SUSCEPTIBILITY AND RESISTANCE STATUS OF DENGUE VECTORS TOWARDS SELECTED PYRETHROIDS IN KOTA BHARU, KELANTAN

AMANI BINTI AHMAD MOKHTAR

UNIVERSITI SAINS MALAYSIA

2021

SUSCEPTIBILITY AND RESISTANCE STATUS OF DENGUE VECTORS TOWARDS SELECTED PYRETHROIDS IN KOTA BHARU, KELANTAN

by

AMANI BINTI AHMAD MOKHTAR

Thesis submitted in fulfillment of the requirements

for the degree of

Master of Science

October 2021

ACKNOWLEDGEMENT

Praises be to ALLAH SWT and peace upon Rasulullah Muhammad SAW. I am grateful to be blessed with ambitions, patience, and perseverance to pursue this study and follow through till the very end. My utmost gratitude is extended to my beloved supervisor who I look up to, Dr Azlinda Binti Abu Bakar. Her knowledge and non-stop encouragement for me has been one of my strengths and aspirations to grow along with the progress of my study. I am also grateful to my co-supervisor, Associate Professor Dr Rafidah Hanim Shomiad @ Shueb for her guidance through some parts of the study that I am not familiar with. I am also thankful to the colleagues, lecturers, and the staffs of the Animal Research and Service Center and the Department of Medical Microbiology and Parasitology, USM for their guidance in the lab work needed to complete the study, especially my close colleagues Tuan Nur Akmalina and Najma Barkhadle. Their demonstrations and insights on the results of my study have helped me learn in-depth about my topic. Special thanks to my family and especially my parents Ahmad Mokhtar Bin Zakaria and Che Nora'zah Binti Noor Din whose words of encouragement and resources have greatly motivated me in furthering my studies.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF SYMBOLS AND UNITS

Symbols/ Units	Definition
%	Per cent
±	Plus-minus
°C	Degree Celsius
™	Trademark
®	Copyright
× g	Times gravity (unit)
bp	Base pairs
cm	centimetres
f	Forward primer
µL	Microliter
g	Gram
min	Minutes
mL	Millilitre
mM	Millimolar
ng	Nanogram
r	Reverse primer
s	seconds

LIST OF ABBREVIATIONS

Abbreviations	Definitions
AEP	*Aedes aegypti* (Panji population)
Ae. aegypti	*Aedes aegypti*
Ae. albopictus	*Aedes albopictus*
ALK	*Aedes albopictus* (Kubang Kerian population)
ALP	*Aedes albopictus* (Panji population)
ARASC	Animal Research and Service Center
ATSDR	Agency for Toxic and Disease Registry
CDC	Centre of Diseases Control
COMBI	Communication for Behavioural Impact
DDT	Dichlorodiphenydichloroethane
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic triphosphate
EPA	Environmental Protection Agency
GDHO	Gombak District Health Office
kdr	Knockdown resistance
MMD	Malaysia Meteorology Department
MOH	Ministry of Health
PCR	Polymerase chain reaction
RR	Resistance ratio
SPSS	Statistical Package for Social Sciences
spp.	Species (plural)
TBE	Tris-borate ethylenediamine tetraacetic acid
ULV	Ultralow-volume
USMKK	Universiti Sains Malaysia, Kampus Kesihatan
VGCC	Voltage-gated calcium channels
VGSC	Voltage-gated sodium channels
VGPC	Voltage-gated potassium channels
WHO	World Health Organization

