I, bioallethrin, cismethrin, bifenthrin, tefluthrin, and permethrin (Figure 2.6). Type II pyrethroids have α-cyno-3-phenoxybenzyl molecules, including deltamethrin, cypermethrin, β cyfluthrin, and ʌ cyhalothrin (Figure 2.6) (Soderlund, 2012).

A



B

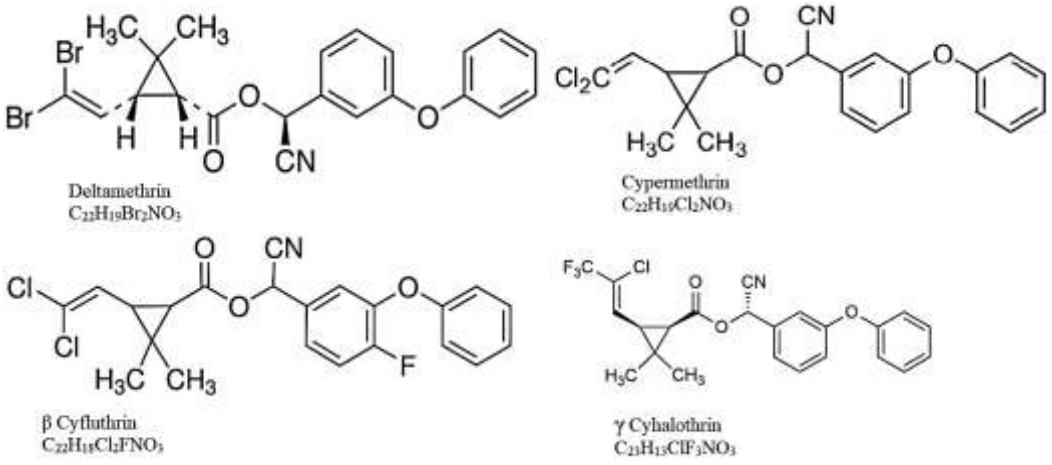


Figure 2.6: Pyrethroid structures based on the two broad classification. A. Type I pyrethroid deficient in α-cyno-3-phenoxybenzyl molecule. B Type II pyrethroid with α-cyno-3-phenoxybenzyl molecule.

Pyrethroid insecticides are neurotoxins. They alter the average nerve conductivity in insects, causing electric current disruption at the voltage-sensitive sodium channel gene. The blackout leads to depolarising neurons, eventually paralyzing and killing the insects (Amelia-Yap et al., 2018; Narahashi, 2002).

2.7.1(b) Organophosphates (OP)

OPs are chemical compounds produced by the esterification of phosphoric acid and alcohol. It goes through hydrolysis and removes alcohol from the ester-bond. OPs are the critical constituent of herbicides, pesticides, and insecticides (Adeyinka et al., 2018). OP is an acetylcholinesterase (AChE) inhibitor in the nervous system. AChE is a cholinergic enzyme at the postsynaptic neuromuscular junctions of muscles and nerves (Trang & Khandhar, 2020). The primary role of OP is to eliminate the catalytic properties of AChE by catalytic serine residue phosphorylation resulting in phophinyl adduct production (Rathnayake & Northrup, 2016). The OP insecticides either or both undergo activation and detoxification metabolic pathways. The activation pathways of OP are mostly more potent than the original compound, as reported in the conversion of malathion to malaoxon. On the other hand, detoxification produces less harmful compounds and insecticide resistance manifestations (Gupta & Crissman, 2013).

OPs are applied against vectors of diseases such as malaria and dengue mosquitoes. They are used as an indoor spraying compound. OPs and CXs have similar chemical structures but vary extensively in details, properties, and uses (Moretto, 2014). Typical OP insecticides used include malathion, parathion, diazinon, fenthion dichlorvos, chlorpyrifos, ethion, and pirimiphos-methyl.

