EFFICACY ASSESSMENT OF COMMERCIAL MOSQUITO COILS AND ASSOCIATED PYRETHROID RESISTANCE MECHANISMS IN *Aedes aegypti* FROM INDONESIA

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2018

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DISSERTATION SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF PHILOSOPHY

INSTITUTE FOR ADVANCED STUDIES
UNIVERSITY OF MALAYA
KUALA LUMPUR

2018

UNIVERSITY OF MALAYA

ORIGINAL LITERARY WORK DECLARATION

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Matric No : **HMA170001**

Name of Degree : **Master of Philosophy**

Title of Project Paper/Research Report/Dissertation/Thesis ("this Work"):

Efficacy Assessment of Commercial Mosquito Coils and Associated Pyrethroid Resistance Mechanisms in *Aedes aegypti* from Indonesia

Field of Study : **Biology and Biochemistry (Entomology)**

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EFFICACY ASSESSMENT OF COMMERCIAL MOSQUITO COILS AND ASSOCIATED PYRETHROID RESISTANCE MECHANISMS IN *Aedes aegypti* FROM INDONESIA

ABSTRACT

Aedes aegypti Linnaeus is the main vector of several arthropod-borne viral infectious diseases globally. Relentless vector control efforts have been performed to curtail disease transmissions, with insecticides remain as the first line of defence in Indonesia. With a dearth of published information, this is the first related report on the efficacy of mosquito coils in Indonesian *Aedes aegypti*. *Aedes aegypti* mosquitoes were sampled from nine regencies in Indonesia and tested against commercially available local pyrethroid-based mosquito coils containing d-allethrin, transfluthrin and metofluthrin to assess insecticide susceptibility profile. In accordance with the WHO resistance indicators, mosquito populations from Denpasar, Mataram, Kuningan, Padang, Samarinda and Sumba Timur were resistant (< 90% mortality rate) whereas populations from Manggarai Barat, Dompu and Pontinak were susceptible (≥ 98% mortality rate) to the active ingredients assayed. Additionally, the knockdown rates between d-allethrin and transfluthrin, d-allethrin and metofluthrin, as well as transfluthrin and metofluthrin displayed significant associations, portraying the presence of cross-resistance within the pyrethroid insecticides. The presence of resistance led to biochemical and molecular studies to characterize the mechanisms involved. In enzyme assays, esterases (ESTs), glutathione-S-transferases (GSTs) and mixed function oxidases (MFOs) were examined. Inconsistent trends in enzyme activities were demonstrated in Indonesian *Ae. aegypti*. MFOs were found to be associated with the pyrethroid resistance of Indonesian *Ae. aegypti*. No significant correlations were shown between pyrethroid resistance phenotype and α-ESTs, suggesting the marginally-exceeded enzyme levels relative to the reference strain in some pyrethroid susceptible populations were causative factor for insecticide resistance in other

groups of insecticides. However, significant correlations were demonstrated between β-ESTs and pyrethroid resistance phenotype. The lowest enzyme levels in GSTs indicated this enzyme was not predominant in causing pyrethroid resistance, despite the presence of significant correlations. Metabolic-mediated resistance did not comprehensively explain the elevated resistance status to pyrethroids in mosquito coil bioassay because of the existence of another mechanism commonly detected in pyrethroid-resistant *Ae. aegypti*. Target-site alterations to pyrethroids in mosquitoes is caused by mutations in voltage-gated sodium channel (*Vgsc*), usually known as knockdown resistance (*kdr*). Three point mutations were screened, namely S989P, V1016G and F1534C mutations. Both S989P and V1016G mutations showed higher frequency of homozygous resistant (RR) than homozygous susceptible (SS) genotype whereas for F1534C mutation, most of them were homozygous susceptible (SS). The co-occurrence of the S989P and V1016G mutations were discovered. Additionally, significant correlations were demonstrated between the allele frequencies of the V1016G mutation and the survivability rates as well as resistance ratios in pyrethroid adult bioassays. This signifies the V1016G can contribute more to the insensitivity of *Vgsc* than the F1534C. In conclusion, this study reveals the first evidence of inefficacy of mosquito coils to some *Ae. aegypti* populations from Indonesia, urging for a revamping of the vector control system. Metabolic-mediated resistance and target-site alterations were also proven to cause pyrethroid resistance in Indonesian *Ae. aegypti*, providing insights into the evolution and adaptation of Indonesian *Ae. aegypti*.

Keywords: *Aedes aegypti*, Mosquito coil, Pyrethroids, Knockdown resistance, Metabolic-mediated resistance

PENILAIAN KEBERKESANAN LINGKARAN UBAT NYAMUK KOMERSIAL DAN MEKANISMA BERKAITAN KERENTANAN PYRETHROID TERHADAP *Aedes aegypti* DARI INDONESIA

ABSTRAK

Aedes aegypti Linnaeus merupakan vektor utama bagi beberapa penyakit berjangkit bawaan artropod di dunia. Pelbagai usaha telah dilakukan bagi mengekang penularan penyakit, di mana penggunaan racun serangga masih merupakan kawalan utama di Indonesia. Walaupun penggunaan lingkaran ubat nyamuk sangat meluas di Indonesia, namun maklumat tentang keberkesanannya masih terhad dan ini merupakan laporan pertama yang dihasilkan di negara ini. Pensampelan *Aedes aegypti* dijalankan di sembilan wilayah di Indonesia dan diuji dengan lingkaran ubat nyamuk tempatan yang mengandungi bahan aktif seperti d-alletrin, transflutrin dan metoflutrin. Kajian membuktikan nyamuk daripada Denpasar, Mataram, Kuningan, Padang, Samarinda dan Sumba Timur adalah rentan (kadar kematian < 90%), manakala, nyamuk daripada Manggarai Barat, Dompu dan Pontianak menpunyai kerintangan yang rendah (kadar kematian ≥ 98%) terhadap bahan aktif yang dikaji. Tambahan, kadar rebah di antara d-alletrin dan transflutrin, d-alletrin dan metoflutrin, serta transflutrin dan metoflutrin menunjukkan perkaitan yang signifikan, membuktikan kewujudan rintangan silang antara racun serangga pyrethroid. Dengan kehadiran kerintangan, kajian biokimia dan molekul telah dijalankan untuk mengenalpasti mekanisma yang terlibat. Dalam kajian mikroassai enzim, penggunaan enzim seperti esterases (ESTs), glutation-S-transferases (GSTs) dan oxidase fungsi campuran (MFOs) telah dijalankan. Keputusan kajian mikroassai enzim menunjukkan corak yang tidak konsisten dalam aktiviti enzim, di mana hanya enzim MFOs terlibat dalam kerintangan pyrethroid di Indonesia. Tiada perkaitan yang signifikan di antara fenotip kerintangan pyrethroid dan α-ESTs. Terdapat perkaitan yang signifikan di antara β-ESTs dan finotip kerintangan pyrethroid. Kadar enzim terendah

yang direkodkan oleh GSTs, menandakan enzim ini tidak dominan dalam menyebabkan kerintangan pyrethroid, walaupun terdapat perkaitan yang signifikan. Walau bagaimanapun, keputusan mikroassai enzim tidak mampu menjelaskan status kerintangan *Ae. aegypti* terhadap pyrethroid, kerana terdapat kewujudan mekanisma utama yang lain. Perubahan tapak sasaran pada pyrethroids dalam nyamuk disebabkan oleh mutasi di voltage-gated sodium channel (*Vgsc*), atau dikenali sebagai kerintangan kerebahan (*kdr*). Tiga poin mutasi telah disaring, iaitu S989P, V1016G dan F1534C. Kedua-dua S989P dan V1016G menunjukkan frekuensi kerentanan homozigot (RR) yang tinggi berbanding kerintangan homozigot (SS). Manakala, bagi F1534C kebanyakannya merupakan kerintangan homozigot. Oleh itu, S989P dan V1016G dapat diklasifikasikan sebagai poin mutase dalam *Vgsc* yang menyebabkan kerentanan pyrethroid pada *Ae. aegypti* di Indonesia, walau bagaimanapun, F1534C tidak memainkan peranan. Hasil kajian juga menunjukkan kombinasi antara S989P dan V1016G juga mampu menyebabkan kerintangan. Kesimpulannya, kajian ini membuktikan ketidakberkesanan lingkaran ubat nyamuk pada populasi *Ae. aegypti* di Indonesia dan menggesa penambahbaikkan terhadap system kawalan vektor sedia ada. Antara lain, kajian ini mampu memberi gambaran kewujudan evolusi dan adaptasi *Ae. aegypti* di Indonesia, dengan terbuktinya kehadiran kerintangan pada enzim mikroassai dan perubahan pada tapak sasaran.

Kata kunci: *Aedes aegypti*, Lingkaran ubat nyamuk, Pyrethroids, Kerintangan kerebahan, Kerintangan berasaskan metabolisma

ACKNOWLEDGEMENTS

I thank all who in one way or another contributed in the completion of this thesis.

I would like to express my sincerest gratitude to my supervisors, Dr. Low Van Lun, Prof. Dato' Dr. Mohd Sofian Bin Azirun and Dr. Chen Chee Dhang for giving me the opportunity to carry out this project and providing invaluable guidance throughout this research. I owe a very important debt of gratitude to Dr. Low whom I have always regarded as an excellent mentor. My sincere appreciation goes to his constant support throughout the study period as it has contributed to the success of this project. His dynamism, vision, sincerity and motivation have deeply inspired me. I am humbled and appreciative for he was the first few who sparked my interest in the scientific arena. It was a great privilege and an honor to work with him and have a share of his exceptional scientific knowledge. I would also like to thank him for his empathy and priceless life lesson, all of which I will never forget.

I thank Prof. Dato' Dr. Mohd Sofian Bin Azirun and Dr. Chen Chee Dhang for providing a good research environment with decent infrastructures which significantly hastened my progress. Their extensive, professional social networks with collaborators from Indonesia have opened up new worlds of possibilities in my research. My highest gratitude and appreciation for their genuine supports throughout this research work.

The unfailing supports from my seniors are deeply appreciated. Ms. Amirah Haziqah Binti Rashid and Ms. Nur Elia Amira Binti Mohd Roslin have been lending their hands whenever I need help; I have learnt a great deal from them, including a range of laboratory skills, statistical assistance and tips on becoming an excellent graduate student. I also thank Mr. Lau Koon Weng and Ms. Chin Ai Choon, for their teachings and advice given throughout my project.

Some special words of gratitude go to my friends who have always been a major source of support when things would get a bit discouraging: Ng Ling Hui, Cindy Chee Jia Jia, Low Jia Xin, Leslie Png Boon Hian, Raphael Lee Kuok Lung and Kenny Chong Khai Siang. I thank them for always being there for me through the ups and downs. I am beyond blessed to have friends who are like family in my life.

I would also like to thank University of Malaya for the research grant support (RP021D-16SUS).

Last but not least, I am extremely grateful to my beloved parents, Yap Ah Chong and Chong Min Fah for their constant care, love and sacrifices. They never fail to provide inspirations and motivations over the past few months, helping me through tight spots during my studies. I thank them for their constant reminders of commitment, patience, endurance and hard work. Also I express my gratitude to my sisters, Yap Sin Yee, Yap Chia Ping and Yap Sean Ann for their supports and valuable prayers. Their contribution may have been less academic but no less important to me. For that, I truly cherish their presence in my life.

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LIST OF SYMBOLS AND ABBREVIATIONS

&	:	and
=	:	equal to
≤	:	less than or equal to
<	:	less than
>	:	greater than
°C	:	degree Celcius
%	:	percent
et al.	:	et alia (“and others”)
i.e.	:	id est (“that is”)
WHO	:	World Health Organization
DNA	:	deoxyribonucleic acid
Ae.	:	*Aedes*
Cx.	:	*Culex*
An.	:	*Anopheles*
ULV	:	ultra low volume
DDT	:	dichlorodiphenyltrichloroethane
DF	:	dengue fever
DHF	:	dengue hemorrhagic fever
DENV	:	dengue virus
MFOs	:	mixed function oxidases
ESTs	:	esterases
GSTs	:	glutathione-S-transferases
GSH	:	glutathione
kdr	:	knockdown resistance

GABA	:	gamma aminobutyric acid
LC_{50}	:	50% lethal concentration
LT_{50}	:	50% lethal time
KT_{50}	:	50% knockdown time
RR	:	resistance ratio; homozygous resistance
RS	:	heterozygous susceptible
SS	:	homozygous susceptible
R	:	resistant
S	:	susceptible
PCR	:	polymerase chain reaction
AS-PCR	:	allele-specific polymerase chain reaction
HOLA	:	Heated oligonucleotide ligation assay
Vgsc	:	voltage-gated sodium channel
pH	:	potential of hydrogen
IGRs	:	insect growth regulator
CSIs	:	chitin synthesis inhibitors
JHs	:	juvenile hormone mimics
PBO	:	piperonyl butoxide
CDNB	:	1-chloro-2, 4-dinitrobenzene
TMBZ	:	3,3'5,5'-tetramethylbenzidine
DTNB	:	5, 5'-dithiobis -(2-nitrobenzoic acid)
ANOVA	:	analysis of variance
df	:	degree of freedom
P	:	possibility value
r	:	correlation coefficient
α	:	alpha

β	:	beta
S	:	south
E	:	east
SIRIM	:	Standards and Industrial Research Institute of Malaysia
cm	:	centimeter
ml	:	milliliter
g	:	gram
rpm	:	revolutions per minute
µl	:	microliter
nm	:	nanometer

CHAPTER 1: INTRODUCTION

1.1 Scope of Study

Many countries are at risks of dengue fever (DF) and dengue hemorrhagic fever (DHF) infections. With an estimation of 50–100 million reported cases annually (WHO, 2017), dengue viruses (DENV) are the most important human arboviruses found worldwide. In Indonesia, dengue was first described in 1779 (Hanley & Weaver 2010) and the disease has since then dramatically expanded in distribution over the years. Dengue fever remains to be a major public health concern in Indonesia with the presence of recurrent epidemic cycles. In 2015, there was a total of 126,675 reported dengue cases with 1,229 deaths (Ministry of Health Republic of Indonesia, 2015), signifying an appalling fact that the disease burden in the country has never ceased. Contributory factor of this issue may very much ascribed to the high availability of man-made or natural containers as breeding sites for *Aedes aegypti* Linnaeus, the anthropophilic primary dengue vector in Indonesia that lives in close proximity with humans to obtain blood meals. Effective control of *Ae. aegypti* is utterly challenging when both vector and viruses have already deep-rooted and spread rapidly in disease-endemic regions. Due to an absence of safe and effective treatment or vaccine for dengue to date, vector control strategies against *Ae. aegypti* continue to be the cornerstone in preventing dengue transmission and outbreak.

The bottom line of inhibiting dengue transmission is to decrease human-vector contact with the use of synthetic chemicals. The use of multiple classes of synthetic insecticides has been largely practiced in vector control strategies, with pyrethroid-based formulations dominating the insecticides market massively. In Indonesia, pyrethroids are widely incorporated into household insecticide products to control mosquitoes, provided that mosquito coils are the most commonly used personal protection products especially in residential areas to shield users against mosquito bites. The choice of main active ingredient of mosquito coils is pyrethroid as suggested by World Health Organization

(WHO) due to the quick knockdown effect on mosquitoes with low mammalian toxicity. It is extensively introduced because of the low cost, ease of use without the need of equipment or electricity and wide cultural acceptance properties, since smoke is widely used in many cultures in eradicating mosquitoes (Biran et al., 2007). Despite the presence of other delivery formats like vaporizer mats, aerosols and liquid vaporizers, mosquito coils are still the most popular choice globally (Yap et al., 2003). These continuing efforts in fighting dengue in Indonesia may not be sustainable when control method of the vector is dealing with many challenges that insecticides are deemed to be decreasing in susceptibility due to the issue of insecticide resistance development. When insects are exposed to certain insecticides for a period of time, selection pressure occurs among them throughout the generations, producing insecticide-resistant offspring (Lee et al., 2003).

Despite coils provide a means of disease control in preventing mosquito bites and the strong dependence on this tool for vector control, the insecticide resistance status of *Ae. aegypti* in Indonesia to mosquito coils has never been monitored. Extensive usage and over-reliance on pyrethroids have contributed to the development of insecticide resistance which is commonly underestimated and easily overlooked. Undoubtedly, insecticide resistance is not a new phenomenon and has been a nuisance at the expense of public health worldwide. Specifically, pyrethroid resistance was reported in different parts of the world (Brengues et al., 2003; Chuaycharoensuk et al., 2011; Lee et al., 2014; Chin et al., 2017; Marcombe et al., 2017).

To carry out effective and sustainable vector control measures, mosquito coil bioassays are the pre-requisite studies for timely detection and monitoring insecticide resistance caused by pyrethroid-based mosquito coils. In Indonesia, information on the resistance of the primary dengue vector *Ae. aegypti* to mosquito coils is absent due to the unavailability of carefully designed studies. Therefore, this research was initiated to examine the

efficacy of pyrethroid-based mosquito coils with the most common active ingredients (i.e., d-allethrin, transfluthrin and metofluthrin) against adult *Ae. aegypti* populations from a total of nine regencies in Indonesia. To identify the underlying mechanism(s) in pyrethroid resistance, enzyme assays are crucial to examine the presence of mixed function oxidases (MFOs), non-specific esterases (ESTs) and glutathione-S-transferases (GSTs) (Hemingway et al., 2004). On top of biochemical analysis, molecular characterizations of insecticide resistance have also been determined. According to Narahashi (1996), resistance to pyrethroids is associated with the voltage-gated sodium channel (*Vgsc*) of insect neurons and single or combinations of amino acid substitutions in the sodium channel. This phenomenon is known as knockdown resistance (*kdr*) and it has been observed in *Ae. aegypti* globally. Owing to the unavailability of large-scale biochemical and molecular analyses on insecticide resistance mechanisms in Indonesian *Ae. aegypti*, the present study aims to fill these gaps.

1.2 Statement of Problems

Pyrethroid-based mosquito coils are broadly used in Indonesia to control *Ae. aegypti*, the primary vector of dengue viruses (DENV). However, vector control programs are facing operational challenges due to the development of insecticide resistance to pyrethroids worldwide. All over Indonesia, there is no data available on the resistance status of *Ae. aegypti* to mosquito coils containing pyrethroids. Hence, the present study was the first attempt to determine the resistance status of Indonesian *Ae. aegypti* against pyrethroid-based mosquito coils. This pioneer study would serve as a timely reminder in order to alert local authorities on the significance of well-structured insecticide resistance management for the improvement of existing vector control operations in Indonesia.

Pyrethroid resistance in *Ae. aegypti* can arise through alterations in the target site of the insecticides or through differential efficacy of metabolic genes. To date, the

characterization of metabolic-mediated mechanisms of Indonesian *Ae. aegypti* against pyrethroids has been restricted to only Java and Sumatra (Ahmad et al., 2007; Astuti et al., 2014). With regard to a dearth of such information, the biochemical mechanisms in Indonesian *Ae. aegypti* populations could be underestimated. In this study, enzyme assays were carried out in nine regencies of Indonesia to confirm the biochemical mechanisms of *Ae. aegypti* in resisting pyrethroids.

Resistance to insecticides is a multi-factorial trait affected by environmental, operational and genetic factors (Lima et al., 2011). Since Indonesia is divided into 34 provinces with numerous islands scattered across Indian and Pacific oceans separating them geographically, *Ae. aegypti* populations from the country may possess different genetic backgrounds. These differences will give rise to discrepancies in susceptibility level of *Ae. aegypti* against pyrethroids. Thus, molecular study was attempted to screen for the mutation genes that confer pyrethroid resistance in *Ae. aegypti* in selected provinces of Indonesia. Identification of the mechanisms contributing to the insecticide resistance could be advantageous in developing effective mosquito control programs in Indonesia.

1.3 Significance of Study

In view of the alarming spate of dengue cases in Indonesia over the past decades, the findings of this study would deliver refined data to generate beneficial information for various fields of profession such as chemists, toxicologists, policymakers, medical entomologists, biochemists, molecular biologists, public health authorities, epidemiologists and more. The continuation use of insecticides urges for a comprehensive study in order to ensure in-depth identification of the degree of insecticide resistance so that the information can be used in re-evaluating current control practices in Indonesia. In light of the importance of assessing the efficacy of mosquito coils, the outcomes also

provide baseline data of *Ae. aegypti* in resisting pyrethroids in different localities of Indonesia for local authorities so as to accustom appropriate dosage of active ingredients incorporated into mosquito coils. Furthermore, the findings of this study could also notify the communities to get involved in gauging the extent of mosquito problem through their participations in environmental aspect such as getting rid of breeding sites.

In addition, understanding the biochemical and molecular mechanisms responsible for the resistance phenotype can assist in the development of tools to control the development and spread of resistant mosquito populations. This will further aid in the design of appropriate resistance management strategies for the control of *Ae. aegypti* in Indonesia. The findings of this study will be essential in reversing the spread and evolution of this resistance issue in Indonesia before insecticide resistance compromises all control measures.

1.4 Aims and Objectives

The main purposes of this study were to investigate the insecticide susceptibility status of *Aedes aegypti* and the underlying mechanism(s) in residential areas across nine regencies in Indonesia. Herein, the present study was carried out to address the following specific objectives:

1. **To evaluate the susceptibility status of field collected *Ae. aegypti* populations against mosquito coils containing pyrethroids in Indonesia.**
    i. To determine the susceptibility status of Indonesian *Ae. aegypti* against pyrethroid-based mosquito coils containing three active ingredients (i.e., d-allethrin, transfluthrin and metofluthrin) by mosquito coil bioassays.