LIST OF APPENDICES

STATUS KERENTANAN DAN KERINTANGAN VEKTOR DENGGI KEPADA PIRETROID TERPILIH DI KOTA BHARU, KELANTAN

ABSTRAK

Nyamuk *Aedes* sp. memainkan peranan penting dalam penyebaran penyakit bawaan vektor seperti denggi, chikungunya, dan Zika di Malaysia. Penggunaan produk insektisid isirumah secara kerap dan aktiviti semburan kabus berkala di kawasan perumahan membangkitkan isu mengenai status kerentanan *Aedes* sp. terhadap insektisid di kawasan tersebut. Objektif kajian ini adalah untuk menilai status kerentanan dan kerintangan *Aedes* sp. terhadap piretroid di Panji, Kota Bharu, Kelantan. Sejumlah 100 nyamuk betina dewasa *Aedes* sp. generasi F1 telah diuji mengikuti garis panduan bioasai WHO terhadap permethrin 0.75% dan lambda-cyhalothrin 0.05%. Analisis PCR juga dijalankan pada alel khusus F1534C dan V1016G dalam nyamuk yang telah diuji untuk menentukan kehadiran mutasi *kdr*. Penentuan komposisi spesis nyamuk *Aedes* di kawasan kajian juga telah dijalankan. Hasil keputusan yang diperolehi menunjukkan bahawa spesies *Ae. albopictus* dijumpai di kedua-dua lokaliti; Panji, Kota Bharu dan Kubang Kerian manakala spesies *Ae. aegypti* hanya dijumpai di Panji, Kota Bharu. Ujian bioasai menunjukkan populasi *Ae. aegypti* di Panji, Kota Bharu mempunyai kerintangan tinggi terhadap kesemua insektisid kajian (piretroid jenis I permethrin 0.75%, 18.41±1.74 & piretroid jenis II lambda-cyhalothrin 0.05%, 14.58±9.45. Manakala, populasi *Ae. albopictus* di kawasan sama mempunyai kerintangan sederhana terhadap lambda-cyhalothrin 0.05% (93.82±3.01), tetapi menunjukkan kerintangan tinggi terhadap permethrin 0.75% (87.79±2.76). Populasi *Ae. albopictus* di Kubang Kerian pula mempamerkan kerentanan kepada lambda-cyhalothrin 0.05% (98.97±1.03) dan kerintangan terhadap

permethrin 0.75% (90.72±3.01). Hasil analisis PCR, populasi *Ae. albopictus* di Kubang Kerian menunjukkan terdapat dua kumpulan genotip yang berbeza iaitu genotip F/C1534 dan genotip kerentanan F/F1534. Manakala bagi populasi nyamuk di Panji, Kota Bharu kedua-dua genotip F/C1534 dan genotip kerintangan C/C1534 dapat dikesan dalam populasi *Ae. aegypti* dan *Ae. albopictus* masing-masing. Pada masa yang sama, kesemua sampel *Ae. aegypti* menunjukkan genotip kerentanan V/V1016. Berdasarkan kajian ini, kedua-dua analisis bioasai dan analisis PCR menunjukkan kerintangan telah terbentuk dalam populasi *Aedes* sp. terhadap insektisid yang diuji. Walau bagaimanapun kajian frekeuensi alel untuk menentukan peranan mutasi *kdr* selain kajian kerintangan metabolik juga diperlukan terhadap kerintangan nyamuk *Aedes* terhadap insektisid piretroid di dalam populasi tersebut.

SUSCEPTIBILITY AND RESISTANCE STATUS OF DENGUE VECTORS TOWARDS SELECTED PYRETHROIDS IN KOTA BHARU, KELANTAN

ABSTRACT

In Malaysia, *Aedes* sp. plays a significant role in the transmissions of vector-borne diseases such as dengue, chikungunya, and Zika. Regular usage of household insecticide products and occasional fogging events mainly in residential areas have raised an issue on the *Aedes* susceptibility status against insecticides. The objective of the study was to evaluate the susceptibility and resistance status of *Aedes* sp. mosquitoes against pyrethroids in Panji, Kota Bharu, Kelantan. A total of 100 F1 generations of adult female *Aedes* sp. were tested following WHO bioassay guidelines against permethrin 0.75% and lambda-cyhalothrin 0.05%. In addition, PCR analysis at the specific alleles of F1534C and V1016G of the tested mosquitoes were carried out to determine the presence of *kdr* mutations. Determination of the *Aedes* mosquito composition at the sampling areas was also done. From the results obtained, *Ae. albopictus* was found at both localities; Panji, Kota Bharu and Kubang Kerian whereas *Ae. aegypti* can only be found in Panji, Kota Bharu. The population of *Ae. aegypti* in the Panji area was highly resistant against all insecticides tested (type I pyrethroid permethrin 0.75%, 18.41±1.74 & type II pyrethroid lambda-cyhalothrin 0.05%, 14.58±9.45). On the other hand, *Ae. albopictus* was moderately resistant against type II pyrethroid lambda-cyhalothrin 0.05% (93.82±3.01). However *Ae. albopictus* showed to be highly resistant against type I pyrethroid, permethrin 0.75% (87.79±2.76). The Kubang Kerian *Ae. albopictus* population was susceptible against lambda-cyhalothrin 0.05% (98.97±1.03) and resistant against permethrin 0.75% (90.72±3.01). From the PCR analysis, the population of *Ae. albopictus* in Kubang