2.8 Insecticide resistance in *Ae. aegypti*

The primary purpose of vector control is to act on the pathogen by decreasing the vector life span, eradicating possibly dangerous vectors, and stopping them from flourishing. The desirable outcome of vector control is to disrupt or reduce transmission to the degree that the disease is no longer a public health threat (Ndiath, 2019).

Insecticides must contact the mosquito, enter its body, and transform into active metabolites to get through to their target sites. The alteration of such flow may result in resistant development (Ndiath, 2019). Insecticide resistance is a pre-adaptive condition. Certain mosquitoes carry rare resistant alleles before being exposed to an insecticide stressor. Such alleles have been proposed to be polymorphisms in the allele sequence and their increased or abundant expression. Typically, the proportion of mosquitoes with resistant polymorphism increase after insecticide exposure. The subsequent generations predictably survive for an extended time, and resistant individuals endure to become the dominant population (Roush, 1993; Xu et al., 2012).

There are three types of insecticide resistance. Behavioural resistance is seen when irregular behaviour prevents possible contact with an insecticide. Physiological resistance is characterized by decreased penetration or increased excretion of insecticides. On the other hand, increased enzymatic activity in the detoxification system and decreased affinity in insecticide target sites describe biochemical resistance. These mechanisms minimize the toxic effects of the insecticide (Ndiath, 2019).

2.8.1 Behavioural resistance

Behavioural insecticide resistance involves modifying mosquitoes' feeding patterns and resting attitudes to avoid insecticides. The mechanism for behavioural resistance is stimulus-dependent, indicating toxic substance recognition by the insect's sensory receptors, leading to irritability and repulsion (Chareonviriyaphap et al., 2013). Behavioural avoidance could be direct contact excitation or non-contact spatial repellency (Amelia-Yap et al., 2018).

In direct contact irritancy, mosquitoes flee the insecticide exposed surface as soon as contact occurs. In non-contact spatial repulsion, mosquitoes fly from the insecticide exposed surfaces before contact. Behavioural avoidance in mosquitoes has shown irritability to insecticides, resulting in mosquitoes escaping treated homes and shifting night feeding habits to broad day blood-feeding in *An. Funestus* (Amelia-Yap et al., 2018; Paeporn et al., 2007; Thanispong et al., 2010).

2.8.2 Physiological resistance

Insecticides enter insects through their cuticle or digestive tract walls to reach their target sites. The rate of insecticide penetration for the same compound differs from one species to another. Insecticides with slow penetration kinetics undergo degradation rapidly. Thus, insects become selected by the insecticide and produce resistant offspring (Ndiath, 2019). In mosquitoes, cuticular thickening leading to resistance occurs instantaneously with other mechanisms, resulting in multiple insecticide resistance (Amelia-Yap et al., 2018; Kasai et al., 2014; Nkya et al., 2013).

2.8.3 Biochemical resistance

Certain enzymes initiate insecticide degradation as insects ingest the compounds. This biochemical mechanism of insecticide resistance involves modifying or changing insecticide into a less sensitive form. The enzymes mainly involve acetylcholinesterase, gamma butyric acid receptor, and VGSC mutation. CYP450, GSTs and esterases significantly change potent insecticides into less sensitive forms (Liu, 2015; Ndiath, 2019).

2.8.3(a) Cytochrome P450 CYP450

CYP450 completes various physiological and biological functions in living cells. They are the largest class of gene superfamilies in all living organisms. They bring oxygen into their respective substrate and are critical for detoxifying and activating xenobiotic and endogenous compounds. The enzymatic complex occurs on the endoplasmic reticulum of a cell. CYP450 genes are deposited mainly in digestive tract cells, Malpighian tubules, and fatty insect tissues (Ndiath, 2019; Hilary Ranson et al., 2011; Sangba et al., 2017). It metabolizes insecticide leading to either bioactivation or detoxification. Furthermore, it is involved in pyrethroids, CM, DDT, and OP resistance (Feyereisen, 1999; Ndiath, 2019).