2. **To characterize metabolic-mediated mechanisms of pyrethroid resistance in *Ae. aegypti*.**
    i. To determine the levels of enzyme activities in Indonesian *Ae. aegypti*.
    ii. To understand the degree of resistance phenotype with the levels of enzyme activities in Indonesian *Ae. aegypti*.
3. **To investigate target-site alterations of pyrethroid resistance in *Ae. aegypti*.**
    i. To determine the prevalence of the V1016G, S989P and F1534C mutations in knockdown resistance (*kdr*) gene of Indonesian *Ae. aegypti*.
    ii. To assess the associations between the frequency of resistance alleles and the degree of resistance phenotype in Indonesian *Ae. aegypti*.

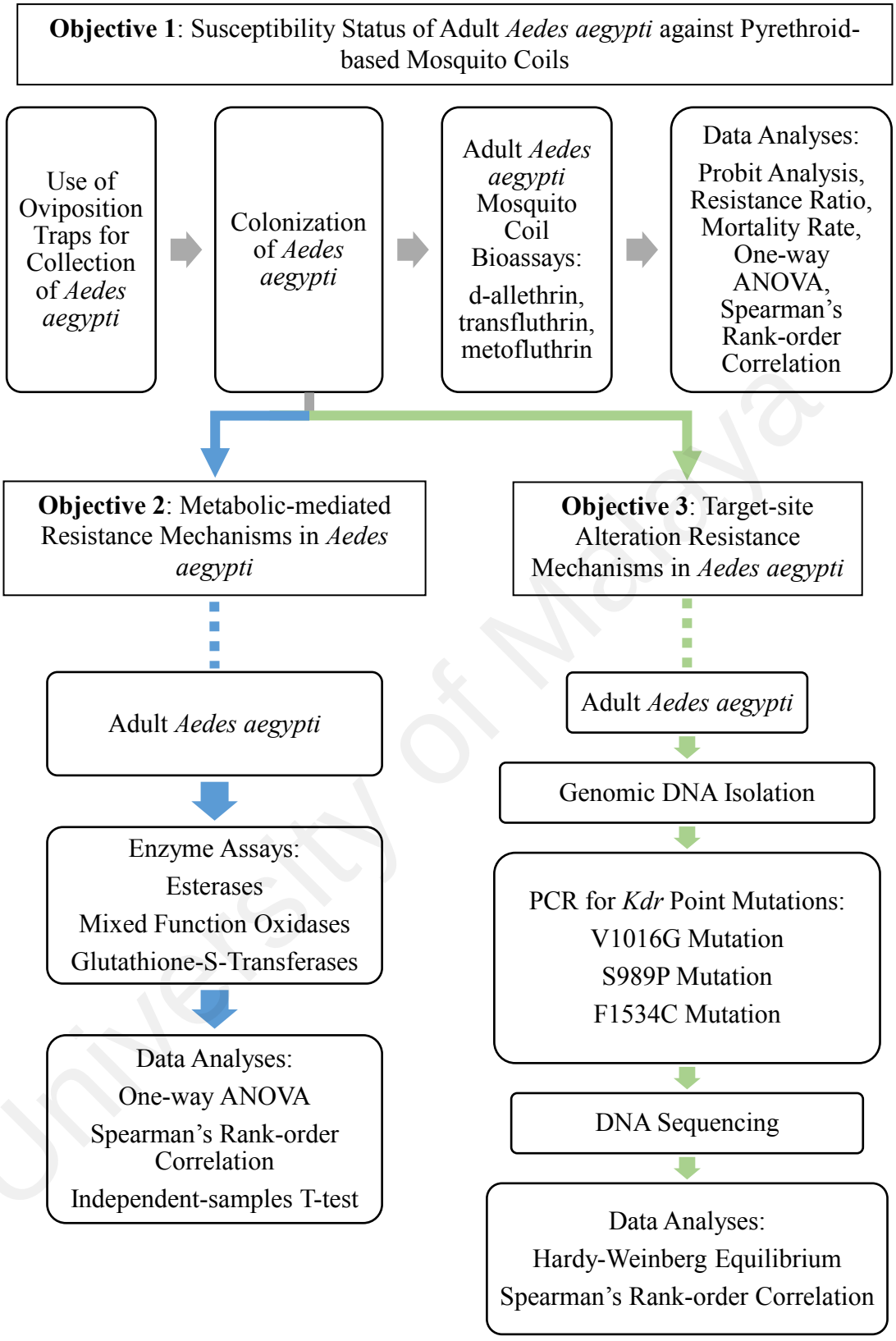

Figure 1.1: Schematic diagram of "Efficacy Assessment of Commercial Mosquito Coils and Associated Pyrethroid Resistance Mechanisms in *Aedes aegypti* from Indonesia"

CHAPTER 2: LITERATURE REVIEW

2.1 Importance of *Aedes aegypti*

Aedes aegypti Linnaeus, one of the world's most deadly creature among the 3,500 species of mosquitoes, is mostly found in subtropical and tropical regions. Native of this mosquito species is believed to be originated from sub-Saharan Africa (Tabachnick, 1991) but disease infection was only observed after the Europeans first stepped foot into the New World (Powell & Tabachnick, 2013). A domestic species like *Ae. aegypti*, thrives vigorously in urban and suburban areas (Saleeza et al., 2011; Ho et al., 2014). The highly adaptable characteristic of this species also potentially makes it more dangerous than other species.

Aedes aegypti is the main vector of four antigenically related serotypes of the dengue viruses (DENV-1, DENV-2, DENV-3 and DENV-4) (Gubler, 1998). This resilient species flourishes in urban and suburban habitats because they can simply breed in water storage containers everywhere. In recent years, the geographical expansion of *Ae. aegypti* has fostered the incidence of dengue fevers to gain an apparent increase worldwide, which put an estimation of almost half of the world's population at stake for the disease (WHO, 2017). Therefore, mitigation is required against the perpetual global transmission of arboviruses. A few possible factors that lead to widespread of dengue cases over the years include poor water and waste management caused by unprecedented urbanization in developing tropical countries (i.e., Southeast Asia); scattered human-made containers that provided more breeding habitats for *Ae. aegypti*; increased international travel that induced further dispersion of viral infections across borders; rapid population growth with sparse and ineffective public health services; and absence of effective methods for mosquito control (Gubler & Clark, 1996; Louis et al., 2016; WHO, 2017).

WHO (2017) has reported that the annual rate of dengue cases worldwide has increased noticeably since 1950. The recurrence of this outbreak in the recent years is caused by several factors, mainly due to the fact that *Ae. aegypti* can efficiently breed anywhere with artificial containers which is often close to susceptible hosts. Thus, this species has become an effective vector of dengue disease. In order to suppress this endemic disease, profound studies about the habitat, physiology and behavior of *Ae. aegypti* will help to foster suitable insights for a more persuasive vector control strategy.

2.2 Biology and Life Cycle of *Aedes aegypti*

Aedes aegypti is usually dark brown to black in colour with white stripes visible from its tarsus. Like all the other arthropods, *Ae. aegypti* is covered by a hard exoskeleton and is bilaterally symmetrical. The three main sections that form the anatomy of an adult mosquito are the head, thorax and abdomen. The adult is easily identified by the white scales that looked like the shape of a lyre on the dorsal side of the thorax whereas the abdomen has three segments out of ten segments which are designated for excretion and reproduction (Rueda, 2008). Subsequent to blood feeding, adult female mosquitoes will deposit several batches of eggs, up to 200 eggs per batch, spreading out to various locations (Rodhain & Rosen, 1997), usually on damp surfaces above the water line. The soft white elliptical eggs that were just laid will turn black and hard within a short amount of time (Schlaeger & Fuchs, 1974). After maturity of the embryo, these eggs are capable of coping with desiccation for months and yet, still hatch when submerged in water. This ability is due to the rigid, proteinaceous shell that reduces water loss (Lehane, 2005).

Aedes aegypti, at the adult stage has a life span of two weeks to a month. It undergoes a complete metamorphosis to attain its adult form (Goma, 1966). This holometabolous life cycle includes evolving from egg to larvae, then pupae, and finally the adult stage. Larvae, also known as “wrigglers” or “wigglers”, are commonly found in clean water and

will dive to the bottom of the container when agitated (Goma, 1966). Larval development involves four phases of instars which takes as little as five days to fully develop (Goma, 1966). The first four phases of instars develop rather quickly, while the fourth instar will take up to three days, in which it also increases more in size and weight (Nelson, 1986). The larvae usually feed on microorganisms or organic matter but they can endure conditions with limited nourishment and low temperature considering that water must be adequate. The pupal stage, is the metamorphosis phase to the adult stage after the fourth instar completed the ecdysis. The pupae, also known as "tumblers", require one to three days to develop (Nelson, 1986) and upon completion of metamorphosis, the adult mosquitoes will emerge from the pupae cuticle. Unlike the pupae of many other insects, mosquito pupae are active and respond to external stimuli. The adult stage is the reproductive stage for mosquitoes (Nelson, 1986). Female mosquitoes are required to take blood meal and mate merely once in a lifetime to lay eggs. Mating is possible within 24 hours of the transformation to adult stage as the females can start taking blood meals. Mating for this species mostly occurs during flight.

2.3 Behavior and Characteristics of *Aedes aegypti*

Aedes aegypti can be frequently found in areas lacked of adequate piped water system. It favours urban areas close to human population; usually staying in the same house where it emerged (Nelson, 1986). However, they will migrate in search for suitable locations to lay eggs. *Aedes aegypti* is commonly found resting indoors, therefore clean water in indoor areas is more likely to be infested with their larvae (Braks et al., 2003). Ideal breeding grounds for *Ae. aegypti* are moist substrates that are prone to momentary inundation, such as natural and man-made containers like tin cans, vases or flower pots, worn out tires, clogged drains, open water tanks, waterhole, and also tree holes. Apart from that, wet season in warmer climate countries will support in the adequacy of oviposition activity (Rozilawati, 2007). As reported previously, seasonal patterns of

dengue outbreak in tropical countries like Thailand, Myanmar and Malaysia usually coincide with the rainy season (Rozilawati, 2007).

Aedes aegypti is a daytime feeder. They are active several hours after dawn and before dusk. Males feed on plants nectar but females require blood meals for eggs development. Each blood meal taken is usually 2.5 times its own body weight (Gonçalves, 2009). Females respond to different stimuli from the vertebrate hosts and select the suitable site to feed on. Currently, not all chemicals attractants have been identified yet, nonetheless, the top recorded are carbon dioxide, lactic acid and octenol (Bowen, 1991). Mosquitoes feed on several types of skin surfaces as long as their proboscis can penetrate. Although *Ae. aegypti* generally feed on any vertebrate host, it is more attracted to human blood as part of their diet due to the nutrition benefits (Harrington et al., 2001). Once punctured, saliva containing anticoagulants will cause blood to flow into the surrounding tissue space which speeds up the feeding time. At the same time, water is also extracted from the host and may leave urine droplets on the skin before fleeing. In addition to the irritation due to saliva, mosquito bites are also important vectors of diseases (Cox et al., 2012).

2.4 *Aedes* Control

Since the exact cure for dengue is yet to be discovered even though significant advancements are made to develop vaccines, vector control will be the only viable approach to deal with this endemic disease (Nagpal et al., 2016). An effective vector prevention to transiently remove *Ae. aegypti* involves several strategies such as eliminating potential mosquito breeding sites, applying chemical insecticides and biological control. However, the most extensively practiced control for *Ae. aegypti*, owing to its high efficacy in regulating the populations with relatively rapid action, is the application of chemical insecticides (Bisset et al., 2009). These continuing efforts in

getting rid of arboviral diseases may lose their efficacies when vector-control methods decreased in susceptibility because of insecticide resistance development (Ranson et al., 2010). The occurrence of insecticide-resistant mosquito populations urges users to apply all of the on-shelf insecticides available cautiously to delay worldwide evolution of insecticide resistance in mosquitoes.

2.4.1 Chemical Control

Among a wide array of *Aedes aegypti* control approaches, use of chemical-based insecticides seems to be the most common tactic in both household and public pest control activities. The four main classes of insecticides used to control *Ae. aegypti* are pyrethroids, organophosphates, carbamates, and organochlorines (David et al., 2013). Of these insecticides, pyrethroids are by far the most commonly used insecticide class in suppressing *Ae. aegypti*. Some frequently used pyrethroids as recommended are permethrin, alpha-cypermethrin, cypermethrin, S-bioallethrin, cyfluthrin and lambda-cyhalothrin (Wuliandari et al., 2015). Adulticides are particularly used to bring down adult mosquito densities or other transmission parameters, categorized into surface residual spray for resting mosquitoes or space spray for flying mosquitoes. Pyrethroids (i.e. deltamethrin, lambda-cyhalothrin, cyfluthrin and permethrin) are alternatives to DDT to be used in surface residual spray. For space spray (i.e. cyfluthrin, deltamethrin, lambda-cyhalothrin, resmethrin and permethrin), uses of ultra low volume (ULV) and thermal fogging are indisputably a conventional method of vector control by not only every household but also for the public health. Usually fogging operations are executed right after the first report of dengue case. Pyrethroid insecticides are one of the most preferred adulticides used during dengue outbreaks (Rodríguez et al., 2002) because this active ingredient has shown to be more specific and effective on the control of adult *Ae. aegypti* and even their larvae in a short-term effect, without intoxicating the non-targeted organisms. It is unquestionably that many factors must be taken into considerations for

effective implementation of space spray operations, such as the impact on environment and the proper dosage used. Larvicides particularly aimed to suppress mosquito larvae, and are usually complementing environmental control. An example of larvicide is insect growth regulators (IGRs), such as chitin synthesis inhibitors (CSIs) and juvenile hormone mimics (JHs). Although they pose relatively low environmental impact, IGRs basically perform slowly that their usage can be narrow. Temephos is another popular larvicide belongs to the organophosphate family, mainly used in standing water where *Ae. aegypti* breeds. Temephos disturbs the central nervous system through inhibition of cholinesterase which this gives rise to death before larvae reaching the adult stage. However, temephos-resistant *Ae. aegypti* populations have already been documented (Mulyatno et al., 2012; Ikawati & Wahyudi, 2017).

2.4.1.1 Household Insecticide Products

Community-oriented strategies emphasize preventing or decreasing human-mosquito contact at the personal or household level. These approaches come in different formulations, involving the uses of household insecticide products (mosquito coils, electric vaporizing mats, electric liquid vaporizers, aerosols and fan emanator), insecticide impregnated nets or curtains, and repellents. Of all these personal protection measures, household insecticide products are the most active and sustainable form of community involvement. To ensure safety, most of these products contain synthetic pyrethroids as active ingredients which own the property of being less hazardous to users.

Mosquito coil is a mosquito-repelling incense with synthetic pyrethroids which by burning it, mosquito-repellent smoke will be produced. The use of mosquito coils is prevalent in Africa, Asia and the Western Pacific region (Becker et al., 2003). Mosquito coils, being one of the most common mosquito control methods used in household level, owe to the low costs, ease of use without the need of equipment or electricity and cultural

acceptance, since smoke is extensively used in many cultures in eradicating mosquitoes (Biran et al., 2007). To ensure safety, most of the mosquito coils contain pyrethroids as active ingredients that provide personal protection by repelling or knockdown mosquitoes and are less hazardous to users. Electric vaporizing mats or liquid vaporizers basically share the same principal to confer personal protection against *Ae. aegypti*. Heat generated from electricity is necessary to drawn up active ingredients from either mat or liquid form. Insecticide vapor will be released to the surroundings and induced behavioral changes in the mosquito populations via a series of sublethal effects like knockdown and biting inhibition.

As reported, Malaysia is one of the two countries in Southeast Asia tested on the efficacies of the commercial mosquito coils against *Ae. aegypti*. The mosquito coil is one of the most extensively-used anti-mosquito household spatial repellents in Malaysia, accounting for the low costs and easy availability on-shelf countrywide (Yap et al., 1996; Mulla et al., 2001; Yap et al., 2003). In 1996, the first study assayed on the efficacies of mosquito coils containing active ingredients like d-allethrin (0.2% w/w) and d-trans allethrin (0.1% w/w) against laboratory *Ae. aegypti* were accomplished, revealing adequate knockdown effects but with minimal mortalities for both coils (Yap et al., 1996). After almost a decade, a similar study was conducted with the use of d-allethrin (0.2% and 0.3% w/w) and d-trans allethrin (0.1% and 0.2% w/w) on laboratory strain of *Ae. aegypti* (El-garj et al., 2015). Dosage increment of the active ingredients in mosquito coils, which are 0.3% w/w of d-allethrin and 0.2% w/w of d-trans allethrin were proven to show higher repellent efficacies and mortality (El-garj et al., 2015). However, this result requires further verification on field strains in natural settings. In the past decades, efficacy studies were performed on the laboratory strains of *Ae. aegypti*. Until recently, insecticidal activities of Malaysian commercial coils have been assessed on field strains of *Ae. aegypti* in a nationwide-scale. *Aedes aegypti* from a total of 11 states was

discovered to be resistant to mosquito coils containing active ingredients of d-allethrin, d-trans allethrin, metofluthrin and prallethrin according to the resistance index recommended by the WHO (2009) (Chin et al., 2017). Since there is a wide array of insecticides used intensively in the country to curtail disease transmission, either operations conducted by the Ministry of Health, private companies or even at household level (Rohani et al., 2011), it is in utmost important to assess regularly on the efficacies of insecticides used to guarantee effective vector control program.

In Thailand, mosquito coils are also extensively used but these products have never been thoroughly studied. Although literature presents on the efficacies of commercially available mosquito coils, up to now, information about detailed assessment on these coils to field populations of *Ae. aegypti* remains to be sparse. These studies are irrefutably important when mosquito coils are the top choice of household insecticide formulations worldwide other than aerosols, vaporizing mats and liquid vaporizers. The first report was completed in 2008 in Thailand, showing a phenomenon of tolerance of *Ae. aegypti* to dl, d-T80-allethrin, d, d-Tprallethrin and methoxymethyl-tetrafluorobenzyl tetramethyl-cyclopropanecarboxylate (K-3050) under semi-field condition (Katsuda et al., 2008a). A similar study was conducted with a total of four field strains of *Ae. aegypti* tested against the same active ingredients, employing 25 m^3 semi-field test system, topical application test and field efficacy test (Katsuda et al., 2008b). Most of the strains were recorded to be tolerant to the mosquito coils tested but pyrethroid resistance may have developed since noticeably large gaps were observed between the timeframe of sample collection and the course of the study, with up to 31 years gap. It is very much recommended that, when possible, mosquito test populations should be acquired with shorter gap to the study to prevent bias in the outcomes. The latest related study was accomplished in 2009 using 25 m^3 semi-field test and topical application test, demonstrating extremely low susceptibility to dl, d-T80-allethrin in some field strains (Katsuda et al., 2009) that they may have

already developed resistance now. All three studies yielded similar outcomes with low allethrin susceptibility but it should be noted that direct comparison of results should be avoided due to different testing approaches used. Standardization of method is needed to prevent significant data deviation from a study to another. The knockdown time may vary in regard to types of method employed, that according to (Chadwick et al., 1975), results carried out in cylinders, small chambers and large rooms are distinct. Moreover, associated assessments on the efficacy of mosquito coil to *Ae. aegypti* are needed.

2.5 Pyrethroids

Pyrethroids available in market are synthetic analogs of pyrethrins, the compounds found in the pyrethrum extract of white-flowered *Chrysanthemum* plants. Although pyrethrins were used since 160 years ago, there was time when pyrethrins were completely being replaced by synthetic insecticides like DDT due to the low photostability (Wakeling et al., 2012) and inconsistency supply from plants (Schleier III & Peterson, 2011). However, organophosphates, organochlorines and carbamates were then ruled out due to significant non-target toxicity and biomagnification (Schleier III & Peterson, 2011). This led to the formulation of pyrethroids, which can alter the structures of the pyrethrins to resist photodegradation (Schleier III & Peterson, 2011) and to enhance insecticidal activity (Wakeling et al., 2012). To date, the use of synthetic pyrethroids is still growing as alternatives for natural pyrethrins (Katsuda, 1999; Housset & Dickmann, 2009). This can be attributed to the rapid knockdown effect on insects, effective on insects with carbamate and/or organophosphate-resistant strains, low mammalian toxicity and quick degradation rate in the environment (Katsuda, 1999). This class of insecticides constitutes approximately 17 % of the worldwide insecticide market share and 1.4 USD billion of global trades (Housset & Dickmann, 2009) despite the presence of numerous classes of chemical. Pyrethroids are categorized into type I (absent of a cyano group) and type II (present of a cyano group) pyrethroids based on their chemical structures,

electrophysiological responses and poisoning symptoms (Valentine, 1990; Schleier & Peterson, 2011). Type I encompasses tetramethrin, resmethrin, allethrin, d-allethrin, phenothrin, permethrin, transfluthrin, bifenthrin, metofluthrin and tefluthrin whereas type II includes cyfluthrin, cypermethrin, cyhalothrin, deltamethrin, fenvalerate, fluvalinate, fenpropathrin, flumethrin, flucythrinate and tralomethrin (Ray, 1991; Klaassen et al., 1996). In general, pyrethroids are neurotoxins which modify the normal function of insect nerves and cause disruption at the voltage-gated sodium channel (*Vgsc*) by depolarizing neurons (Scott, 1988; Narashi, 2002), paralyzing and eventually killing the insect.

Pyrethroids are currently being used extensively to control *Aedes aegypti* adults worldwide. Deltamethrin, cypermethrin, cyfluthrin. λ-cyhalothrin, permethrin, α-cypermethrin, pyrethrum, bifenthrin, d-phenothrin, z-cypermethrin and etofenprox are the major types of pyrethroids used, and their treatments usually involve either residual or space spray (Smith et al., 2016). Narrowing down to Southeast Asia, deltamethrin, permethrin, α-cypermethrin, cyfluthrin and λ-cyhalothrin are commonly employed in suppressing *Ae. aegypti*, particularly in Malaysia (Wan-Norafikah et al., 2010; Loke et al., 2012; Ishak et al., 2015), Indonesia (Astari & Ahmad, 2005; Ahmad et al., 2007; Wuliandari et al., 2015), Singapore (Lai et al., 2001; Koou et al., 2014a; Koou et al., 2014b) and Thailand (Chareonviriyaphap et al., 1999; Yaicharoen et al., 2005; Jirakanjanakit et al., 2007).

2.5.1 Burden of Pyrethroid Resistance in *Aedes aegypti*

Currently, insecticide resistance is one of the most severe issues facing mosquito control agencies. The loss of efficacy of pyrethroids may consequently bring high possibility of operational failure of *Ae. aegypti*-borne diseases control and subsequently lead to increased disease transmission. This will cause even larger capital to be invested into mosquito control program by the government in return, for examples, the

development of new insecticides in terms of labors and costs, which may cause direct socio-economic impact to a country. Other than the commonly-reported adverse impacts on public health and environment, reports particularly on the economic cost of pyrethroid resistance in *Ae. aegypti* in Southeast Asia have not been presented. This is in large part due to the progression of operating and resource costs throughout the vector control program since the first occurrence of control problems until complete control failures have not been described. To expedite the progress and execution of scientifically-sound policies of insecticide use, economic costs in Southeast Asian context are vital to be studied.

A need of new paradigm or choice to control *Ae. aegypti* is urgent, provided that the current vector control strategies in Southeast Asia resulted in the discovery of insecticide-resistant mosquito populations. The ultimate goal of vector control program should focus on the establishment of an efficient government-community partnerships that the community plays a fundamental character in implementing these programs. However, it should also be stressed that the success of these programs can only be accomplished if both parties are committed to implement procedures in assisting community participation in *Ae. aegypti* control.

2.5.2 Pyrethroid Resistance Mechanisms

Physiological resistance and behavioral avoidance of *Ae. aegypti* to insecticides are two integral responses ascribed to the intensified implementations of chemical-based control programs. Physiological resistance implies the survival of mosquito victims under the exposure to chemical insecticides which would normally lead to complete death, comprising the most well-documented mechanisms conferring resistance against pyrethroid insecticides in *Ae. aegypti* such as insensitivity of target site which causes knockdown resistance (*kdr*) (Brito et al., 2013); and metabolic detoxification via mixed

function oxidases (MFOs), non-specific esterases (ESTs) and glutathione S-transferases (GSTs) (Chareonviriyahpap et al., 2013). Behavioral avoidance is the development of any change in the behavior of a mosquito to decrease the exposure of toxic substances or to escape lethal effect of insecticide, but this mechanism is easily ignored (Chareonviriyahpap et al., 2013). Other than the aforementioned resistance mechanisms, insecticide resistance in *Ae. aegypti* is also mediated by cuticular resistance. Notes on each mechanism are described as follows.

2.5.2.1 Behavioral Avoidance

In-depth behavioral avoidance studies in *Ae. aegypti* are scarce across Southeast Asia until now. A change in mosquitoes' feeding or resting behavior to minimize exposure to insecticides, this mechanism is generally categorized into direct contact excitation and non-contact spatial repellency (Chareonviriyahpap et al., 2013). Direct contact excitation (irritancy) is a phenomenon when mosquitoes escape from insecticide-exposed environment upon physical contact, while non-contact spatial repellency is when mosquitoes leaving the toxic area even before getting contact with a treated surface. Related behavioral avoidance studies of *Ae. aegypti* exposed to a range of pyrethroids have been inspected in Thailand, projected that average to strong irritancy have demonstrated in all tested populations when compared to repellency (Grieco et al., 2005; Chareonviriyahpap et al., 2006; Paeporn et al., 2007; Thanispong et al., 2010;). Despite the importance of behavioral responses of mosquitoes to insecticides contribute to the efficacy of chemicals, the relative complications of the assay designs, for instance, problems in differentiating between contact excitation and non-contact spatial repellency responses exerted in mosquitoes, lacking ideal statistical methods in analyzing data, and difficulties in introducing and removing test specimens, cause a dearth of information in this mechanism.

2.5.2.2 Cuticular Resistance

Cuticle thickening is implicated in insecticide resistance by reducing the uptake of the insecticide that reaches the target site in response to the modification of chemical composition of the cuticle. In mosquitoes, cuticular resistance is often pointed out but related studies are insufficient. A recent study revealed that this mechanism may play a major role in the development of resistance where it normally happens simultaneously with other mechanism(s) (Kasai et al., 2014), causing resistance to single or multiple insecticides (Nkya et al., 2013). It was reviewed elsewhere that cuticle thickening is associated with metabolic detoxification whereby thicker cuticle causes gradual insecticide absorption rate that will increase the effectiveness of metabolic detoxification in *Anopheles funestus* (Wood et al., 2010). Moreover, it is crucial to take note that insects with cuticular resistance will display resistance level of not more than 3-fold in comparison to susceptible insects, but the co-occurrence of other resistance mechanism will lead to a surge in insecticide resistance level markedly (Lee et al., 2003). This is demonstrated by *Anopheles gambiae* in Benin (Djouaka et al., 2008) through which over-expression of cuticular genes and P450 genes gave rise to a relatively high resistance level. Cuticular resistance to pyrethroids is also characterized in *Ae. aegypti* (David et al., 2010) but there is a lack of similar report documented in Southeast Asia. The fact that this least-understood mechanism may play substantial role in resistance urges for immediate attention in investigating, particularly in pyrethroid-resistant *Ae. aegypti*.

2.5.2.3 Knockdown Resistance (*Kdr*)

Target site resistance in mosquitoes is related to either single or multiple mutations in target genes; for example, the voltage-gated sodium channel (*Vgsc*) which leads to knockdown resistance (*kdr*), mutations in the acetylcholinesterase (*Ace-1*) gene and GABA receptors (Hemingway & Ranson, 2010; Ishak et al., 2017). The most well-studied target site resistance for *Ae. aegypti* is *kdr* because it confers resistance against

pyrethroids. The *Vgsc* gene comprises four homologous domains and each of them contains six hydrophobic subunits (segment 1–6). In the past few decades, numerous mutations in the *Vgsc* gene have been documented to be linked to *kdr* in many insect disease vectors. The single point L1014F mutation in the domain II segment 6 (IIS6) was the foremost mutation associated with pyrethroid resistance identified. Thereafter, different substitutions of C, F, H, S or W at this position were reported in a few mosquito species in the genera of *Anopheles* and *Culex* mosquitoes (Torres et al., 1999; Enayati et al., 2003; Martinez- Xu et al., 2005; Verhaeghen et al., 2010).

In *Ae. aegypti* mosquitoes, the L1014F mutation has yet to be identified but several different *kdr* mutations have been detected in Southeast Asia; for examples, S989P, I1011M/V, V1016G/I and F1534C based on the mutation positions of house fly sodium channel (Brengues et al., 2003; Saavedra-Rodriguez et al., 2007; Rajatileka et al., 2008; Bingham et al. 2011; Kawada et al., 2014; Wuliandari et al., 2015). It is no surprise that more sodium channel mutations in resistant insects have been discovered taking the advantage of the convenience of molecular approach. Beside the identification of single point mutations, novel co-occurrence of mutations was also detected. As firstly reviewed by Brengues et al. (2003), V1016G was closely related to S989P in Southeast Asian countries such as Indonesia, Myanmar, Thailand and Malaysia (Srisawat et al., 2010; Kawada et al., 2014; Ishak et al., 2015; Wuliandari et al., 2015), and this common phenomenon was believed to confer higher resistance. Co-occurrence of triple mutations V1016G, S989P and F1534C would result in even higher resistance to pyrethroids, as seen in *Ae. aegypti* populations from Myanmar (Kawada et al., 2014). The detection of these specific mutations, such as V1016G, S989P and F1534C were affirmed to confer sodium channel resistance to pyrethroids and other associated mutations which are still yet to be inspected. It is very likely for the emergence of new *kdr* mutations when pyrethroids remained to be the primary insecticide-based interventions in the control of

Ae. aegypti. Hence, these detections act as a crucial role in resistance management of *Ae. aegypti* by observing the occurrence of pyrethroid resistance.

It is remarkable that despite two distinct substitutions occur at the same amino acid position, V1016G and V1016I display different geographical variations with dissimilar response of *Ae. aegypti* sodium channel to pyrethroids. V1016G has been detected in Southeast Asia (Brengues et al., 2003; Rajatileka et al., 2008; Kawada et al., 2014; Hamid et al., 2017a; Marcombe et al., 2017) while V1016I has been found in North and South America (Linss et al., 2014; Alvarez et al., 2015; Dolabella et al., 2016). In recent times, the V1016I mutation was detected in Vietnam (Bingham et al., 2011) and V1016I along with F1534C was detected in Ghana that there may be presence of migration event (Kawada et al., 2016). The detection of these point mutations in *Ae. aegypti* strains in continents they have never existed before requires further validation to confirm if the resistance is caused by migration or *de novo* (new) mutations. The evolution and distribution of pyrethroid resistance in *Ae. aegypti* populations is relevant when their eggs can endure desiccation for an extended period that permit them to put human travel to good use.

2.5.2.4 Metabolic-mediated Insecticide Resistance

Metabolic resistance in *Aedes aegypti* involves alterations in a series of the expression of a complex group of enzymes, causing a rise in the detoxification process of insecticides. This resistance mechanism facilitates increased biodegradation of insecticides, with the help of enzymes such as GSTs, ESTs and MFOs (cytochrome P450 monooxygenases). A total of 160 P450 genes, 49 GSTs and 26 ESTs have been revealed in an international genome project of *Ae. aegypti* (Strode et al., 2008). Among these, cytochrome P450 genes are the enzyme family highly responsible for the development of pyrethroid resistance in *Ae. aegypti*. Therefore, cytochrome P450 genes are of particular

interest to study since they are capable in metabolizing xenobiotics such as drugs, plant toxins and insecticides. One of the most noteworthy characteristics of P450 genes linked to enhanced metabolic detoxification of insecticides is the detection of a significant rise in P450 proteins and P450 activity due to the overexpression of insecticide-resistant insects P450 genes, which is closely associated with the development of insecticide resistance (Feyereisen et al., 1999; Zhu et al., 2008). However, identification of enzymes associated with insecticide resistance is difficult because of the presence of diverse group of P450 genes and high structural similarity in isoforms that makes identifying isoform related to resistance to be challenging. While molecular approaches in detecting *Vgsc* gene mutations in pyrethroid-resistant *Ae. aegypti* are broadly documented, they are rare for P450 genes. *CYP9* family has been involved in pyrethroid resistance in the dengue vector *Ae. aegypti* (Marcombe et al., 2009). *CYP9J22* and *CYP9M9* were the two P450 genes found with the highest transcript level of the resistant populations (Marcombe et al., 2009). On a global scale, the most frequently found P450 genes are *CYP9M6*, *CYP6BB2*, *CYP9J26* and *CYP9J28*, which are commonly detected to be over-expressed in resistant strains in many studies, projecting their capability in metabolizing permethrin (Pavlidi et al., 2012; Stevenson et al., 2012; Kasai et al., 2014).

ESTs hydrolysis of pyrethroids resulting in the detoxification process has assumed to involve in metabolic resistance in some cases. This mechanism encompasses a series of reaction, such as gene amplification, up-regulation, and/or coding sequence mutations. However, published data on which exact ESTs in contributing to the presence of pyrethroid-resistant *Ae. aegypti* populations in Southeast Asia continues to be scarce. Elevated levels of GSTs are also commonly associated with insecticide resistance. Most of the GSTs are made of a super family structure of cytosolic-dimeric enzymes that are grouped into a total of six classes, namely Delta, Epsilon, Omega, Sigma, Theta and Zeta (Lumjuan et al., 2005). The two largest classes of GSTs for insects are Delta and Epsilon,

provided that Epsilon class is often involved with resistance (Lumjuan et al., 2007). In *Ae. aegypti*, there are basically a cluster of eight sequentially arranged genes that mapped genetically to chromosome 2, supercontig 1.291 in the Epsilon GSTs class (Lumjuan et al., 2007). Recently, microarray studies have discovered some additional members of this gene cluster with elevated levels in *Ae. aegypti* populations resistant to insecticides (Marcombe et al., 2009). However, their roles in metabolizing insecticides like pyrethroids are yet to be determined. Although ESTs and GSTs are found over-expressed in pyrethroid-resistant *Ae. aegypti*, the causal of specific enzymes to the resistant populations from each of the enzyme family are still largely unverified up to now especially in Southeast Asia.

It is important to investigate metabolic-mediated insecticide resistance of *Ae. aegypti* to pyrethroids since co-existence of more than one mechanism is likely to occur, especially the largest endemic sites of dengue fever Southeast Asia. In addition, efforts should be put in to the development of new insecticides, taking these enzymes related to insecticide resistance into consideration in the span of design procedure.

2.6 Present Situation of Pyrethroid Susceptibility in *Aedes aegypti* in Southeast Asia

Published literature with regards to the insecticide resistance profiles of *Ae. aegypti* against pyrethroids in most of the Southeast Asian countries are detailed as follows. Information were extracted from all publications documenting mortality, lethal concentration (LC_{50}) and knockdown time (KT_{50}) from bioassays of *Ae. aegypti* mosquito populations using merely pyrethroids. Since the main emphasis was chiefly placed on studies assaying pyrethroids against *Ae. aegypti*, other classes of insecticides tested and species other than *Ae. aegypti* found in the same publication were excluded and not reviewed in the present study. *Kdr* assays used to observe the effect of control tactics on

pyrethroid resistance were also studied. Distribution of different *kdr* mutations in *Ae. aegypti* in Southeast Asia were summarized in Table 2.1. Knowledge gaps which required attentions were also identified in this chapter.

2.6.1 Cambodia

Although dengue is a major health concern in Cambodia (Seng et al., 2008), there are merely two known studies evaluating insecticide resistance status of *Ae*. *aegypti* in the country. The first related study was carried out using temephos (an organophosphate) at Phnom Penh and Kampong Cham in accordance with WHO standard bioassays (Polson et al., 2001). Mosquito populations from Phnom Penh revealed temephos resistance whereas the latter populations were susceptible (Polson et al., 2001). It is believed that temephos resistance in Phnom Penh populations led to another more comprehensive study encompassing Phnom Penh, Siem Reap, Kampong Cham and Battambang using both temephos and pyrethroids (permethrin and deltamethrin). Resistance to permethrin and deltamethrin were reported in *Ae. aegypti* populations from all the aforementioned study sites (Boyer et al., 2016). Therefore, a nationwide assessment is recommended to examine pyrethroid resistance in *Ae*. *aegypti* since Cambodia is a country bordering the dengue-endemic countries Thailand, Laos and Vietnam, so as to develop effective vector control methods to halt the spread of dengue fever.

In Cambodia, the point mutations of the *kdr* gene related with pyrethroid resistance of *Ae. aegypti* were analyzed alongside with samples from other Southeast Asia countries in two international studies. The F1534C mutation was detected in the first study (Yanola et al., 2011) whereas C1534 mutant alleles (with VV/CC patterns) were detected in the second study (Saingamsook et al., 2017). Further detection is highly sought after due to a deprived of nationwide studies that may possibly lead to the discovery of other unreported point mutations of the *kdr* gene. This helps to understand the nature of

pyrethroid resistant in *Ae. aegypti* in this country, given that dengue is endemic year-round in Cambodia. Biochemical mechanisms of *Ae. aegypti* to pyrethroid resistance have not been reported.

2.6.2 Indonesia

After a first occurrence in 1968, countless outbreaks of dengue fever (DF) and dengue hemorrhagic fever (DHF) cases transmitted by the primary vector *Ae. aegypti* mosquitoes have dramatically increased in recent years. The latest statistic showed 71,668 DF human cases in 2015 (Ministry of Health Republic of Indonesia, 2015), covering all 34 provinces of the country. In Indonesia, pyrethroids have been broadly used since the 1980s against *Ae. aegypti* but there has been a dearth of knowledge on published information. Earlier, some pioneer works reported that *Ae. aegypti* populations from West Java (Ciamis, Purwakarta and Bogor) and Bandung were resistant to permethrin, lambda-cyhalothrin, cypermethrin and d-allethrin (Brengues et al., 2003; Astari & Ahmad, 2005;). Despite high LT_{90} values being recorded (Astari & Ahmad, 2005), their resistant status could not be concluded. The standard susceptible laboratory strain used as a control in the study appeared to be resistant, led to an uncertainty of the resistant status. More recently, published data showed the presence of pyrethroid resistance in *Ae. aegypti* even after approximately 10 years of the pioneer studies (Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a). Their susceptibility profiles suggested similar trends of resistance to pyrethroids (Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a), signifying comparable patterns of insecticide usage in these sampling sites.

Over the years, biochemical studies have acknowledged the role of detoxifying enzymes in the development of pyrethroid resistance in *Ae. aegypti*. The first small-scale test in determining the resistance mechanisms involved in pyrethroid-resistant *Ae. aegypti* was accomplished in 2007, comprising strains from Bandung, Palembang and Surabaya

(Ahmad et al., 2007). Adult *Ae. aegypti* from Bandung demonstrated the highest resistance level to both permethrin and deltamethrin, corresponding to the high levels of activity of enzymes tested, i.e., MFOs, α-ESTs and β-ESTs, that play roles in the development of resistance to pyrethroids. In contrast, another study accomplished by Astuti et al. (2014) showed that larvae from most sampling sites in Cimahi were still susceptible to pyrethroids although Bandung situates in close proximity to Cimahi. Adult susceptibility profile may greatly correlate with larval susceptibility profile in some cases (Nazni et al., 2005; Wan-Norafikah et al., 2010). However, it should be remembered that there is a difference between resistance score in larvae and adults because they may develop dissimilar resistant mechanisms through different mechanism pathways from larval to adult stages (Koou et al., 2014b). Thus, adult bioassay is still required to verify the resistance status.

Other than the responsibility of detoxifying enzymes in the insecticide resistance mechanisms, mutations in the *Vgsc* gene should also be highlighted because they may act as markers for resistance monitoring. In Indonesia, *kdr* profiling of *Ae. aegypti* had recorded three point mutations related to pyrethroid resistance, namely the S989P, V1016G and F1534C mutations (Brengues et al., 2003; Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a; Hamid et al., 2017b). The discovery of the V1016G mutation was first reported by Brengues et al. (2003) in the Semarang strain. Later, some findings were in agreement that all three mutations in *Vgsc* gene were detected in *Ae. aegypti* populations from Yogyakarta, Central Java Province, Denpasar and Jakarta (Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a; Hamid et al., 2017b). The V1016G mutation was most frequently detected in high frequencies, remarkably associated with permethrin and deltamethrin resistance in *Ae. aegypti*. Previous reports discovered relatively low frequency of the F1534C mutation, which did not demonstrate significant contribution to the resistance development of pyrethroids in *Ae. aegypti*

(Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a; Hamid et al., 2017b). As for the S989P point mutation, it generally co-occurred with the V1016G mutation, showing association on pyrethroid resistance development in *Ae. aegypti*. Furthermore, the frequency of the V1016G mutation occurred in *Ae. aegypti* in the Central Java has increased two-fold in just a decade, compared to a report generated earlier (Brengues et al., 2003). These preliminary detections of insecticide resistance will fundamentally aid in initiating appropriate control measures in delaying development of mosquito resistance, for instance, rotation of insecticide or addition of catalyst to enhance the efficacy of insecticides in suppressing *Ae. aegypti* in Indonesia.

2.6.3 Laos

Compared to the neighboring countries, there is a scarcity of study on pyrethroid resistance in *Ae. aegypti* in Laos. The first dengue fever outbreak was recorded in 1998 in Khammouane Province (Tsuda et al., 2002). Although *Ae. aegypti* has been stated to be the main vector of numerous mosquito-related diseases in Laos (Jennings et al., 1995; Tsuda et al., 2002) with 22,772 cases of dengue reported during 2010 (Hiscox et al., 2013), there is only a single report investigating pyrethroid resistance status of *Ae. aegypti* in the country thus far. Samples were collected from five provinces and populations involved in the study identified high level of resistance against permethrin, which contrast with deltamethrin that exhibited high susceptibility in *Ae. aegypti* (Marcombe et al., 2017). Higher quantities of P450 genes were detected in field compared to reference strains but no particular *CYP* was mentioned. The frequencies of *kdr* mutations were low for the V1016G mutation (< 0.36) but relatively higher for the F1534C mutation (> 0.6 for majority of places). With a total of 18 provinces available in Laos and a reliance upon pyrethroids in *Ae. aegypti* control after the official ban of DDT in 2010 (Ministry of Health LP, 2010), more comprehensive studies are needed in Laos, as this knowledge is fundamental in managing vector control programs.