Eleven families of CYP450 reported having been highly expressed in DDT or pyrethroid-resistant mosquitoes, including CYP4G, CYP4H, CYP6N, CYP6M, CYP6P, CYP6Z, CYP9K, CYP12F, CYP314A, CYP325A, and CYP325D (David et al., 2014). The most identified CYP450 in insecticide resistance *An. gambiae* include CYP6M2 and CYP6P3 (Müller et al., 2008; Stevenson et al., 2011). At the same time,

CYP6M7 and CYP6P9a were mainly expressed in pyrethroid resistance in *An. funestus* (Amenya et al., 2008; Irving et al., 2012).

2.8.3(b) Glutathione S-transferases GST

GSTs are a dimeric group of multifunctional enzymes detoxifying xenobiotics (Hemingway & Ranson, 2000). Two GST forms exist in insects, GST1 and GST2. Both interact in insecticide-resistant interplay. GSTs are found in the cytoplasm of the cells (Ndiath, 2019). However, their expression varies from one species to another or even within species. For example, insecticide-resistant *An. gambiae* overexpresses GST1 (Prapanthadara et al., 1995), while GST2 has overexpressed in insecticide-resistant *Ae. aegypti* (Grant & Hammock, 1992).

GSTs are involved in DDT resistance. They metabolize the toxin into a non-toxic product, which is DDE. For example, in *An. funestus*, a single amino acid modification of L119F, exerts GSTs metabolic resistance to DDT and cross-resistance to pyrethroids (Paine & Brooke, 2016; Riveron et al., 2014).

2.8.3(c) Esterases

Esterases are enzymes that hydrolyse ester bonds. In insects, esterases perform several functions, including reproduction, hormone metabolism, digestion, and neurotransmission. Esterases are found in the cytoplasm and on the endoplasmic reticulum of the digestive tract cells, the Malpighian tubules and the reproductive systems, and the fatty insect tissues. There are two types of esterases. Esterases are classified based on the favourable hydrolase α or β naphthyl acetate. They are also classified based on the nucleotide sequences and their molecular weight when separated by polyacrylamide. Most of the esterase gene families are

carboxylesterase/carboxyl/cholinesterase gene families (Hemingway & Ranson, 2000; Montella et al., 2012; Ndiath, 2019; Paine & Brooke, 2016).

Esterase hydrolysis ends in the detoxification of xenobiotics through mutations changing the amino acid sequence. This initial detoxification covers specific reaction sequences, including gene amplification and overexpression (Amelia-Yap et al., 2018).

2.8.3(d) Acetylcholinesterase (AChE)

AChE is an enzyme protein that hydrolyses acetylcholine concealing the neurotransmitter receptor channels. It occurs in the insects' central nervous systems and targets synthetic OP and CX insecticides. This enzyme is essential for inhibiting the neurotransmitter acetylcholine at the neuronal junction. The electric nerve impulse reaching the neuronal joint produces AChE, allowing receptor binding on the postsynaptic membrane. This process enables the sodium and potassium channel to open and generates an electric charge distribution shift at the beginning of the nerve impulse on the postsynaptic membrane. Synthetic OP and CX successfully obstruct AChE action, resulting in insect death (Ndiath, 2019). Monooxygenases transform synthetic OPs into oxon derivatives before acting as AChE inhibitors.

AChE modification in insecticide-resistant mosquitoes transforms insensitivity to the enzyme's inhibition properties by OP and CX. AChE1 has been identified in several mosquitoes, such as *An. Gambiae*, *An. albimanus*, *Cx vishnui*, *Cx pipiens*, and *Cx. quinquefasciatus* controlled by OP and CX, with a mutation in the G119S AChE1 enzyme (Alout et al., 2008). Only AChE1 conferred insecticide resistance against OP and CX insecticides (Hemingway & Ranson, 2000). In other studies, Liu (2015) and Weill et al., (2002) have reported AChE2 in mosquitoes and other insects.