2.6.4 Malaysia

Dengue or dengue hemorrhagic fever has been a critical public health concern in Malaysia at all times since the first outbreak in 1973. Regardless of extensive fogging operations with malathion in Malaysia as early as the 1970s, and followed by the replacement of pyrethroids like permethrin and deltamethrin in early 1998 until today (Low et al., 2013), dengue remains number one infectious disease with a total of 101,357 cases reported in the country (Ministry of Health Malaysia, 2017). Numerous surveillance activities have been conducted to investigate insecticide susceptibility status of *Ae. aegypti*. The first study examining on the pyrethroid resistance level of *Ae. aegypti* in Malaysia was accomplished in 2001, using field strains from Kuala Lumpur, Selangor, Negeri Sembilan, Johor, Kelantan and Pahang. The urban strain of *Ae. aegypti* from Kuala Lumpur showed the highest resistance to permethrin, validating the high levels of ESTs compared to the laboratory strain (Rohani et al., 2001). This result was in agreement with the findings performed after almost a decade. Permethrin resistance persisted in field strains of *Ae. aegypti* collected from dengue-endemic areas of Kuala Lumpur (Wan-Norafikah et al., 2010). Both larval and adult bioassays of the field strains confirmed the development of tolerance towards permethrin with several folds higher than the laboratory strain (Wan-Norafikah et al., 2010), indicating high reliability on chemical insecticides to control *Ae. aegypti* in an unprecedented scale at the dengue hotspots. Piperonyl butoxide (PBO) was also reported to be effective in enhancing the effectiveness of permethrin through which strong correlations were confirmed between LC_{50} or LT_{50} values and MFOs levels for all strains (Wan-Norafikah et al., 2010), implying the involvement of MFOs activity in causing permethrin resistance in this mosquito species.

Another similar study performed at dengue-prone sites of Selangor documented resistance and incipient resistance of *Ae. aegypti* to permethrin (mortality ≤ 80%) and cyfluthrin (mortality 45–97.8%) respectively (Loke et al., 2012). Although cyfluthrin was

not used by the municipal in vector control program, fluctuated resistance was detected and this may be attributed to the role of *kdr*. Screening of *kdr* is yet to be performed in Selangor but it has already accomplished and confirmed on the presence of *kdr* in Kuala Lumpur (Ishak et al., 2015) which is just a stone's throw away from Selangor. DDT resistance was also detected over the course of the study (Loke et al., 2012), further supporting the role of *kdr* in both pyrethroid and DDT resistance due to the shared *Vgsc* target site. GSTs activity exerted on DDT resistance has not been discovered in Malaysian mosquitoes including *Ae. aegypti* (Lee & Chang 1995; Low et al., 2013). The widespread of insecticide resistance pays full responsibility to failures in vector control program. *Aedes aegypti* populations from mainland Penang were also highly resistant to lambda-cyhalothrin (Hasan et al., 2016). These outcomes were supported by the fact that pyrethroids have been broadly sprayed in the study areas for more than 10 years. Of several studies conducted in the country, insecticide susceptibility status of permethrin was often tested against *Ae. aegypti* in Malaysia because this insecticide is one of the main adulticides used in Malaysia (Ministry of Health Malaysia, 2016). Moreover, the development of permethrin resistance at a higher rate than malathion and temephos (Hamdan et al., 2005) might be closely associated with gene activation due to the exposure to insecticidal pressure.

More recently, despite discrepancies of insecticide susceptible status found in the field strains of *Ae. aegypti* from Kuala Lumpur, Penang, Johor Bharu and Kota Bharu tested against permethrin and deltamethrin, the mosquito strains from Kuala Lumpur remained to exhibit the highest resistance levels (Ishak et al., 2015). Synergist assays with PBO showed that metabolic resistance mechanisms played key role in certain strains. This particular study is the first report in Malaysia in characterizing the *kdr* resistance in Malaysian populations of *Ae. aegypti*. The V1016G and the F1534C mutations were detected, with the F1534C mutation closely associated with pyrethroid resistance whereas

the V1016G mutation co-occurred to contribute to the additive effect of pyrethroid resistance in Malaysia (Ishak et al., 2015). A microarray-based genome-wide transcriptional analysis discovered that metabolic resistance of pyrethroid resistance in *Ae. aegypti* populations is predominantly caused by over-expressed of the cytochrome P450 genes (*CYP9J27*, *CYP6CB1*, *CYP9J26* and *CYP9M4*) (Ishak et al., 2017). However, more characterization work on the cytochrome P450 family must be carried out to understand the precise roles of these genes in contributing pyrethroid resistance in *Ae. aegypti*.

2.6.5 Myanmar

The first documented outbreak of DHF was in 1969 and the outbreaks gradually increased to 12 out of 14 states in Myanmar (Aung et al., 1996). This disease was documented with a high of 83,381 cases, causing 3,243 deaths from 1970 to 1991 (Aung et al., 1996). No sign of decrement in DHF incidence with 5,621 cases in 2005 to an approximate two-fold of 11,049 cases in 2006 (Oo et al., 2011) despite persistent control efforts made by the Myanmar government since 1968 (Aung et al., 1996). Although the significance of the disease requires immediate attention, there is only a single report on the ecological study of *Ae. aegypti* focusing on the southern part of Myanmar (Oo et al., 2011). There is still an unavailability on the bioassay studies of the status of pyrethroid resistance in *Ae. aegypti* in the country.

There is an absence of study to date, regarding the biochemical mechanisms of *Ae. aegypti* to pyrethroid resistance. In 2014, a project was initiated to evaluate the types and frequencies of point mutations of the *kdr* gene related with pyrethroid resistance of *Ae. aegypti*. It was revealed that V1016G and S989P mutations were extensively scattered in Yangon city with high frequencies of 84.4% and 78.8% respectively (Kawada et al., 2014). Another widely scattered point mutation identified with relatively low allelic

frequency of 21.2% was F1534C (Kawada et al., 2014). Other than three single point mutations were detected in Yangon city, three patterns of co-occurrence of point mutations were also identified with widely distributed homozygous V1016G/S989P mutations (65.7%) as well as a small number of homozygous V1016G/F1534C mutations (2.9%) and homozygous V1016G/F1534C/S989P (0.98%) (Kawada et al., 2014). It should be noted that the observed types and frequencies of resistance alleles may be very well representing the Yangon city, as samples were collected from seven townships. However, it may not be a decent approximation of types and genotype frequencies of the country because the size of Yangon city (598.75 km^2) is relatively small compared to Myanmar (676,578 km^2). Therefore, samplings is needed throughout the country to ensure the surveillance of resistance genes in wild *Ae. aegypti* populations are comprehended.

2.6.6 Philippines

There appears to be an agreement in literature that *Ae. aegypti* was introduced into the Philippines approaching the end of 19^{th} century and followed by the proliferation of this dengue vector (Powell et al., 2013). However, there are only two known studies evaluating the insecticide resistance of *Ae. aegypti* to pyrethroids in the Philippines. In the first study, *Ae. aegypti* from Luzon Island, Manila was confirmed to be susceptible to dieldrin even after eight years of insecticide application in the country since 1959 (Johnsen et al., 1967). Currently, the latest susceptibility test of *Ae. aegypti* with the use of pyrethroid was completed in 1997 in Cebu city. It was proven that treatment of curtains with permethrin was an effective vector control measure (Madarieta et al., 1999). These preliminary studies facilitated in verifying the development of resistant strains in the country but the information may not be up to date that ample related studies are recommended in the future because dengue fever is endemic in neighboring countries like Malaysia. Susceptibility tests of different pyrethroids and related resistance mechanisms

should be attempted on different field populations of *Ae. aegypti* in the country to confirm insecticide susceptibility status and guarantee the representativeness of the information generated since those insecticides have been put to use for several decades.

2.6.7 Singapore

It is clear that *Ae. aegypti* mosquitoes are the primary vectors of dengue fever in Singapore with the first dengue case logged in the 1960s (Lim et al., 1961). In the 1970s, pyrethroids were first introduced to the country to deprive the populations but permethrin resistance was identified in field *Ae. aegypti* later (Tan et al., 1998). Subsequently, an assessment in 1999 revealed a 12.9-fold of RR_{50} of field *Ae. aegypti* against permethrin (Lai et al., 2001). Resistance of *Ae. aegypti* to cypermethrin was also reported in a study from 2004 to 2007 (Lee et at., 2014). More recently, the detections of resistance of *Ae. aegypti* to pyrethroids were further verified by two comprehensive nationwide insecticide resistance studies on larvae tested with permethrin and etofenprox; as well as adults with cypermethrin, deltamethrin and etofenprox (Koou et al., 2014a; Koou et al., 2014b). Despite a few decades of gap between these studies, high resistance to pyrethroids remained to persist in the Singaporean *Ae. aegypti* populations and they continued to thrive, portraying the widespread or probably inappropriate use of pyrethroids ever since they were first introduced in Singapore. The use of synergists (PBO, *S,S,S*,-tributyl phosphorotrithioate, and triphenyl phosphate) was also deemed to be insignificant to the insecticides used in these studies to increase toxicity, signifying negligible roles of metabolic-based resistance when further biochemical investigation discovered that detoxifying enzymes such as MFOs, ESTs, and GSTs failed to contribute to pyrethroid resistance (Koou et al., 2014a; Koou et al., 2014b). The ineffectiveness of synergists proposes that molecular work is recommended to further investigate the mechanisms contribute to the high pyrethroid resistance exhibited in local *Ae. aegypti*.

Other than fogging, space spraying, chemical treatment and source reduction as dengue vector control methods, deltamethrin-treated net is an alternative. However, its efficacy was proven to be unsatisfactory against field *Ae. aegypti* in a recent study (Pang et al., 2015). Three molecular investigations have evaluated the roles of individual and multiple *Vgsc* gene mutations in contributing to the developed pyrethroid resistance in Singaporean *Ae. aegypti*: F1534C, S989P/V1016G and F1534C/V1016G (Hirata et al., 2014; Kasai et al., 2014; Pang et al., 2015). However, in-depth study showed that G1016 alleles independently pay more responsibility to *Vgsc* gene insensitivity than C1534 alleles independently (Hirata et al., 2014; Kasai et al., 2014). To the best of our knowledge, S989P/V1016G/F1534C triple mutations in *Vgsc* gene exhibited in the local *Ae. aegypti* were first reported in Singapore. They exerted the largest pressure in channel sensitivity to permethrin and deltamathrin by 1100-fold and 90-fold (Hirata et al., 2014), implying the likelihood of reduced efficacy to pyrethroid insecticides. There is also a need of an immediate attention to monitor the occurrence of triple mutations in *Vgsc* gene of field *Ae. aegypti* populations worldwide, so that the challenge of mosquito vector control will not further be exacerbated. The significance of P450-mediated resistance is mirrored by the highly-resistant *Ae. aegypti* Singapore (SP) strain, showing 1,650-fold of resistant to permethrin and PBO has reduced the resistance by 48-fold (Kasai et al., 2014). P450 genes (*CYP4C50*, *6BB2*, *6F2*, *6F3*, *6Z7*, *6Z8*, *9M4*, *9M5* and *9M6*) were also discovered to be over-expressed in adult females and males (Kasai et al., 2014). Precise and reliable molecular diagnosis approach to identify metabolic enzymes conferring pyrethroid resistance in *Ae. aegypti* populations should be emphasized to elucidate the contribution degree of P450 genes to field-resistant strains from distinct areas.

2.6.8 Thailand

Thailand is the country with the most studies evaluating on the insecticide resistance of pyrethroids to *Aedes aegypti* in Southeast Asia, reflecting a substantial geographical

bias as this species is widespread in many countries in this sub-region. Thailand first experienced outbreaks of DHF in 1958 and the disease has since then distributed nationwide (Sheppard et al., 1969). To date, DF or DHF remain a severe health threat in Thailand regardless of unrelenting vigilance in vector control programs. Reliance has been on the carbamate, organochlorine and organophosphate insecticides since 1950 (Chareonviriyahpap et al., 1999) and the use of synthetic pyrethroids then dominated the market since 1992. Pyrethroid-based formulations with 12 distinct active ingredients, are commercially available to all household levels countrywide to control mosquitoes in response to their low price, quick knockdown effect and are relatively safe for human contact because of low mammalian toxicity (Chareonviriyahpap et al., 1999). Uncountable years of routine contact with these insecticides have induced high levels of resistance in *Ae. aegypti*.

To understand the insecticide susceptibility profile of Thai *Ae. aegypti* to pyrethroids, there have been several published pioneering works available but specific geographical restrictions were observed whereby they were conducted in small confined areas during previous decades. Increasing tolerance or resistance to different types of pyrethroids, namely deltamethrin, permethrin, dieldrin, bioallethrin, bioresmethrin or alpha-cypermethrin, has been reported in larval and adult *Ae. aegypti* in Thailand (Chadwick et al., 1977; Chareonviriyahpap et al., 1999; Somboon et al., 2003; Paeporn et al., 2004; Ponlawat et al., 2005; Yaicharoen et al., 2005; Sathantriphop et al., 2006; Jirakanjanakit et al., 2007; Thanispong et al., 2008). The absence of large scale evidence-based understandings of the knowledge between the susceptibility profile and insecticides used may largely hinder the effect of dengue control efforts. A comprehensive study evaluating the insecticide susceptibility level will aid in better efficiency in program-planning to target the disease vector. More recently, published data on pyrethroid resistance in Thai *Ae. aegypti* addressed the issue that all 32 strains of *Ae. aegypti* were discovered to be

resistant to permethrin, ranging from 4–56.4% (Chuaycharoensuk et al., 2011). The frequency of susceptibility to deltamethrin in this species showed more than 98% of mortality to deltamethrin in the majority of the populations, with incipient resistance detected in minor populations. Conversely, all 32 strains of *Ae. aegypti* were entirely susceptible to lambda-cyhalothrin with 100% mortality. Significantly high levels of permethrin resistance were documented by Jirakanjanakit et al. (2007) (5% mortality) and Thanispong et al. (2008) (2–9% mortality) which contrast with this particular study. This may attribute to the differences in sampling sites and the alterations in levels of exposure to permethrin.

A simple colorimetric assay based on Heated Oligonucleotide Ligation Assay (HOLA) *kdr* assay from past studies was developed to detect substitutions within domain II, subunit 6 (i.e., Met1011, Val1011, Ile1016, and Gly1016) (Rajatileka et al., 2008). The V1016G mutation was evidently detected in high allele frequency of 0.23 throughout Thailand in pyrethroid-resistant *Ae. aegypti*, and that the I1011V mutation to be the minority with an allele frequency of 0.14 (Rajatileka et al., 2008). Despite only a thermal cycler being involved in this assay, extra reagents are needed which contributes to the increment of cost. An allele-specific PCR assay was then developed to detect the V1016G mutation which was shown to be reliable (Stenhouse et al., 2013). Homozygous 1016G mosquitoes were found to be common in Thailand and showed higher survival rates than either heterozygous or wild-type (1016V) mosquitoes upon deltamethrin exposure, indicating this particular mutation confidently related to deltamethrin resistance. Subsequently, the F1534C mutation was discovered as a novel amino acid mutation in 2010 in permethrin-resistant *Ae. aegypti*, and was reported to play a significant role in contributing permethrin resistance in multiple field strains (Yanola et al., 2010). The wide distribution of this mutation led to the development of high-throughput molecular tools, namely TaqMan SNP genotyping and an AS-PCR assay which proved to be consistent in

detecting the F1534C resistance mutation in the permethrin-resistance *Ae. aegypti* populations (Yanola et al., 2011). Recently, a multiplex PCR was developed to detect both V1016G and F1534C *kdr* mutations in *Ae. aegypti* through a single-reaction protocol (Saingamsook et al., 2017). This method was evidenced to depict high sensitivity and specificity in detecting the aforesaid *kdr* mutations, enabling the monitoring of the frequency of mutant alleles across dengue-endemic countries at ease with reduced time and cost.

Other than the discovery of singly-occurred mutations, the novel co-occurrence of the S989P mutation and the V1016G mutation were detected in Thai deltamethrin-resistant *Ae. aegypti* (Srisawat et al., 2010). The 1016G mutation usually coexists with 989P mutation but there is also an absence of 989P mutation in some Thai populations which were homozygous for 1016G (Brengues et al., 2003). Since some studies showed that the V1016G mutation has been reported in the absence of the S989P mutation, it is likely to hypothesize that this point mutation acts as an additive mutation (Srisawat et al., 2010). Furthermore, the V1016G/F1534C/S989P mutations have been confirmed in Thailand, demonstrating an additive effect on deltamethrin sensitivity which gives high level of resistance (Plernsub et al., 2016a). Recent work has also emphasized that the variations of point mutations exerted on pyrethroid-resistant *Ae. aegypti* revealed different response to insecticides. The thermal fogging spray with deltamethrin and PBO synergist killed all resistant genotypic mosquitoes in indoors (Plernsub et al., 2016b). In contrast, the outdoor spray merely displayed minor impact on the G1016 homozygous mosquitoes, partially killed the G1016/C1534 double heterozygous mosquitoes and triggered high mortality in the C1534 homozygous mosquitoes (Plernsub et al 2016b). More associated studies should be conducted to understand other polymorphisms complementing the *Vgsc* mutation and how they are related to the resistance phenotype when selection pressure is extrapolated to act on the survival of pyrethroid-resistant *Ae. aegypti*. Moreover, further

verification is also needed to make implications that the aforesaid mutations are linked to deltamethrin resistance. Other than the target site insensitivity mechanism acts upon the pyrethroid resistance in *Ae. aegypti*, a previous study addressed the absence of the 1016G mutation in deltamethrin resistance mosquito strains and revealed the role of MFOs in conferring the resistance (Yaicharoen et al., 2005). Thus, the contributions of metabolic mechanisms in these wild-type individuals upon deltamethrin exposure earn further attentions. This overlaps the study of Somwang et al. (2011) which described oxidative enzyme systems also involve in pyrethroid resistance in *Ae. aegypti* in Thailand. Another study showed that biochemical assays are very much required when a difference of low *kdr* resistant allele frequency of S989P and V1016G with high level of permethrin resistance was reported in field strains of *Ae. aegypti*, which this implies detoxification enzymes show the possibility to be involved in insecticide resistance mechanisms (Srisawat et al., 2012).

Biochemical assays used to detect enzymes metabolizing insecticides have been available for over three decades to monitor insecticide resistance of *Ae. aegypti* in various countries. In Thailand, the first related study was accomplished in 2002 using laboratory bred artificially selected resistant *Ae. aegypti*. GSTs and ESTs activities were detected to be only marginally higher relative to the susceptible strain, signifying that both enzyme groups show no major role in permethrin resistance (Prapanthadara et al., 2002). In contrast, the subsequent biochemical assay of metabolic enzymes tested with field *Ae. aegypti* to pyrethroid resistance revealed ESTs, MFOs and GSTs are closely associated with permethrin resistance whereas deltamethrin resistance is related to ESTs and MFOs (Paeporn et al., 2004). Later, a number of studies proposed that an elevated level of MFOs was usually in association with pyrethroid resistance (Yaicharoen et al., 2005; Pethuan et al., 2007). Moreover, considerable escalated levels of ESTs and GSTs were stated in some field strains of pyrethroid-resistant *Ae. aegypti* (Pethuan et al., 2007). The latest study

demonstrated that the aforementioned enzymes were either unrelated or merely contributed partially in pyrethroid resistance (Choovattanapakorn et al., 2017). In regards to the discrepancies of these enzymes as contributory factors to pyrethroid resistance in *Ae. aegypti*, it should be noted that *in vitro* experiments may not certainly reflect the *in vivo* circumstances of insect metabolizing insecticide molecules and thus, field studies including numerous variables should be considered in the future.

2.6.9 Timor-Leste

Timor-Leste is not excluded from vector-borne diseases transmitted by *Aedes* mosquitoes due to its generally warm and humid climate which is conducive for their growth. Dengue cases were first reported in 2003, followed by an outbreak resulted in 933 cases and 37 deaths in 2005 in Dili, Bobonaro and Baucau (Whelan & Petitt, 2007; WHO, 2009). Although *Ae. aegypti* was extensively spread in the country (WHO, 2009), there was only single international project carried out, by Frances et al. (2016) who employed bottle bioassays to examine insecticide resistance in *Ae. aegypti* and the respective biochemical mechanisms using pyrethroids (permethrin, resmethrin, lambda-cyhalothrin). These insecticides were in line with the other insecticides used more than a decade ago for vector control programs in the country as stated by Whelan & Petitt (2007). Since pyrethroid resistance was detected in *Ae. aegypti* collected from a single site in Dili with elevated levels of ESTs, there is a need in investigating the efficacies and mechanisms contributed to the resistance of pyrethroids towards the *Ae. aegypti* populations in Timor-Leste.

2.6.10 Vietnam

DHF was first documented in Hanoi, Vietnam in 1958 (Mihov et al., 1959), followed by the first and second outbreaks recorded in 1960 and 1963 respectively in South Vietnam (Halstead et al., 1965). Vietnam, like all the other Southeast Asian countries,

utilizes chemical insecticides as the primary tool in ceasing spread of the deadly vector of DHF, *Ae. aegypti*. Although *Ae. aegypti* was introduced to the country in 1915 (Stanton et al., 1920) and DHF has been reported to be endemic in Vietnam for decades (Huong et al., 1999), there are insufficient amount of studies available in evaluating the efficacies of pyrethroids to *Ae. aegypti* and their related mechanisms (Huong et al., 1999; Huber et al., 2003; Huong et al., 2004; Kawada et al., 2009a; Kawada et al., 2009b; Bingham et al., 2011).

The first insecticide resistance study of *Ae. aegypti* was conducted in 1998 and 1999, presenting that the mosquitoes were resistant to few pyrethroid insecticides in several locations of Central Highlands and Nam Bo (Huong et al., 1999). Later, a similar but more comprehensive study was completed in 2004, encompassing four regions of Vietnam and revealed that *Ae. aegypti* from numerous locations in the Centre and North regions were susceptible to pyrethroids but those from South and Central Highlands were more resistant (Huong et al., 2004). This was believed to be related to the frequent and prolonged uses of pyrethroids in the highlands for the *Ae. aegypti* control program (Huong et al., 2004). Apart from the aforementioned factor that may influence the susceptibility status of *Ae. aegypti* to pyrethroids, mosquitoes from places with high populations developed higher resistance than those from outskirts. Huber et al. (2003) showed that *Ae. aegypti* in cities with high populations, like Ho Chi Minh City, developed higher resistance than those from the outskirts or Long An Province. Other than inspecting previously reported dengue hotspots, these pioneer studies provided insights into pyrethroid resistance in *Ae. aegypti* and reported that *Ae. aegypti* populations in the country had already developed resistance to some pyrethroids.

The most extensive nationwide assessment of pyrethroid resistance in *Ae. aegypti* was completed in 2009, approximately a century after this disease vector made its way to

Vietnam. A total of 527 collection points were involved from northern to southern Vietnam (Kawada et al., 2009b). A simple bioassay of fourth instar larvae using glass vials was developed to detect knockdown susceptibility (Kawada et al., 2009b). The most notable outcome of this study demonstrated a pronounced increment of resistance in *Ae. aegypti* to d-allethrin with decrease in the latitude of sampling sites (Kawada et al., 2009b), corresponding to the findings of an earlier study (Huong et al., 2004). Analysis of point mutations was further investigated and the F1534C mutation was verified to be the chief point mutation that gave rise to high resistance in the specimens collected from the South whereas the heterozygous V1016G mutation was very low in frequency (Kawada et al., 2009a). Although merely low frequencies were detected in the North and the percentage of homozygous F1534C remained low (7.4%), unrestrained use of photo-stable pyrethroids that persists in the environment may induce selection pressure for this point mutation which will result in more resistance offspring. With 21,000 liters of pyrethroids used in dengue control operations in 20 southern provinces in 2007 (Pasteur Institute, 2008), it is no surprise that *Ae. aegypti* populations from Vietnam have developed insecticide resistance. Therefore, the use of PBO as a synergist was considered in order to increase the efficacy of detalmethrin to *Ae. aegypti* from Nha Trang (Bingham et al., 2011). The study subsequently proved that synergists might play crucial role in *Ae. aegypti* control program (Bingham et al., 2011). Moreover, Nha Trang strain housed multiple resistance, the overexpressed P450 gene *CYP9J32* relative to the susceptible strain was found capable in metabolizing deltamethrin effectively; as well as two homozygous *kdr* mutations which were the I1011V (100%) mutation and the V1016I (67%) mutation. Attention should be paid to the detection of the V1016I mutation in Vietnam when this point mutation was only circumscribed in the continent of America in the past (Saavedra-Rodriguez et al., 2007; Garcia et al., 2009; Alvarez et al., 2015). Thus, consistent monitoring of insecticide resistance is required.

Table 2.1: Distribution of different *kdr* mutations in *Aedes aegypti* in Southeast Asia

Country	Mutation	Reference
Cambodia	F1534C	Yanola et al., 2011; Saingamsook et al., 2017
	V1016G	Saingamsook et al., 2017
Indonesia	V1016G	Brengues et al., 2003; Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a; Hamid et al., 2017b; Saingamsook et al., 2017
	F1534C	Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a; Hamid et al., 2017b; Saingamsook et al., 2017
	S989P	Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a
	V1016G/S989P	Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a
Laos	V1016G	Marcombe et al., 2017
	F1534C	Marcombe et al., 2017
Malaysia	V1016G	Ishak et al., 2012
	F1534C	Ishak et al., 2012
Myanmar	V1016G	Kawada et al., 2014; Saingamsook et al., 2017
	S989P	Kawada et al., 2014
	F1534C	Kawada et al., 2014; Saingamsook et al., 2017
	V1016G/S989P	Kawada et al., 2014
	V1016G/F1534C	Kawada et al., 2014
	V1016G/F1534C/S989P	Kawada et al., 2014

Table 2.1 continued

Country	Mutation	Reference
Philippines	N/A	
Singapore	F1534C	Kasai et al., 2014; Pang et al., 2015
	V1016G	Rajatileka et al., 2008; Kasai et al., 2014; Pang et al., 2015
Thailand	I1011V	Rajatileka et al., 2008
	F1534C	Yanola et al., 2010; Yanola et al., 2011; Saingamsook et al., 2017
	S989P/V1016G	Srisawat et al., 2010; Srisawat et al., 2012
	V1016G	Brengues et al., 2003; Rajatileka et al., 2008; Saingamsook et al., 2017
	V1016G/F1534C/S989P	Plernsub et al., 2016
Timor-Leste	N/A	
Vietnam	V1016I	Bingham et al., 2011
	I1011V	Kawada et al., 2009a
	F1534C	Kawada et al., 2009a
	V1016G	Kawada et al., 2009a

N/A: Not Available

CHAPTER 3: METHODOLOGY

3.1 Mosquito Coil Bioassays

3.1.1 Study Sites

Aedes aegypti was sampled from a total of nine regencies in Indonesia using mosquito-oviposition trap (ovitrap). The study sites were selected according to the suggestions by local collaborators, prioritizing the safety of each of the location. The details of each of the sampling site are tabulated in Table 3.1. The collection sites of the mosquitoes are shown in Figure 3.1.

Table 3.1: Geographical description of *Aedes aegypti* collection sites in Indonesia

Sunda Islands	Islands	Provinces	Regencies	Study sites	GPS coordinates
Greater Sunda Islands	Java	West Java	Kuningan	Kuningan	S 6º13'5.260" E 106º50'15.936"
	Sumatra	West Sumatra	Padang	Air Tawar Barat	S 0º53'48.260" E 100º20'45.265"
	Borneo	East Kalimantan	Samarinda	Sidodadi	S 0º28'41.646" E 117º08'46.441"
		West Kalimantan	Pontianak	Bangka Belitung Laut	S 0º3'31.967" E 109º21'19.322"
Lesser Sunda Islands	Bali	Bali	Denpasar	Sanur	S 8º41'10.254" E 115º15'23.634"
	Lombok	West Nusa Tenggara	Mataram	Ampenan	S 8º34'13.911" E 116º05'08.575"
				Pagesangan	S 8º36'2.666" E 116º06'07.080"
	Sumbawa		Dompu	Bada	S 8º32'20.878" E 118º27'28.799"
	Flores	East Nusa Tenggara	Manggarai Barat	Labuan Bajo	S 8º29'34.269" E 119º52'40.889"
			Sumba Timur	Waingapu	S 9º39'49.331" E 120º16'17.321"

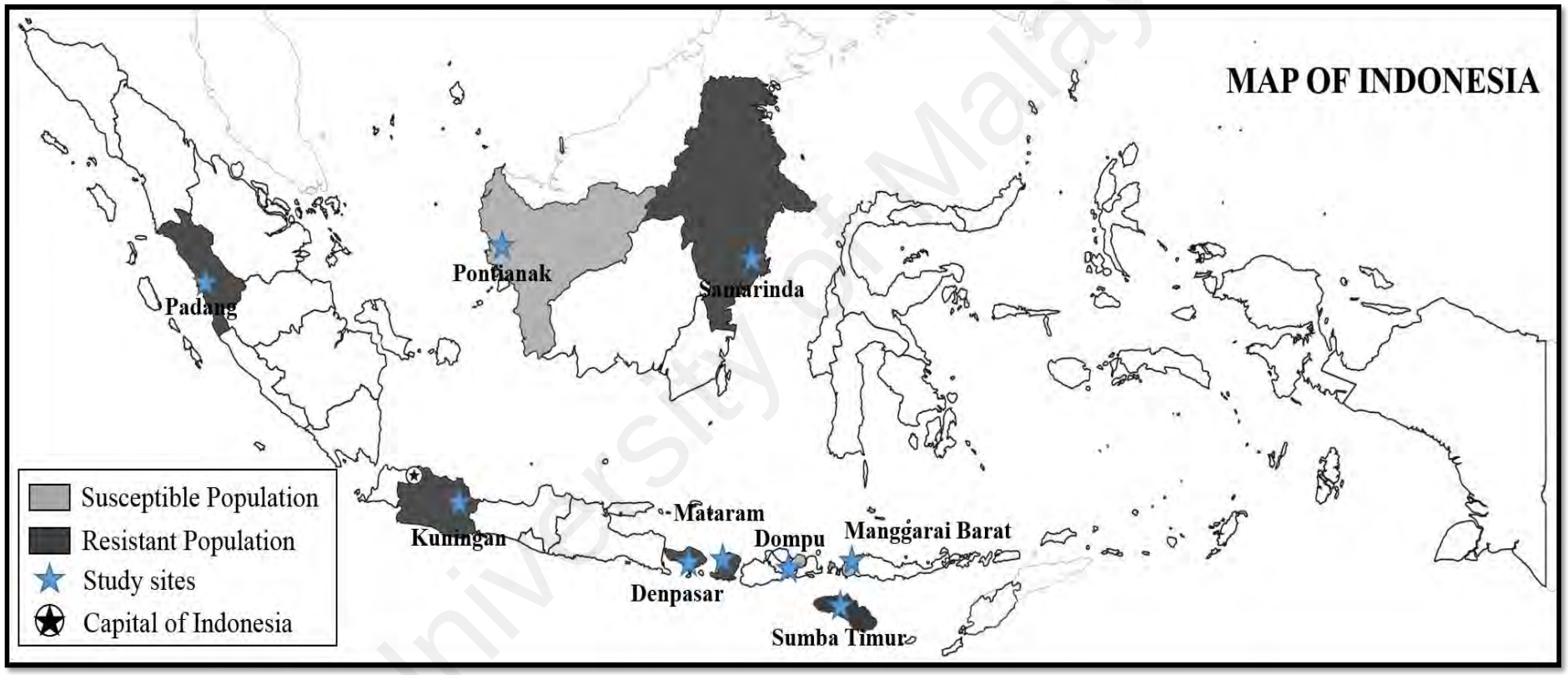

Figure 3.1: Collection sites of *Aedes aegypti* larvae in Indonesia

3.1.2 Ovitrap Preparation and Sample Collection

Ovitraps were set up based on the protocol developed by Lee (1992). An ovitrap consisted of a black-painted 300 ml plastic cup with dimensions of 9.0 cm height, 6.5 cm base diameter and 7.8 cm of opening diameter. A 2.5 cm x 10.0 cm x 0.3 cm paddle made of hardboard was positioned diagonally into each of the ovitrap. The ovitrap was then filled with chlorine-free tap water up to a height of 5.5 cm. A total of 40 ovitraps were placed randomly within the study sites at each of the nine regencies, with the best conditions of close proximity to other potential breeding sites protected from direct sunlight and rainfalls. Five days later, the ovitraps were collected and transported back to the laboratory (Institute of Biological Sciences, Faculty of Science, University of Malaya) for further identification at adult stage.

3.1.3 Mosquito Colonization

Adult *Aedes aegypti* mosquitoes were identified and colonized the insectarium (Institute of Biological Sciences, Faculty of Science, University of Malaya) in respective wooden cages measuring 30 cm x 30 cm x 30 cm covered with netting according to locations. 10% sucrose solution was provided for the adult mosquitoes as food source. Four-to-five day old female adults were supplied with blood meal from mice to produce F1 generation that would be used for subsequent susceptibility test. An oviposition site which was made of plastic cup containing 200 ml of chlorine-free water lined with filter paper was placed into the cage. The collected eggs were allowed to hatch in plastic containers filled with chlorine-free water measuring 25 cm x 30 cm x 5 cm. Larvae were fed with beef liver powder. Larvae that pupated were then transferred into a small plastic cup and introduced into the rearing cage for the adults to emerge. An *Ae. aegypti* laboratory strain from French Polynesia (Bora-Bora) was used as the reference susceptible group.

3.1.4 Mosquito Coil Bioassays

Three easily available commercial mosquito coils from Indonesian market with different content of active ingredients were used in mosquito coil bioassays, specifically d-allethrin 0.25% w/w, transfluthrin 0.03% w/w and metofluthrin 0.0097% w/w. The bioassays were accomplished in accordance with the standardized protocol described by Standards and Industrial Research Institute of Malaysia (SIRIM, 1986), adopting a resistance indicator from WHO (2016). The test was performed in a 70 cm x 70 cm x 70 cm see-through glass chamber, with a sliding window measuring 18 cm x 20 cm located at the bottom middle of the chamber door to ease the placement of coil and the release of mosquitoes. The conditions of the laboratory were controlled at a temperature of 27 ± 2°C and relative humidity of 80 ± 10%. Mosquito coil weighted 0.50 g was fixed on a coil stand, with both ends ignited inside the chamber. Both smoldered remains of coil and coil stand were eliminated after the coil was completely burnt. To ensure the coil smoke distributed evenly throughout the chamber, a small electric fan was switched on in it. A total of twenty, two-to-five-day old sugar-fed adult *Ae. aegypti* females were released into chamber to expose to the aforementioned coils separately in each replicate. The number of knocked-down mosquitoes was counted and recorded for every minute up to 20 minutes. Mosquitoes which were failed to fly or no longer maintained in normal posture would be considered as knockdown. Electric aspirator was used to collect all tested mosquitoes after 20 minutes of exposure time and subsequently transferred into a clean plastic container for 24 hours post-treatment effect. The containers were covered up tightly with netting to prevent mosquitoes from escaping. The mosquitoes were provided with 10% sucrose solution soaked in cotton wool as food source. All the mosquitoes were kept at 27 ± 2°C and 80 ± 10% relative humidity during the 24-hour recovery period. Mortality was recorded 24 hours after the initial exposure period. Subsequently, used chambers were wiped thoroughly using detergent and water before

the next test would be carried out. To ensure the cage is not contaminated by insecticide after cleaning, control experiments were carried out by releasing the adult females into the chamber without exposing to any coils for 20 minutes. An acceptable mortality rate for the control mosquitoes should strictly be 0%. Toxicological tests were repeated three times for each study site and active ingredient. Control experiments were carried out by releasing the adult females into the chamber without exposing to any coils for 20 minutes.

3.1.5 Data Analyses

Mosquito coil bioassays data were pooled and analyzed from at least three replicates of tests. The data within the range from 5–95% knockdown was subjected to probit analysis (Finney, 1971) using the computer software SPSS (version 20) to obtain 50% knockdown time (KT_{50}). The resistance ratios (RR) of all field-collected *Ae. aegypti* mosquitoes were calculated using the formula as follows:

$$\text{Resistance ratio (RR)} = \frac{KT_{50} \text{ of field strain}}{KT_{50} \text{ of reference strain}}$$

RR values < 5 indicate low resistance, 5–10 indicate medium resistance, whereas >10 indicate high resistance (Mazzarri & Georghiou, 1995). Comparative measure of knockdown and mortality between the study sites was performed by one-way ANOVA using SPSS version 20. Tukey's test was used to separate means in significant ANOVAs, $P < 0.05$. Spearman's rank-order correlation analysis between a pair of knockdown rates was performed to determine the presence of cross-resistance (Bisset et al., 1997). The mortality rate after a 24-h post treatment was used to evaluate the susceptibility status of the mosquitoes, with criteria as stated by WHO (2016):

- Mortality rate of ≥ 98% : susceptible to insecticide;
- Mortality rate of < 98% : possible development of resistance to insecticide;
- Mortality rate of < 90% : resistance to insecticide.

3.2 Enzyme Assays

3.2.1 Mosquito Strains

Details of the sampling sites were described previously (Table 3.1). The identified female *Ae. aegypti* mosquitoes were blood-fed for the production of the subsequent generation. The non-blood-fed three-to-five days old female mosquitoes were stored in -20°C freezer prior to the biochemical tests. A total of 720 female *Ae. aegypti* with 24 individual mosquitoes representing each of the population (three enzyme assays for every population, comprised of the laboratory reference strain) were used. All mosquitoes were colonized in an insecticide-free setting.

3.2.2 Enzyme Assays

Non-specific esterases (ESTs) enzyme assays were performed in accordance with previously established protocols (Brogdon et al., 1988; Lee, 1990). A total of 24 individual mosquitoes were homogenized in phosphate buffer solution and were subsequently centrifuged at 15,000 rpm for 10 minutes at 4˚C. Then, four aliquots of supernatant (50 µl) derived from the homogenate of each individual mosquito were obtained in this assay. The 50 µl of substrate solution (either α-naphthyl acetate or β-naphthyl acetate) was placed in a 96-well plate and left to stand for a minute, followed by adding 50 µl of 3 mm indicator solution (fast blue B salt). The reaction was then incubated for 10 minutes and was stopped by the addition of 50 µl of 10% acetic acid. The optical density was measured at 450 nm using absorbance microplate reader (BIO-TEK® ELx800™).

Mixed function oxidases (MFOs) enzyme assays were carried out based on the method described by Brogdon et al. (1997). A total of 24 individual mosquitoes were homogenized in sodium acetate buffer solution. Next, four aliquots of homogenate (100 µl) from each individual mosquito were obtained in this assay. The absorbance was

determined at 630 nm after a five-minute incubation of individual mosquito homogenate in each well with 200 µl of 2 mm 3,3'5,5'-tetramethylbenzidine (TMBZ) and 25 µl of 3% hydrogen peroxide.

Glutathione-S-transferases (GSTs) enzyme assays were accomplished according to the protocol described by Lee & Chang (1995). A total of 24 individual mosquitoes were homogenized in potassium phosphate buffer solution. Centrifugation was subsequently performed at 14,000 rpm for 10 minutes at 4˚C. Then, four aliquots of homogenate (100 µl) from each individual mosquito were placed in a 96-well plate, followed by adding 50 µl of 2 mm glutathione (GSH) and 50 µl of 1 mM 1-chloro-2, 4-dinitrobenzene (CDNB). Lastly, the reaction was allowed to incubate for 30 minutes. The optical density value was then read at 400 nm.

3.2.3 Data Analyses

In order to determine the associations between the survivability rates in mosquito coil bioassays and enzyme activities, Spearman's rank-order correlation was performed. Comparative measure of mean enzyme activities between the study sites was performed by one-way analysis of variance (ANOVA) using SPSS version 20. Tukey's test was used to separate means in significant ANOVAs, $P < 0.05$. Independent-samples t-test was performed to indicate significant decrease or increase in mean differences.

3.3 *Kdr* Gene Screening

3.3.1 Mosquito Strains

Details of the sampling sites were described previously (Table 3.1). Ten non-blood-fed three-to-five days old adult female *Aedes aegypti* from each of the population which were yet to expose to any insecticide were randomly selected to screen for *kdr* mutations. The total number of mosquitoes involved in each of the point mutation were stated below.

3.3.2 Genomic DNA Isolation

The abdomens were dissected and discarded from mosquito specimens before carrying out genomic DNA isolation to avoid contamination. The mosquito genomic DNA was isolated using a commercial kit named G-spin^{TM} Total DNA Extraction Kit (iNtRON Biotechnology, Inc, Korea), according to the manufacturer's protocol (refer Appendix A).

3.3.3 Direct DNA Sequencing

Genotyping of the V1016G, S989P and F1534C mutations were performed by direct DNA sequencing based on a previous publication by Yanola et al. (2011). For V1016G and S989P genotyping, a total of 100 mosquitoes were sequenced using the same pair of primers designed, encompassing domain II segment 6 of *Ae. aegypti Vgsc* gene. Amplification was performed using IIP_F as a forward primer (5'-GGT GGA ACT TCA CCG ACT TC-3') whereas IIS6_R as a reverse primer (5'-GGA CGC AAT CTG GCT TGT TA-3'). A subset of 43 samples from the V1016G and S989P genotyping were subjected to the detection of the F1534C mutation, performed using Ge-IIIS6_F as a forward primer (5'-GCT GTC GCA CGA GAT CAT T-3') while IIIS6_R as a reverse primer (5'-GTT GAA CCC GAT GAA CAA CA-3') designed to include domain III segment 6 of *Ae. aegypti Vgsc* gene (Yanola et al., 2011). Amplification of the sodium channel region was performed in a final volume of 20 µl for each sample, comprising 1

µl of 10 pmol of each forward and reverse primer, 12.5 µl of GeNet Bio ExPrime Taq™ Premix (Global Gene Network, Daejeon, South Korea) and 25–50 ng of genomic DNA of mosquito.

PCR amplifications for both of the V1016G and F1534C mutation regions were carried out using an Applied Biosystems™ Veriti™ 96-well thermal cycler (Thermo Fisher Scientific, Inc., Waltham, MA), programmed with the parameters starting with an initial denaturation of 95°C for 2 min, followed by 35 cycles of 95°C for 30 s (denaturation), 63°C for 30 s (annealing), 72°C for 30 s (extension) and a final extension at 72ºC for 2 min. PCR amplicons were subjected to gel electrophoresis with the use of a 2% agarose gel pre-stained with SYBR Safe™ (Invitrogen, USA) in Tris-Acetate-EDTA (TAE) buffer.

The PCR amplicons were then delivered to a commercial company for DNA direct sequencing. Samples were sequenced using BigDyeH Terminator v3.1 Sequencing Kit™ (Thermo Fisher Scientific, Inc., Waltham, MA) and analyzed on ABI PRISM 377 Genetic Analyzer™ (Thermo Fisher Scientific, Inc., Waltham, MA).

3.3.4 Data Analyses

Heterogenous mutations were quantified based on generated sequences: heterozygous genotype (RS) exhibits double peaks in the mutation point, whereas homozygous genotype (RR/SS) exhibits only one specific peak (Simsek et al., 2001).

The frequencies of the resistant and susceptible *kdr* alleles for each of the point mutation were determined by the Hardy-Weinberg Equilibrium using an online calculator (Rodriguez et al., 2009). Spearman's rank-order correlation analyses between the allele frequencies of the point mutations and the survivability rates as well as resistance ratios

obtained from adult bioassays of all active ingredients tested were performed to examine significant correlations.

CHAPTER 4: RESULTS

4.1 Mosquito Coil Bioassays

The susceptibility status of adult *Aedes aegypti* mosquitoes to the pyrethroid-based mosquito coils are presented in Tables 4.1 and 4.2. In each active ingredient tested, the adult mosquitoes revealed different trends of susceptibility across all study sites. Exposure of Bora-Bora laboratory reference colony to the mosquito coils caused 100% mortality rate in all replicates, with KT_{50} of 0.54 min, 0.76 min and 0.84 min to d-allethrin, transfluthrin and metofluthrin, respectively. For field samples, the KT_{50} of female adult *Ae. aegypti* tested with d-allethrin-, transfluthrin- and metofluthrin-containing coils ranged from 0.65 to 14.32 min (the longest KT_{50} population: Sumba Timur); 0.8 to 16.4 min (the longest KT_{50} population: Samarinda); and 0.78 to 20.57 min (the longest KT_{50} population: Samarinda), respectively.

Mortality percentage for all the populations tested against d-allethrin, transfluthrin and metofluthrin ranged from 11.67 to 100%, 8.33 to 100% and 5 to 100%, respectively. Populations from Manggarai Barat and Pontianak exhibited full mortality to all three active ingredients tested, revealing high susceptibility. Mortality rates in accordance with WHO resistance indicators showed that mosquito strains from Denpasar, Mataram, Kuningan, Padang, Samarinda and Sumba Timur were resistant (< 90% mortality rate) whereas mosquito strains from Manggarai Barat, Dompu and Pontianak were susceptible (≥ 98% mortality rate) to all active ingredients assayed.

In addition, one-way ANOVA demonstrated that the susceptibility status of *Ae. aegypti* to all active ingredients tested were significantly different across all study sites ($P < 0.0001$). Spearman's rank-order correlation showed significant associations between the knockdown rates of d-allethrin and transfluthrin ($r = 0.833$; $P = 0.005$), d-allethrin and metofluthrin ($r = 0.700$; $P = 0.036$), transfluthrin and metofluthrin ($r = 0.950$; $P < 0.001$).

Table 4.1: Knockdown time (KT_{50}) and resistance ratio (RR) of Indonesian *Aedes aegypti* adults against d–allethrin (0.25%), transfluthrin (0.03%) and metofluthrin (0.0097%)

Location	Active Ingredients					
	d-allethrin		Transfluthrin		Metofluthrin	
	KT_{50} (min) 95% CL	RR	KT_{50} (min) (95% CL)	RR	KT_{50} (min) (95% CL)	RR
Reference	0.54 (0.38–0.65)		0.76 (0.65–0.87)		0.84 (0.73–0.95)	
Denpasar	8.91 (7.71–10.40)	16.62	11.74 (10.02–14.17)	15.40	10.32 (9.15–11.80)	12.26
Mataram	4.52 (4.00–5.07)	8.44	5.54 (4.77–6.37)	7.27	11.19 (9.69–13.20)	13.29
Kuningan	10.39 (9.44–11.51)	19.38	15.05 (13.15–17.77)	19.75	14.36 (12.48–17.07)	17.06
Manggarai Barat	1.00 (0.85–1.14)	1.87	1.13 (0.98–1.27)	1.48	1.36 (1.21–1.51)	1.61
Dompu	0.66 (0.53–0.76)	1.22	0.78 (0.62–0.91)	1.02	0.78 (0.66–0.90)	0.93
Padang	4.87 (3.99–5.78)	9.09	4.33 (3.92–4.74)	5.68	4.96 (4.48–5.45)	5.89
Pontianak	0.99 (0.83–1.14)	1.84	0.80 (0.70–0.91)	1.06	0.94 (0.82–1.06)	1.12
Samarinda	8.64 (7.78–9.65)	16.13	16.40 (14.14–19.78)	21.52	20.57 (17.30–26.05)	24.43
Sumba Timur	14.32 (12.63–16.66)	26.72	6.67 (5.67–7.75)	8.76	9.82 (8.92–10.85)	11.66

CL: Confidence Limit.

Table 4.2: Percentages of knockdown and mortality of Indonesian *Aedes aegypti* adults against d–allethrin (0.25%), transfluthrin (0.03%) and metofluthrin (0.0097%)

Regencies	Knockdown			Mortality		
	d-allethrin 0.25%	Transfluthrin 0.03%	Metofluthrin 0.01%	d-allethrin 0.25%	Transfluthrin 0.03%	Metofluthrin 0.01%
Reference	100±0	100±0	100±0	100±0	100.00 ±0.00	100±0
Denpasar	63.33±1.67^{a}	55±0^{a}	61.67±0.33^{a}	^{R}11.67±0.33^{a}	^{R}8.33±0.33^{a}	^{R}21.67±0.33^{a}
Mataram	78.3±0.33^{abce}	78.3±0.33^{abce}	66.67±0.33^{abce}	^{R}50±0.58^{abce}	^{R}56.67±0.67abc^{abce}	^{R}41.67±0.67^{abce}
Kuningan	61.67±0.33^{c}	55±0^{bc}	55±0.58^{abc}	^{R}15±1.53^{bc}	^{R}13.3±0.33^{bc}	^{R}5±0.577^{abc}
Manggarai Barat	100±0^{abcd}	100±0^{abcd}	100±0^{abcd}	^{S}100±0^{abcd}	^{S}100±0^{abcd}	^{S}100±0^{abcd}
Dompu	100±0^{ab}	100±0^{ab}	100±0^{ab}	^{S}98.3±0.33^{ab}	^{S}100±0^{ab}	^{S}100±0^{ab}
Padang	71.67±0.33^{abdef}	91.67±0.33^{acdef}	86.67±0.33^{bcdef}	^{R}30±0.58^{abdef}	^{R}50±1.53^{acdef}	^{R}26.67±0.67^{bcdef}
Pontianak	100±0^{abcfg}	100±0^{abcfg}	100±0^{abcfg}	^{S}100±0^{abcfg}	^{S}100±0^{abcfg}	^{S}100±0^{abcfg}
Samarinda	70±0^{acdeg}	58.3±0.33^{bdefgh}	51.67±0.67^{bdefgh}	^{R}38.3±0.88^{acdeg}	^{R}15±0.58^{bdefgh}	^{R}11.67±0.33^{bdefgh}
Sumba Timur	55±0^{acdeg}	63.3±0.67^{acdegh}	68.3±0.67^{acdegh}	^{R}45±0.58^{acdeg}	^{R}41.67±0.88^{acdegh}	^{R}36.67±1.33^{acdegh}
One-way ANOVA	$P < 0.0001$ F= 126.27 df= (9, 20)	$P < 0.0001$ F= 148.08 df= (9, 20)	$P < 0.0001$ F= 196.69 df= (9, 20)	$P < 0.0001$ F= 126.27 df= (9, 20)	$P < 0.0001$ F= 148.08 df= (9, 20)	$P < 0.0001$ F= 196.69 df= (9, 20)

Means followed by a different letter were significantly different, $P < 0.05$, Tukey's test.
R = resistant (mortality < 90%) and S = susceptible (mortality ≥ 98%) as determined by WHO (2016).

4.2 Enzyme Assays

To correlate the results of both mosquito coil bioassays and enzyme assays, the results from the former were therefore repeated in this section. The mosquito bioassays verified various trends in resistance in adult *Ae. aegypti* against d-allethrin, transfluthrin and metofluthrin. Adult mortality logged 24 h after the initial exposure period of d-allethrin, transfluthrin and metofluthrin ranged from 11.67 to 100%, 8.33 to 100% and 5 to 100%, respectively. Mortality rates in accordance with WHO resistance indicators showed that mosquito populations from Denpasar, Mataram, Kuningan, Padang, Samarinda and Sumba Timur were resistant (< 90% mortality rate) whereas populations from Manggarai Barat, Dompu and Pontianak were susceptible (≥ 98% mortality rate) to the active ingredients assayed.

One-way ANOVA revealed that the means of the enzyme activities of α-ESTs, MFOs and GSTs in Indonesian *Ae. aegypti* were significantly different across all study sites ($P < 0.05$) (Table 4.3).

In Spearman's rank-order correlation, significant correlations between survivability rates in adult bioassays of all insecticides tested against three enzymes activities in Indonesian *Ae. aegypti* were demonstrated. The significant correlations were shown between the d-allethrin survivability rate in adult bioassays and β-ESTs ($r= 0.704$, $P= 0.023$); MFOs ($r= 0.790$, $P= 0.007$); and GSTs ($r= 0.690$, $P= 0.027$). There were also significant correlations between the transfluthrin survivability rate in adult bioassays and β-ESTs activity ($r= 0.743$, $P= 0.014$); MFOs ($r= 0.708$, $P= 0.022$); and GSTs ($r= 0.634$, $P= 0.049$). With respect to the metofluthrin survivability rate in adult bioassays, significant correlations were confirmed in β-ESTs ($r= 0.781$, $P= 0.008$); MFOs ($r= 0.632$, $P= 0.050$); and GSTs ($r= 0.696$, $P= 0.026$). Only α-ESTs showed no significant correlations to the survivability rate in adult bioassays of all insecticides tested— i.e., d-

allethrin (r= 0.475, P= 0.165); transfluthrin (r= 0.468, P= 0.172); and metofluthrin (r= 0.519, P= 0.124).

In non-specific ESTs assays, a significant increase in α-ESTs activity was detected in merely some populations, namely Padang, Pontianak, Denpasar and Manggarai Barat whereas the mean levels of α-ESTs activity in the Kuningan population showed significant decrease. An absence of significant increase in β-ESTs activity was observed in all populations as shown in Table 4.3. Of nine studied populations, four populations (i.e., Kuningan, Denpasar, Dompu and Sumba Timur) displayed a significant increase in MFOs activity. As for GSTs assays, no elevated level of GSTs activity was observed (except for Kuningan and Sumba Timur).

Table 4.3: Mean (±SE) levels of non-specific esterases (α- and β-ESTs), mixed function oxidases (MFOs) and glutathione-S-transferases (GSTs) activities of *Aedes aegypti* adults sampled from different localities in Indonesia

Regencies	α-ESTs	β-ESTs	MFOs	GSTs
Reference	0.16±0.00	0.17±0.00	0.18±0.01	0.04±0.00
Kuningan	**0.15±0.00^{a}	0.18±0.00	*0.35±0.01^{a}	*0.06±0.00^{a}
Padang	*0.19±0.00^{b}	0.18±0.00	0.22±0.01^{a}	0.05±0.00^{a}
Samarinda	0.20±0.00^{ac}	0.20±0.00	0.20±0.01^{ab}	0.05±0.00^{a}
Pontianak	*0.18±0.00	0.14±0.00	0.19±0.01^{ac}	0.04±0.00^{ab}
Denpasar	*0.19±0.00^{d}	0.19±0.00	*0.26±0.01^{abcd}	0.05±0.00^{b}
Mataram	0.17±0.00	0.18±0.00	0.22±0.01^{a}	0.05±0.00^{a}
Dompu	0.14±0.00^{bcd}	0.14±0.00	*0.25±0.01^{ae}	0.05±0.00^{a}
Manggarai Barat	*0.17±0.00	0.16±0.00	0.17±0.01^{adef}	0.05±0.00^{a}
Sumba Timur	0.14±0.00^{bcd}	0.15±0.00	*0.25±0.01^{acf}	*0.05±0.00^{b}
One-way ANOVA	P= 0.005; f= 6.089; df= (9, 10)	P= 0.098; f= 2.369; df= (9, 10)	P < 0.001; f= 22.480; df= (9, 10)	P < 0.001; f= 11.191; df= (9, 10)

Means followed by a different letter were significantly different, P< 0.05, Tukey's test.

Asterisk * indicates significant increase in mean differences compared to the laboratory reference strain, P < 0.05, t-test.

Asterisk ** indicates significant decrease in mean differences compared to the laboratory reference strain, P < 0.05, t-test.

4.3 *Kdr* Gene Screening

To screen for sodium channel mutations in the wild populations of Indonesian *Ae. aegypti*, the direct DNA sequencing method validated the presence of the V1016G, S989P and F1534C mutations (Figure 4.1). Since the sequences of 37 samples were of poor qualities, only total of 63 female *Ae. aegypti* mosquitoes collected from sub-urban areas in nine regencies were included for analysis. In the V1016G genotyping, the RR genotype was identified to be in major among the study sites (six out of nine) with 46 individuals from a total sample size of 63 mosquitoes (Table 4.4). In these six study sites, namely Denpasar, Mataram, Kuningan, Padang, Samarinda and Sumba Timur, the frequencies of the resistant 1016 allele were all equal to 1.0. Out of nine populations, merely three populations (i.e., Pontianak, Dompu, Manggarai Barat) demonstrated the presence of SS genotype with a total of 17 individuals recorded. The frequencies of the susceptible 1016 allele were also documented to be 1.0 in these three populations. None of the RS genotype was detected in the 1016 position across any tested populations in Indonesia. Thus, only two genotypes at the position 1016 were presented (46 RR and 17 SS).

The direct DNA sequencing method also confirmed the presence of serine-proline transversion at the position 989 within domain II (S989P) along with the V1016G mutation. The genotypes detected from the S989P *kdr* mutation in the wild populations of *Ae. aegypti* were summarized in Table 4.5. The RR genotype being the most predominant genotype was found in six out of nine populations, with a record of 41 individuals of adult female *Ae. aegypti* from a sample size of 63. It was subsequently followed by the SS genotype with 20 individuals, whereas only 2 individuals were detected as the RS genotype.

To examine the role of another frequently detected *kdr* mutation that might presumably induce pyrethroid resistance, 43 individuals from all of the populations genotyped earlier

for the V1016G/ S989P mutations were used to determine for the presence of the F1534C mutation. Through verification, all the nine populations exhibited the SS genotype in the 1534 position as shown in Table 4.6. Two individuals were found exhibited heterozygous but no individuals exhibited the mutant homozygous.

Spearman's rank-order correlation showed significant positive correlations between the allele frequencies of the V1016G mutation and the survivability rates in adult bioassays of d-allethrin (r= 0.825, P= 0.006); transfluthrin (r= 0.836, P= 0.005); and metofluthrin (r= 0.836, P= 0.005). In addition, significant positive correlations were also recorded between the allele frequencies of the V1016G mutation and the resistance ratios of d-allethrin (r= 0.822, P= 0.007); transfluthrin (r=0.822, P= 0.007); and metofluthrin (r= 0.822, P= 0.007). With respect to the S989P and F1534C mutations, no significant correlations were detected for all of the active ingredients assayed on the adult female *Ae. aegypti*.

Co-occurrence of the V1016G and S989P mutations in individual *Ae. aegypti* was detected in six out of nine populations (i.e., Kuningan, Padang, Samarinda, Denpasar, Mataram, Sumba Timur) with a high frequency of 68.25% (43 out of 63 individuals) (Table 4.7).

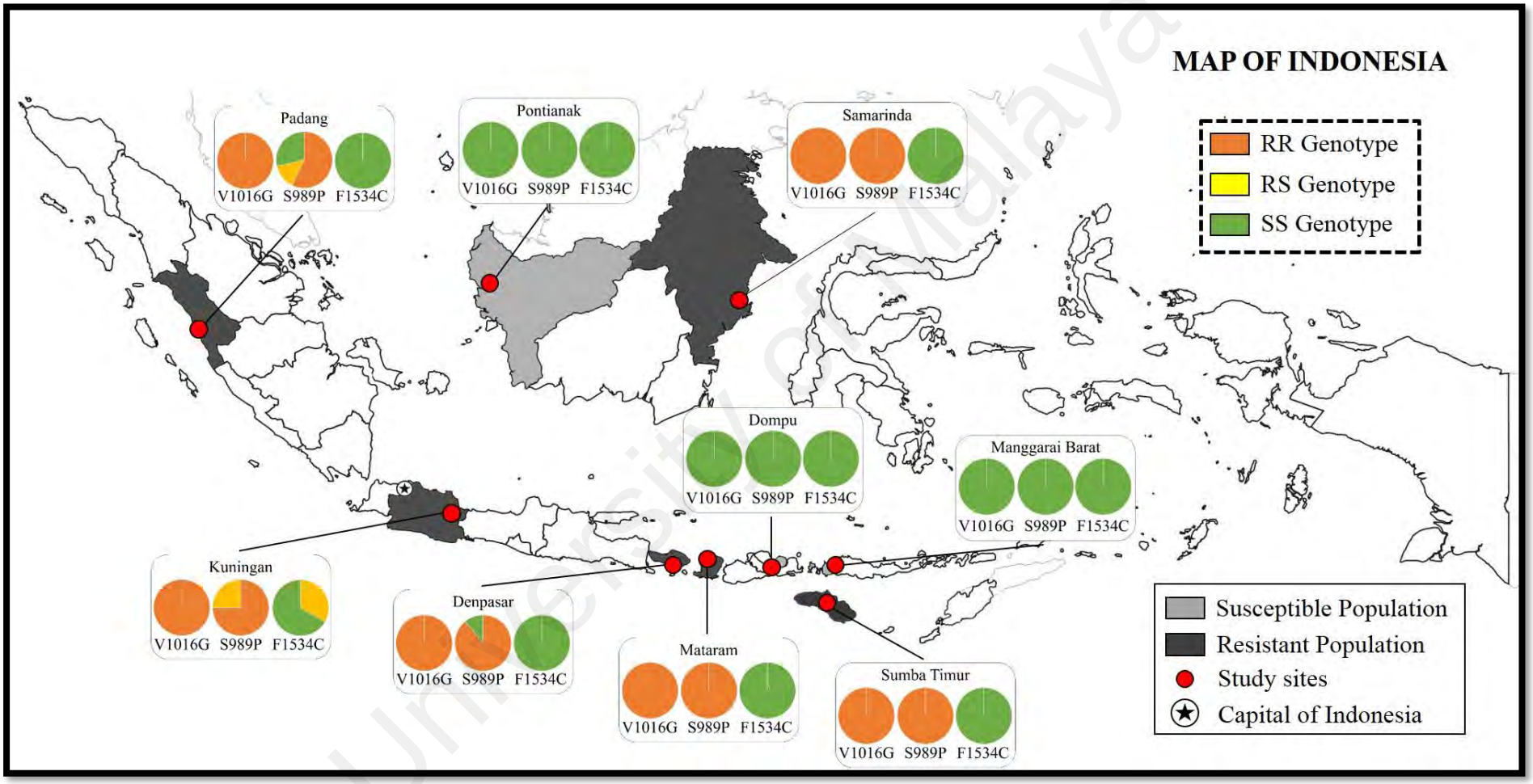

Figure 4.1: Distributions of the V1016G, S989P and F1534C point mutations in Indonesian *Aedes aegypti*

Table 4.4: The V1016G genotyping and frequency of *kdr* alleles in Indonesian *Aedes aegypti*

		Genotype			Allele Frequency	
Regencies	n	SS	RS	RR	S	R
Kuningan	4	0	0	4	0.00	1.00
Padang	7	0	0	7	0.00	1.00
Samarinda	9	0	0	9	0.00	1.00
Pontianak	6	6	0	0	1.00	0.00
Denpasar	9	0	0	9	0.00	1.00
Mataram	7	0	0	7	0.00	1.00
Dompu	4	4	0	0	1.00	0.00
Manggarai Barat	7	7	0	0	1.00	0.00
Sumba Timur	10	0	0	10	0.00	1.00
Total	63	17	0	46	0.27	0.73

Table 4.5: The S989P genotyping and frequency of *kdr* alleles in Indonesian *Aedes aegypti*

		Genotype			Allele Frequency	
Regencies	n	SS	RS	RR	S	R
Kuningan	4	0	1	3	0.13	0.88
Padang	7	2	1	4	0.36	0.64
Samarinda	9	0	0	9	0.00	1.00
Pontianak	6	6	0	0	1.00	0.00
Denpasar	9	1	0	8	0.11	0.89
Mataram	7	0	0	7	0.00	1.00
Dompu	4	4	0	0	1.00	0.00
Manggarai Barat	7	7	0	0	1.00	0.00
Sumba Timur	10	0	0	10	0.00	1.00
Total	63	20	2	41	0.40	0.60

Table 4.6: The F1534C genotyping and frequency of *kdr* alleles in Indonesian *Aedes aegypti*

		Genotype			Allele Frequency	
Regencies	n	SS	RS	RR	S	R
Kuningan	6	4	2	0	0.83	0.17
Padang	4	4	0	0	1.00	0.00
Samarinda	4	4	0	0	1.00	0.00
Pontianak	5	5	0	0	1.00	0.00
Denpasar	6	6	0	0	1.00	0.00
Mataram	3	3	0	0	1.00	0.00
Dompu	5	5	0	0	1.00	0.00
Manggarai Barat	4	4	0	0	1.00	0.00
Sumba Timur	6	6	0	0	1.00	0.00
Total	43	41	2	0	0.98	0.02

Table 4.7: Co-occurrence of the V1016G and S989P mutations in Indonesian *Aedes aegypti*

Regencies	n	Co-occurrence of V1016G and S989P (n)	Percentage (%)
Kuningan	4	4	100.00
Padang	7	5	71.42
Samarinda	9	9	100.00
Pontianak	6	0	0.00
Denpasar	9	8	88.88
Mataram	7	7	100.00
Dompu	4	0	0.00
Manggarai Barat	7	0	0.00
Sumba Timur	10	10	100.00
Total	63	43	68.25

*Co-occurrence denoted whether an individual carries both point mutations showing the same genotypes (RR/RR) or a mutation showing heterozygosity (RR/RS).

CHAPTER 5: DISCUSSION

5.1 Mosquito Coil Bioassays

Pioneer studies in 2005 and 2007 revealed *Aedes aegypti* populations in Indonesia had developed insecticide resistance to permethrin, lambda-cyhalothrin cypermethrin and d-allethrin (Brengues et al., 2003; Astari & Ahmad 2005). Recently, *Ae. aegypti* populations from several main cities of Indonesia remained to show the unrelenting development of insecticide resistance to a wide range of pyrethroids despite a ten years gap in between these studies (Wuliandari et al., 2015; Sayono et al., 2016; Hamid et al., 2017a), reflecting this particular class of insecticide to be the mainstay in mosquito control program. In accordance with the vast majority of reports from past studies, detection of resistance in a few Indonesian *Ae. aegypti* populations to pyrethroid-based mosquito coils in this study is therefore conjecturable. The high reliance and extended use of pyrethroids for *Ae. aegypti* have unquestionably given rise to the prevalence of pyrethroid resistance. In particular, the use of fast-acting chemical insecticides for mosquito control exerts strong selection pressures that may favour the survival of resistant mosquitoes. For instance, the low mortality percentage of *Ae. aegypti* from Denpasar in the present study disclosed high resistance status to all the active ingredients tested (i.e., d-allethrin, transfluthrin and metofluthrin). This can be attributed to the high degree of mosquito control in the island to stop the outbreak when there was a record showing 6,898 DF cases with 38 deaths from 2014 to 2016 (Hamid et al., 2017b). Furthermore, the use of pyrethroid-based spatial repellents other than mosquito coils such as mosquito-repelling lotion or patch is also suspected to be a large pyrethroid-resistance causative factor due to the rocketing growth of tourism in the island over the years. This phenomenon was documented in Belize (Wagman et al., 2015) and Puerto Rican (Agramonte et al., 2016), suggesting the potential of wild mosquito populations conferring resistance to these pyrethroid-based spatial repellents.

In this study, adult *Ae. aegypti* collected from some study sites in Indonesia exhibited various trends in resistance against the three pyrethroid active ingredients assayed. High resistance level of *Ae. aegypti* exerted by the pyrethroid-based interventions in certain provinces is not particularly remarkable because pyrethroid resistance in this species of mosquito has long been logged in some countries, for examples, Malaysia, Thailand and Singapore (Wan-Norafikah et al., 2010; Chuaycharoensuk et al., 2011; Koou et al., 2014a). More recently, a nationwide study has been conducted in Malaysian *Ae. aegypti* and revealed that all strains were resistant to metofluthrin, d-allethrin, d-trans allethrin, and prallethrin (Chin et al., 2017). This suggests pyrethroids only cause minimal insecticidal effect on pyrethroid-resistant *Ae. aegypti* as their efficacies have been compromised. It is also noteworthy to mention that KT_{50} of this study may not display direct representativeness to natural settings when the tested mosquitoes are restrained in a glass chamber where they may be exposed to higher concentrations of insecticides relative to when they are not strictly confined. . Despite the limitations, glass-chamber method allows measurement of the responses of *Ae. aegypti* against a wide range of chemical accurately. This may not be probable in field settings with the presence of many environmental factors that can affect the efficacy of the products.

In this study, recovery was observed in resistant strains after a 24-hour post-treatment period in an insecticide-free setting, indicating the knockdown effect may be a temporary phenomenon for some populations. The discrepancies between the percentages of knockdown and mortality of adult Indonesian *Ae. aegypti* stress that they have been subjected to strong selection pressures after continuous exposure to pyrethroids for an extended period of time, not forgetting the effect from the application of insecticides for crop protection and control of other medically important pests (Sekiyama et al., 2007; Thorburn, 2015). It can also be ascribed to the role of mosquito coil as a spatial repellent that aids in declining mosquito behavior within the insecticide-exposed distance rather

than causing direct mortality (Chin et al., 2017). Despite the high recovery rate of *Ae. aegypti* after the exposure to pyrethroid-based mosquito coil, mosquito-repelling products remain to be popular because they evidently reduce human-mosquito contact. Furthermore, according to Yap et al. (1996), coil formulations exerted sub-lethal effects against *Ae. aegypti* by reducing the longevity and decreasing blood-engorgement activity. With the scientifically-verified efficacy of coil and its user-friendly properties, this points out such mosquito-repelling products can be integrated into national vector control program rather than to be used singly.

Previous studies on insecticide resistance in Indonesia mainly focused on merely several main cities in Java or touristic islands although Indonesia has more than 900 inhabited islands stretched from east to west over 5,000 km with worldwide surge of DF and DHF. This geographical bias exposed the aforementioned cities to be high dengue-prone areas. On the contrary, the findings of this study showed that pyrethroid resistance is present in many localities, suggesting pyrethroid has long been used in the study areas. This also indicates the presence of geographical expansion in this species when resistant mosquitoes from small cities may have migrated from the historical dengue hotspots. The distribution of pyrethroid resistance *Ae. aegypti* populations is applicable when their eggs can undergo desiccation for a prolonged interval that allow them to spread through human-mediated dispersal. Hence, the detection of insecticide resistance in new dengue sensitive areas is foreseeable when vertical transmission of DENV has reported to be present in Indonesian *Ae. aegypti* (Satoto et al. 2014), causing the high reliance of pyrethroid in vector control that triggers the development of pyrethroid resistance in return.

In Indonesia, remarkably alike as most of Southeast Asian countries, many insecticides have been used for mosquito control. It comes as no surprise when the case of resistance

to DDT by *Ae. aegypti* is very common in Indonesia (Brengues et al., 2003), because DDT was the first chemical in widespread use to control *Ae. aegypti* and was introduced to the country since 1950s (Bang et al., 1982). Later, the government has banned the use in 1970s and subsequently substituted by pyrethroids. Since resistances to both DDT (organochlorine) and pyrethroid is conferred by point mutations on the *Vgsc* of insects (Ranson et al., 2000; Hemingway et al., 2004), cross-resistance may happen when DDT-resistant *Ae. aegypti* resulted in the occurrence of pyrethroid resistance. A contemporary example of cross-resistance within the pyrethroid group of insecticides was demonstrated in this study when there were significant correlations within the mosquito knockdown rates of all active ingredients. Cross-resistance happens when there are overlapping of certain mechanisms due to insecticide pressure. It is believed the tolerance to single pyrethroid insecticide might possibly result in cross-resistance to other insecticides resided in this particular class (Lee et al., 2003; Kawada et al., 2009a). The detection of cross-resistance is not unique to Indonesia because it has been reported in several countries among *Ae. aegypti*, i.e., Singapore (Koou et al. 2014a); Mexico (Flores et al. 2013); and Malaysia (Chin et al. 2017). Constant presence of cross-resistance within the insecticides tested therefore suggested that they are no longer effective in controlling the resistance Indonesian Ae. aegypti populations.

The use of pyrethroid in Indonesia since 1980s failed to fulfil its duty when human DF cases demonstrate no sign of decrement in the country (Ministry of Health Republic Indonesia, 2015). This vector-borne disease is recorded in nearly every province of the country although some islands are geographically isolated by oceans. Household insecticide formulations other than mosquito coils, for examples, liquid vaporizer, aerosol and mat have been broadly introduced and easily obtained in Indonesian markets. The formulations of these products usually contained active ingredients from the pyrethroid group of insecticides, namely d-allethrin, tetramethrin, d-trans allethrin, prallethrin,

transfluthrin, s-bioallethrin, permethrin, deltamethrin and d-phenothrin (Yap et al., 2000). In response to the excessive reliance on these pyrethroid-based household insecticide products in indoor setting, high insecticide resistance was therefore detected in this study. This is attributable to the endophilic behaviour of *Ae. aegypti* which there are higher chances for them to expose or get in contact with the chemical released by these products and therefore, the development of resistance via selection pressure (Chen et al., 2005). As the first coil study assessing on the susceptibility status of *Ae. aegypti* against pyrethroid-based mosquito coils in Indonesia, the result provides an insight that varying insecticidal effects were exerted to Indonesian *Ae. aegypti*. This suggests the necessity of rotating different chemicals in different localities and the use of pyrethroids should be abandoned in locations with pyrethroid-resistant *Ae. aegypti*. The findings provided may contribute to the local authorities on the susceptibility data to be referred to in opting for an appropriate vector control program. Moreover, with the fact that the bioassays were conducted under laboratory conditions, there exists the probability that these products may not elicit ideal mortality responses to all strains for end-user. Thus, it is suggested to employ semi-field test system to ensure accuracy of the findings.

Most of the adult *Ae. aegypti* populations tested have developed resistance to pyrethroid-based mosquito coils. The result of this study is contrasted to the results of many pioneer studies done earlier in Indonesia that demonstrated either susceptible or tolerant *Ae. aegypti*. This indicates that pyrethroid resistance has successfully developed in the country due to high reliance of routine application over the past years. Although the resistance ratios from some study sites remained low, the resistance may have built up gradually that provinces with susceptible strains may result in resistant in the future if the same control approach is still in use in a long run. This strongly suggests the abandoning of pyrethroid in vector control program and the reconstruction of well-structured national control measures urgently. However, due to its less hazardous

characteristic to humans than other classes of insecticides, there possesses a possibility for the reintroduction of pyrethroid into the national vector control program in Indonesia later. Thus, the monitoring of the efficacy of pyrethroid against *Ae. aegypti* still has to be adhered closely, with the intention of turning back to its use at a proper time. With a critical need of effective mosquito control tools to combat resting mosquitoes, this study has accomplished in good time in reviewing the efficacies of Indonesian mosquito coils because this aids in gaining valuable knowledge to develop highly effective spatial repellents to complement other control strategies to eliminate dengue. The detection of insecticide resistance in some populations in this study prompted further biochemical and molecular studies to characterize the resistance mechanisms in *Ae. aegypti*.

5.2 Enzyme Assays

Enzyme assays demonstrated that not all detoxifying enzymes (i.e., ESTs, MFOs and GSTs) were involved in pyrethroid resistance in Indonesian *Ae. aegypti*. Although previous studies have described the roles of these enzymes in contributing to pyrethroid resistance in the wild Indonesian *Ae. aegypti* (Ahmad et al., 2007; Astuti et al., 2014), the findings of this study showed discrepancy in the results in some of the populations. The difference in the roles of contributory enzymes may imply the involvement of more than one resistance mechanisms exerted on the local *Ae. aegypti*. This urges the local authorities on an abrupt need in revising vector control strategies in the country.

Previous studies that proved pyrethroid resistance was commonly the outcome of elevated ESTs activity in *Ae. aegypti* (Ahmad et al., 2007; Putra et al., 2016) and *Cx. quinquefasciatus* (Sarkar et al., 2009; Low et al., 2013) contradicted the present findings which showed no correlation pertaining the survivability rates of all tested insecticides against the enzyme activity of α-ESTs. Therefore, the elevated ESTs levels in some of the Indonesian *Ae. aegypti* (i.e., Padang, Samarinda, Pontianak, Denpasar, Mataram and

Manggarai Barat) in comparison to the Bora-Bora reference strain may not solely be related to pyrethroid resistance when statistical analysis showed no significant correlations between pyrethroids resistance and α-ESTs. With regard to the mosquito coil bioassays, pyrethroid-susceptible populations from some study sites showed marginally-exceeded enzyme levels when compared to the reference strain. This points out the possibility that the enzyme activity detected, act as a contributory factor accounted for DDT resistance, as reported in the past by Hemingway & Ranson (2000). In addition, elevated levels of ESTs are also often correlated with organophosphates. Thus, it could be inferred that elevated ESTs level detected in adult *Ae. aegypti* in this study may suggest the presence of DDT and organophosphate insecticide resistance which could be observed in previous studies in DDT-resistant *Ae. aegypti* (Marcombe et al., 2012), organophosphate-resistant *Cx. quinquefasciatus* (Vaughan et al., 1997) and organophosphate-resistant *Ae. aegypti* (Bisset et al., 2001; Pethuan et al., 2007). The development of organophosphate and DDT resistance came as no surprise in Indonesia after malathion was recommended by the government for *Aedes* control (Elyazar et al., 2011) and DDT was in widespread use in Indonesia several decades ago. However, future toxicological studies on the organophosphates and DDT susceptibility will be needed to reveal the true explanations behind the elevated ESTs activity in some mosquito populations.

Despite significant correlations were observed between the survivability rates of pyrethroids in adult bioassays and GSTs activity in the Indonesian *Ae. aegypti* populations, this enzyme may not be predominant resulting in the pyrethroid resistance reported. This is because the GSTs enzyme levels recorded were the lowest relative to other groups of enzymes in the present study even in the resistant populations. As reported by Hollingworth & Dong (2008) and Che-Mendoza et al. (2009), an elevated level of GSTs activity is usually associated with resistance to several insecticide classes in a wide

range of arthropods but it is chiefly accounted for DDT resistance. However, even previous studies had put in efforts to ascertain the role of GSTs in DDT resistance in *Anopheles maculatus* (Lee & Chang 1995), *Ae. aegypti* (Lee & Chang 1995; Prapanthadara et al., 2002), and *Cx. quinquefasciatus* (Lee & Chang 1995; Low et al., 2013). In this study, it appeared that DDT resistance status of these species of mosquitoes failed to correlate with this enzyme. Thus far, the relationships between insecticide resistance and the enzyme activity of GSTs are still inadequately studied in various mosquito species across the world. In order to correlate the significant increases in the mean enzyme activities of GSTs in the Kuningan as well as Sumba Timur populations to the presence of DDT resistance in these field populations, the use of suggested diagnostic doses of DDT in WHO adult bioassays will be sufficed to accomplish. The detection of negligible mortality rate in the bioassays indicates the likelihood that the pyrethroid resistance previously described might be a consequence of cross-resistance with DDT. Since 1950s, DDT was the first chemical in widespread use to control *Ae. aegypti* in Indonesia (Bang et al. 1982). Later, the government has banned the use in 1970s and subsequently pyrethroid is used as a substitute. Surprisingly, DDT resistance phenotype can still be detected despite several decades of abandoning DDT in the country (Brengues et al., 2003), owing to the persistence of DDT in the environment that may have caused continuous selection pressure of resistance. However, as previously discussed, the role of GSTs was limited as seen in the low enzyme activity in most of the populations in this study. In addition to DDT resistance, some field populations were exerted with high levels of resistance to all of the pyrethroids tested. Therefore, a dearth of associations reported between GSTs activity and DDT resistance could be ascribed to a mutation in the sodium channel protein, which is the target site for both pyrethroid and DDT insecticides resulting in a *kdr* phenotype (to be discussed further). As a result, it can be extrapolated that

pyrethroid resistance detected in some of the Indonesian *Ae. aegypti* was because of the combined action of metabolic detoxification and target-site insensitivity.

The enzyme assays performed in the present study revealed elevated MFOs levels in some of the *Ae. aegypti* populations from Indonesia, inferred that MFOs could be the predominant enzyme triggering pyrethroid resistance in this species. It is true that elevated level of MFOs has been associated with resistance to pyrethroids in *Ae. aegypti* as previously described (Pethuan et al., 2007; Paeporn et al., 2004). However, such elevation of MFOs activity in most of the mosquito populations spread out to distinct regencies where pyrethroid resistance was detected may lead to a loss in the efficacy of insecticides, placing the public health of Indonesia at stake especially those with high mean values of enzyme activity recorded. To further reassure the resistance detected in some Indonesian mosquito populations is in large part due to metabolic detoxification, MFOs inhibitors such as PBO which can increase insecticide action can be used. This allows for the verification the impact of MFOs on the toxicity of all active ingredients tested. Moreover, MFOs are the enzymes most frequently linked to cross-resistance between pyrethroids and DDT (Scott et al., 1998). This stresses the importance of assessing the DDT susceptibility status of Indonesian *Ae. aegypti* again in the future.

Inconsistent trends in enzyme activities were demonstrated in Indonesian *Ae. aegypti*. Interestingly, many of the populations had enzyme levels lower than that of the reference strain. Moreover, *Ae. aegypti* from some regencies, which demonstrated high RR to all active ingredients assayed, did not reflect high activity profile for all the enzyme classes involved, signifying the complexity between these enzymes and pyrethroids. Hence, metabolic detoxification fails to comprehensively explain the elevated resistance status to pyrethroids, unless in rare cases that enzymes expressed are at very low levels, which are below the detection limit of the biochemical assays. Additional mechanisms may also

be accounted for the high pyrethroid resistance exhibited by the wild *Ae. aegypti*, for examples, behavioral avoidance, cuticular resistance or knockdown resistance. Furthermore, insecticide resistance caused by more than one mechanism are also likely to happen. This phenomenon has previously been reported, causing a stir in mosquito control programs worldwide. Thus, additional investigations with the use of synergists will give more information on metabolic-mediated resistance mechanisms.

In general, the pyrethroid resistance mechanism in Indonesian *Ae. aegypti* is conferred partially by MFOs. Resistance to chemical insecticides is indeed a multi-factorial trait incorporating several aspects such as genetic (modifications at target sites and metabolic detoxification), environmental (biotic and abiotic factors of breeding sites) and operational (types and durations of control measures) factors. Likewise, the large geographic regions of Indonesia may cause diverse genetic backgrounds in different *Ae. aegypti* populations, closely associated with the occurrence of different resistance mechanisms to the same insecticide in distinct populations. This fortifies the importance that control measures is ought to be tailored to the distinctiveness in each of the population. Although *in-vitro* experiments employing the use of model substrates may not directly reflect the *in-vivo* conditions of mosquitoes in metabolizing insecticide molecules, findings from this study is capable in delivering baseline data and accentuating the necessity of in-depth investigations into metabolic resistance in *Ae. aegypti* populations from Indonesia. Ultimately, the results also serve as a stepping stone to understand the enzyme-mediated physiological processes in *Ae. aegypti*. This will open up the possibility thoroughly studying the biology of this mosquito species, which is indispensable in developing effective vector control strategy for its major disease vector.

5.3 *Kdr* Gene Screening

The S989P, V1016G and F1534C mutations are the most commonly detected *Vgsc* mutations found in pyrethroid-resistant *Ae. aegypti* in Southeast Asia. To confirm whether these point mutations play major roles in contributing pyrethroid resistance detected from the WHO mosquito coil bioassays against Indonesian *Ae. aegypti*, genotyping of mutations using PCR and direct DNA sequencing were performed at position 989 and 1016 in IIS6 region and 1534 in IIIS6 region of the *Vgsc* in mosquito specimens sampled. The sampling locations encompassed larger geographical expansions to screen for *kdr* in *Ae. aegypti* relative to past studies completed in the country, making the present study to be the largest in respect of geographical coverage in Indonesia thus far. In accordance with the mosquito coil bioassays accomplished earlier in this study, most of the Indonesian *Ae. aegypti* populations revealed high levels of resistance against all the pyrethroids assayed, namely, d-allethrin, transfluthrin and metoflurthrin. With the emergence of such alarming issue, scientific findings from Southeast Asia (i.e., Malaysia) still displayed effectiveness of the mosquito coils against the field strains of *Ae. aegypti* (Chin et al., 2017). In this study, mosquitoes from some localities showing somewhat low *kdr* mutation frequency, were in accordance with their low resistance phenotype.

Based on the results, three mutations, V1016G, S989P and F1534C were detected in Indonesian *Ae. aegypti*. Direct DNA sequencing of IIS6 region of *Vgsc* of adult *Ae. aegypti* collected across some of the regencies in Indonesia demonstrated the presence of both V1016G and S989P mutations. The presence of the V1016G mutation was within expectation when this statement can be supported by a fund of evidence through which it was commonly discovered in Southeast Asia, namely, Malaysia (Ishak et al., 2015), Thailand (Brengues et al., 2003), Vietnam (Kawada et al., 2009a), Singapore (Rajatileka et al., 2008) and Myanmar (Kawada et al., 2014). Previous studies in some parts of Indonesia such as Yogyakarta (Wuliandari et al., 2015), Semarang (Bregues et al., 2003),

Jakarta (Hamid et al., 2017a) and Denpasar (Hamid et al., 2017b) also concurred with the findings from the current study. This point mutation, on the other hand, is yet to be reported in South American *Ae. aegypti* despite its prevalence in Southeast Asian countries (Bregues et al., 2003; Saavedra-Rodriguez et al., 2007; Rajatileka et al., 2008).

From the findings of this study, the V1016G mutation revealed the RR genotype to be the most predominant through the extensive dispersal across all of the study sites involved, whereas a low frequency of the SS genotype was shown. The absence of the heterozygosity in the V1016G mutation could be because of polymorphism, as reported by Martins et al. (2013) that other than amino acid changes, nucleotide and insertion or deletion polymorphisms can also be involved in a gene duplication event. Since heterogeneous (RS) duplications had an intermediate phenotype with lower resistance and fitness cost which is in opposed to homogeneous (RR) duplications that enabled increment in both pesticide resistance and fitness costs (Bass et al., 2017), the frequency of the RS genotype may reduce rapidly after a few generations when strong selection pressure exerted. Thus, the gene duplication of the mutant homogeneity in the *Vgsc* of *Ae. aegypti* may be arisen in response to adaptation due to the extensive use of pyrethroids in the country. This aids in maintaining the genotype with high fitness cost in order to enhance such trait for the continuity of the development of insecticide resistance and decrease the likelihood of deleterious effects concerning fitness cost.

Additionally, great differences were detected between number of the mosquitoes being the 1016G homozygous mutant and the 1016V wild-type homozygous. It was also discovered that the highest frequency of *kdr* resistance allele in *Ae. aegypti* populations were those from Kuningan, Padang, Samarinda, Denpasar, Mataram and Sumba Timur. This result corresponded to the result of the mosquito coil bioassays performed previously, indicating those categorized as resistant were with 1.0 resistant allele

frequency whereas those grouped under susceptible were with 1.0 susceptible allele frequency. In this case, it can be concluded that homozygous mutant females were resistant whereas wild-type homozygous were susceptible. This is indeed true when Wuliandari et al. (2015) verified 1016G homozygote was more largely related to the resistance of both Type I and Type II pyrethroids than other genotypes.

Inversely, among all of the adult mosquitoes selected for the F1534C mutation screening, SS genotype was discovered in majority. The F1534C mutation was the least frequently found relative to the S989P and V1016G mutations in this study. This outcome conformed to the past studies conducted in Jakarta and Denpasar (Hamid et al., 2017a; Hamid et al., 2017b) but in contrast to studies performed in other parts of Southeast Asia such as Thailand (Yanola et al., 2011), Vietnam (Kawada et al., 2009a), Malaysia (Ishak et al., 2015) and Singapore (Pang et al., 2015). The discrepancies from these findings may very much linked to a more recent emergence of the F1534C mutation in Indonesian *Ae. aegypti* when gene flow occurs through the occurrence of migration events. Interestingly, such incongruities in result can also be attributed to the extensive geographical distribution of Indonesia with geographic expansion extending 5,120 km from east to west and 1,760 km from north to south, causing the difference in findings across Southeast Asia countries when gene expression is mediated in response to environmental dissimilarity. Thus, this point mutation is thought to be yet significant in leading to the development of pyrethroid resistance in *Ae. aegypti* populations from Indonesia.

By comparing the relationships between the allele frequencies of the V1016G mutation and the pyrethroid survivability rates as well as resistance ratios to all of the active ingredients examined, significant correlations were detected. This points out the higher the frequency of the V1016G mutation, the greater the level of resistance to d-allethrin, transfluthrin and metofluthrin in these tested Indonesian *Ae. aegypti* populations. In

contrast, no significant correlations were demonstrated between the allele frequencies of the S989P as well as F1534C mutations and the status of the insecticide susceptibility mosquito coil bioassays. Such lack of significant correlations may indicate that both S989P and F1534C mutations do not involve in the development of pyrethroid resistance. This may also suggest only the V1016G mutation is closely associated with pyrethroid resistance in Indonesian *Ae. aegypti* populations. Nevertheless, the possibility of the involvement of other resistance mechanisms in *Ae. aegypti*, should be taken into consideration and thoroughly investigated before any one-off conclusion is made.

Brengues et al. (2003) pointed out knockdown resistance against pyrethroids in *Ae. aegypti* may possibly be conferred by one or more point mutations in the *Vgsc* locus. In the present study, a pattern of co-occurrence of point mutation, specifically V1016G/S989P was detected. The co-occurrence of the S989P and V1016G mutations were often found in pyrethroid-resistant *Ae. aegypti* populations, indicating they may have resulted in an increment of the insensitivity of *Vgsc* to pyrethroids. Although the S989P mutation was detected in all of the study sites, it may not stand alone as the main cause in leading to the occurrence of insecticide resistance. This is because the S989P mutation has always been associated with the V1016G mutation, whereas the S989P mutation has yet to be found occurring alone (Kawada et al., 2014, Ishak et al., 2015). However, the V1016G has been discovered in spite of an absence of the S989P mutation in Thai *Ae. aegypti* (Brengues et al., 2003; Rajatileka et al., 2008). Therefore, it can be inferred that the S989P mutation may likely result in synergizing mutation, escalating the effect of pyrethroid resistance. Du et al. (2013) further proved that the S989P mutation did not show any additive effect to the V1016G mutation but later, a contradictory study showed that the existence of the S989P mutation can highly decline the sensitivity to pyrethroid (Hirata et al., 2014). It is also worth mentioning the high frequency of the co-occurrence of the V1016G and S989P mutations is in line with past studies in Indonesia.

The presence of the debating concern if the S989P mutation was responsible in causing additive role in pyrethroid resistance development in *Ae. aegypti* is of controversial. Thus, it is of critical need to conduct further studies with regards to the additive effect of the S989P mutation to the V1016G mutation.

Many controversies were shown on the accuracies of both direct DNA sequencing and PCR in detecting the point mutations in the *Vgsc* of *Ae. aegypti*. Despite the availabilities of allele-specific PCR (AS-PCR) and multiplex PCR that enable the detection of few point mutations in one or two reactions at our conveniences, the sensitivity and specificity of these detection methods may not be ideal. In the present study, there were deviations in the results when scored visually on gel electrophoretograms. Multiplex PCR showed inconsistency on the presence of internal control bands whereas negative control bands were observed for AS-PCR throughout the entire span of optimization process although the protocols were strictly adhered to. Thereafter, direct DNA sequencing was opted for to obtain the results in the present study. The use of the direct DNA sequencing was further in approval when incongruence of results from both direct DNA sequencing and AS-PCR were documented in previous studies in several species of mosquitoes such as *Ae. aegypti* (Yanola et al., 2011) and *Cx. quinquefasciatus* (Low et al., 2013). Due to budget constraint, only a certain amount of mosquitoes per population was randomly selected for direct DNA sequencing.

The genotyping of mutations with regard to insecticide resistance can contribute as an advantageous surveillance tool in tracking resistance and involving in the intervention of novel chemical insecticides for mosquito control. With such, the V1016G, S989P and F1534C mutations in *Vgsc* were detected in Indonesian *Ae. aegypti*. Being the first study consisted of large biogeographical areas in Indonesia, the current report has documented

the V1016G mutation as the predominant genotype in Indonesian *Ae. aegypti*. This situation requires immediate attention before all of the control tactics were compromised.

5.4 Future Challenges and Perspective

When development of resistance has become a worldwide issue, there are alternatives to control *Ae. aegypti*. Environmental control involves source reduction and the use of mosquito traps or screen net covers (Kittayapong et al., 2012; Lee et al., 2013; Ponlawat et al., 2013; Lau et al., 2015b). Standing water and unnecessary containers, in both indoor and outdoor conditions are ought to be eliminated to prevent breeding of mosquitoes, specifically *Ae. aegypti* and other container breeders (Yap et al., 2003). Containers that performed functions in daily life should be screened or properly covered. Source reduction should also include getting rid of natural habitats that collect water, for instances bamboo stumps and tree holes to avoid the breeding of *Ae. aegypti*.

To diversify the choices of vector control, research has been reinforced in developing pathogenic organisms to combat dengue vector. In biological control, the natural enemies are either predators, microbes or parasites (Yap et al., 2003). Examples of biological control Asia include the participation of predaceous aquatic insects as natural enemies to suppress mosquito populations by predating on mosquito larvae as food source (Seng et al., 2008; Tun-Lin et al., 2009; Lazaro et al., 2015;) or the use of the entomopathogenic bacteria *Bacillus thuringiensis* var. *israelensis* to destroy the gut lining of mosquito larvae (Kittayapong et al., 2012; Saiful et al., 2012). Hypothetically, predaceous animal species should result in reduction of mosquito populations but there is limited evidence regarding tangible proof of declining related disease burdens. Therefore, it should be noted that these methods would work well alongside with chemical insecticides but should never be employed as a sole control tactic during endemic outbreaks of dengue when immediate elimination of disease vectors should be prioritized.

Another biological control method involves the release of *Ae. aegypti* infected with *Wolbachia* sp. These naturally-occurring bacteria infect a wide range of arthropods and nematodes but are absent in *Ae. aegypti* (Jeyaprakash & Hoy, 2000). To decrease dengue transmission, *Wolbachia* from naturally infected organisms are vertically transmitted into *Ae. aegypti*. This will cause sterility via cytoplasmic incompatibility, resulting in eggs without progeny when an uninfected female *A. aegypti* mates with a *Wolbachia*-infected male (Vythilingam et al., 2016). *Wolbachia* has drawn much attentions that field trials are ongoing in several countries such as Australia (Hoffmann et al., 2011), Vietnam (Nguyen et al., 2015) and Indonesia (Rašić et al., 2015). Despite wMel *Wolbachia* showed positive result in lowering the incidence of dengue in human populations in northern Australia (Hoffmann et al., 2011), wMelPop *Wolbachia* with even more significant resistance to DENV infection failed to established successfully in Australia and Vietnam. Hence, more progressive studies pertaining to the large-scale field release of *Wolbachia*-infected *A. aegypti* are essential before this approach displays high success in dengue control program throughout dengue-endemic countries.

It is irrefutably the fact that chemical insecticides remain to hold a place primarily in vector-control interventions, specifically during an emergency endemic period. When *Ae. aegypti* exhibited a propensity to develop resistance to countless groups of chemical insecticides leading to loss of functions, insect growth regulators (IGRs) may likely reduce resistance developing. The discovery of IGRs is in accordance with the knowledge of their growth, development, function and behavior. Thus, the intelligent use of IGRs has the likelihood to conquer the market of many other insecticides when Lau et al. (2015a) reported these group of insecticides pose encouraging results to control the field populations of *Ae. aegypti*, specifically cyromazine.

Pyrethroid resistance in Southeast Asian *Ae. aegypti* should never be overlooked. Many countries rely on the interventions of chemical insecticides to control the mosquito vectors of dengue. In the past chapters of this thesis, several issues that highlight the need for immediate attention were addressed. Undeniably, these studies demonstrated a strong tendency of lopsided geographical distribution in published reports with more than half literatures published from Thailand, Indonesia and Malaysia. Although studies retrieved from the database reflected the burden of dengue fever, it is believed that some dengue-endemic countries such as Vietnam and Myanmar may have restricted the accessibility of the database without releasing to the public. This purports difficulty in deciding on suitable insecticides for dengue control and therefore, a platform with insecticide resistance status of different *Ae. aegypti* populations in a homogenous format would critically solve related challenges and ease future vector control planning. In certain cases, uniformity of protocols was neglected. A broad range in the methodologies utilized to record and analyze resistance or susceptibility data affecting cross-examination of various studies to be very challenging. Thus, only some attempts were made to specify the level of susceptibility that the standardization of methods in all of the studies done would contribute to reliable comparison of the outcomes. Method of recording insecticide resistance should be consistent, taking $LC_{50}/LT_{50}/KT_{50}$ or other similar set of data for instances, that actual values may still have to be provided for comparison in some circumstances.

In addition, the use of the revised version of WHO guidelines was not practiced in all of the studies. WHO has initially published two different databases on the diagnostic dosages of distinct insecticides (WHO, 1981; WHO, 1992) but there were still some studies employed the original diagnostic doses to examine the susceptibility status of *Ae. aegypti* (Jirakanjanakit et al., 2007; Loke et al., 2012; Koou et al., 2014) while some have already opted for the newly revised guideline (Somboon et al., 2003; Sathantriphop et al.,

2006). In recent times, WHO has released the latest version of test procedures to detect insecticide resistance for both *Aedes* larvae and adults (WHO, 2016). Thereafter, all researchers are recommended to adhere to the changes made by WHO on the guidelines to evaluate the resistance status of field *Aedes* population. Furthermore, future studies should pinpoint on widening the number of sampling sites on a wide array of active ingredients of the pyrethroid group that are yet to be tested in most cases, including resmethrin, bifenthrin, flumethrin, tralomethrin that they may have potentials in effective control of *Ae. aegypti* or cross-resistance within the active ingredients of pyrethroid group may have already occurred whereby the last resort would be abandoning of the pyrethroid group of insecticides. The prevalence use of pyrethroid-based mosquito coils calls for further validation on their efficacies across Southeast Asia. As of now, little has been reported on the efficacies of these mosquito coils to *Ae. aegypti* populations.

Efforts of understanding the aforementioned mechanisms on *Ae. aegypti* are urgently required, especially some dengue-endemic countries with unavailability of associated reports because the absence of these data poses the vector control against *Ae. aegypti* remains to be the basis in combating dengue transmission and outbreak control. Bioassays are only capable in detecting resistance that has already existed in a strain while diagnostic assays can detect resistance once it appears that this may avoid the failure in vector management. *Kdr* assays have long been used for a couple of years in detecting point mutations to observe the effect of control tactics on insecticide resistance but it is utterly crucial to note that these molecular assays of target site resistance should also not replace bioassays until other resistance mechanisms, for example, metabolic resistance can be complemented by comparable assays. A major advantage of molecular approach worth-mentioning is the sequencing of *Ae. aegypti* genome aids in progressive study on insecticide resistance mechanisms and more rewarding research will be looking forward to searching for new diagnostic markers of pyrethroid resistance in dengue vectors.

Biochemical assays in detecting enzymes linked to pyrethroid resistance have been utilized in several Southeast Asian countries because these assays deliver noteworthy information for estimating resistance. However, the short of specificity and sensitivity of some assays may lead to complications in analyzing data. As for the addition of synergist such as PBO used to examine the significant of metabolic resistance, the protocol can be very problematic since a huge amount of alive *Ae. aegypti* populations will be needed. Hence, the above-mentioned challenge in biochemical assays demand even more cautious act when analyzing the use of synergist data.

Across Southeast Asia, dengue appears to be endemic. Thus far, unavailability of data regarding pyrethroid resistance in *Ae. aegypti* in some parts of Southeast Asia such as Brunei Darussalam, Christmas Island of Australia and The Andaman/Nicobar Islands of India necessitates immediate research works to be conducted. As for the reported countries, resistance status of many *Ae. aegypti* populations to pyrethroids demonstrated the need for more effective control strategies, possibly the intervention of new insecticides. The fact that either migration or *de novo* mutations in posing issue on the spread of *kdr* mutations that were once occurred locally are now discovered in different continents despite geographical separations should be highlighted. More efforts are bound to combat insecticide resistance to terminate the spread of diseases and vectors. However, it is understandable that this could be a difficult undertaking because a substantial amount of funds is needed. Therefore, vector control success will very much count on policymakers, academicians and scientists.

CHAPTER 6: CONCLUSION

1. Indonesian adult *Aedes aegypti* demonstrated inconsistent susceptibility against mosquito coils containing d-allethrin, transfluthrin and metofluthrin across all study sites.
2. *Aedes aegypti* from Denpasar, Mataram, Kuningan, Padang, Samarinda and Sumba Timur was resistant to d-allethrin-, transfluthrin- and metofluthrin-based mosquito coils.
3. *Aedes aegypti* from Manggarai Barat, Dompu and Pontianak was susceptible to d-allethrin-, transfluthrin- and metofluthrin-based mosquito coils.
4. Correlations between d-allethrin and transfluthrin resistance; between d-allethrin and metofluthrin resistance; as well as between transfluthrin and metofluthrin resistance in adult mosquito coil bioassays were discovered.
5. Mixed function oxidases contributed pyrethroid resistance in *Aedes aegypti*.
6. Activities of all enzymes tested (except for α-esterases) were associated with d-allethrin, transfluthrin and metofluthrin resistance.
7. The V1016G, S989P and F1534C mutations of voltage-gated sodium channel were discovered in Indonesian *Ae. aegypti*. In the V1016G genotyping, RR genotype was predominant with 46 individuals (out of 63). Two genotypes at the position 1016 were found, with only RR genotype was detected in resistant populations whereas only SS genotype was detected in susceptible populations.
8. The RR genotype was the most predominant genotype with 41 individuals (out of 63) detected from the S989P *kdr* mutation. A total of 20 individuals were detected as the SS genotype while two individuals were confirmed as the RS genotype.
9. The F1534 genotyping revealed the occurrence SS genotype in all populations. Merely two individuals exhibited heterozygous genotype whereas no RR genotype was observed across all study sites in Indonesia.

10. Correlations between the d-allethrin, transfluthrin and metofluthrin resistance phenotypes (in adult bioassays) and allele frequencies of the V1016G mutation were demonstrated.
11. Correlations between the resistance ratios of d-allethrin, transfluthrin and metofluthrin (in adult bioassays) and allele frequencies of the V1016G mutation were also discovered.
12. In six out of nine populations (i.e., Kuningan, Padang, Samarinda, Denpasar, Mataram, Sumba Timur) of *Aedes aegypti*, co-occurrence of the V1016G and S989P mutations was detected.
13. It is at utmost importance in monitoring the insecticide resistance status of various populations of *Aedes aegypti* in Indonesia to guarantee effective vector control. In respect of the insecticide resistance detected in the present study, more efforts are required to combat insecticide resistance to decrease the spread of diseases and vectors.

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LIST OF PUBLICATIONS AND PAPERS PRESENTED

Publications

1. Amelia-Yap, Z. H., Chen, C. D., Sofian-Azirun, M., & Low, V. L. (2018). Pyrethroid resistance in the dengue vector *Aedes aegypti* in Southeast Asia: present situation and prospects for management. *Parasites & Vectors*, 11(1), 332.
2. Amelia-Yap, Z. H., Chen, C. D., Sofian-Azirun, M., Lau, K.W., Suana, I.W., Harmonis, Syahputra, E., Razak, A., & Low, V. L. (2018). Efficacy of mosquito coils: Pyrethroid resistance in *Aedes aegypti* from Indonesia. *Journal of Economic Entomology* doi: 10.1093/jee/toy296.
3. Amelia-Yap, Z.H., Chen, C. D., Suana, I. W., Lau, K. W., Elia-Amira, N. M. R., Haziqah-Rashid, A., Tan, T. K., Lim, Y. A. L., Sofian-Azirun, M., & Low, V. L. (2018). Metabolic-mediated mechanisms of pyrethroid resistance in the primary dengue vector *Aedes aegypti* in Indonesia. (Submitted)
4. Amelia-Yap, Z. H., Chen, C. D., Sofian-Azirun, M., Lau, K.W., Suana, I.W., Harmonis, Syahputra, E., Razak, A., & Low, V. L. (2018). The V1016G point mutation: the key mutation in the voltage-gated sodium channel (*Vgsc*) gene of pyrethroid-resistant *Aedes aegypti* in Indonesia. (Submitted)

Poster Presentations

1. Amelia-Yap, Z. H., Low, V. L., Chen, C. D., Lau, K.W., Suana, I.W., Harmonis, Syahputra, E., Razak, A., Sofian-Azirun, M. (2018). Efficacy of Mosquito Coils: Emphasizing Cross-resistance to Pyrethroids in *Aedes aegypti* from Indonesia. 54th Annual Scientific Conference of the Malaysian Society of Parasitology and Tropical Medicine (MSPTM) "Tropical and Zoonotic Diseases: Stemming the Tide". 14-15th March 2018, Connexion Conference and Event Centre, Bangsar South City, Kuala Lumpur, Malaysia. (Abstract: page 59)
2. Amelia-Yap, Z. H., Chen, C. D., Sofian-Azirun, M., Lau, K.W., Suana, I.W., Harmonis, Syahputra, E., Razak, A., Low, V. L. (2018). The V1016G point mutation: the key mutation in the voltage-gated sodium channel (*Vgsc*) gene of pyrethroid-resistant *Aedes aegypti* in Indonesia. Malaysian Society of Parasitology and Tropical Medicine (MSPTM) Mid-year Seminar in Honour of Professor Mak Joon Wah. 7th July 2018, International Medical University, Bukit Jalil, Kuala Lumpur, Malaysia. ***Second Prize**
3. Amelia-Yap, Z.H., Chen, C. D., Suana, I. W., Lau, K. W., Elia-Amira, N. M. R., Haziqah-Rashid, A., Tan, T. K., Lim, Y. A. L., Sofian-Azirun, M., Low, V. L. (2018). Metabolic-mediated mechanisms of pyrethroid resistance in the primary dengue vector *Aedes aegypti* in Indonesia. 2nd Asian Simuliidae and 1st National Veterinary Parasitology Symposium. 23-24th July 2018, Bogor Agricultural University, Bogor, Indonesia.