Kerian showed two different genotype groups, F/C1534 and susceptible F/F1534 genotypes. On the other hand, both genotype groups of F/C1534 and resistant C/C1534 were detected in the mosquito population of *Ae. aegypti* and *Ae. albopictus* in Panji, Kota Bharu respectively. While all *Ae. aegypti* samples showed the V/V1016 genotype. Based on this study, both the bioassay and the PCR analysis showed that the *Aedes* sp. population had developed resistance against the insecticides tested. However, a study on allele frequency to confirm the role of the *kdr* mutations as well as a study on metabolic resistance are needed towards the *Aedes* mosquito resistance against pyrethroid insecticide in the population.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 *Aedes* mosquitoes

Aedes aegypti and *Aedes albopictus* are from the class Insecta under the phylum Arthropoda, specifically of the order Diptera, which are identified by their distinctive characteristic of having one pair of wings. They are among the 950 known species of mosquitoes in the world (Britannica, 2020). The *Aedes* mosquito belongs to the Culicidae family (Musesbeck, 1952; Goma, 1996 as cited by Sivanathan, 2006), which can be divided into three subfamilies; Toxorhynchitinae, Anophelinae and Culicinae. *Aedes* spp. are known to inhabit regions with tropical and temperate climates in the world, but some species can be found in regions beyond that range due to changes in environmental influences or being introduced by humans to a new place (Britannica, 2020).

1.1.1 Taxonomic classification

The classifications of the two *Aedes* spp. are as follows:

Ae. aegypti (Linnaeus, 1762)	*Ae. albopictus* (Skuse, 1894)
Kingdom: Animalia	Kingdom: Animalia
Phylum: Arthropoda	Phylum: Arthropoda
Class: Insecta	Class: Insecta
Order: Diptera	Order: Diptera
Family: Culicidae	Family: Culicidae
Subfamily: Culicinae	Subfamily: Culicinae
Genus: *Aedes*	Genus: *Aedes*
Species: *aegypti*	Species: *albopictus*

1.1.2 Description and life cycle

Ae. aegypti and *Ae. albopictus* are identified by having white marks on the abdomen and legs and can be differentiated by the markings on the thorax of the individuals. *Ae. aegypti* has a lyre-shaped white marking (Figure 1.1) while *Ae. albopictus* has a straight line that starts from the head that goes two thirds on the thorax (Figure 1.2). According to Klowden (1993), both species of *Aedes* mosquitoes, occupy similar ecological niches as they are sympatric species. *Ae. albopictus* has a high preference for human residential area with a lot of vegetation, therefore, is more abundant in a rural area (Chen et al., 2006). Meanwhile, Sucharit et al. (1978) and Foo et al. (1985) demonstrated that *Ae. aegypti* prefer less vegetation and indoors (cited in Rozilawati et al., 2007)). Lenhart et al. (2005) and Perich et al. (2003) found that both *Ae. aegypti* and *Ae. albopictus* tends to breed in any variety of water-holding containers with *Ae. albopictus* has natural containers as their oviposition preferences.

In the case of ovitrap methods that are widely used by mosquito researchers, Rozilawati et al. (2007) found that *Ae. albopictus* will most likely colonize the ovitraps placed in the outdoors compared to other container-breeders such as *Culex, Toxorhyncites*, *Armigeres,* and even *Ae. aegypti* due to preference of habitat.

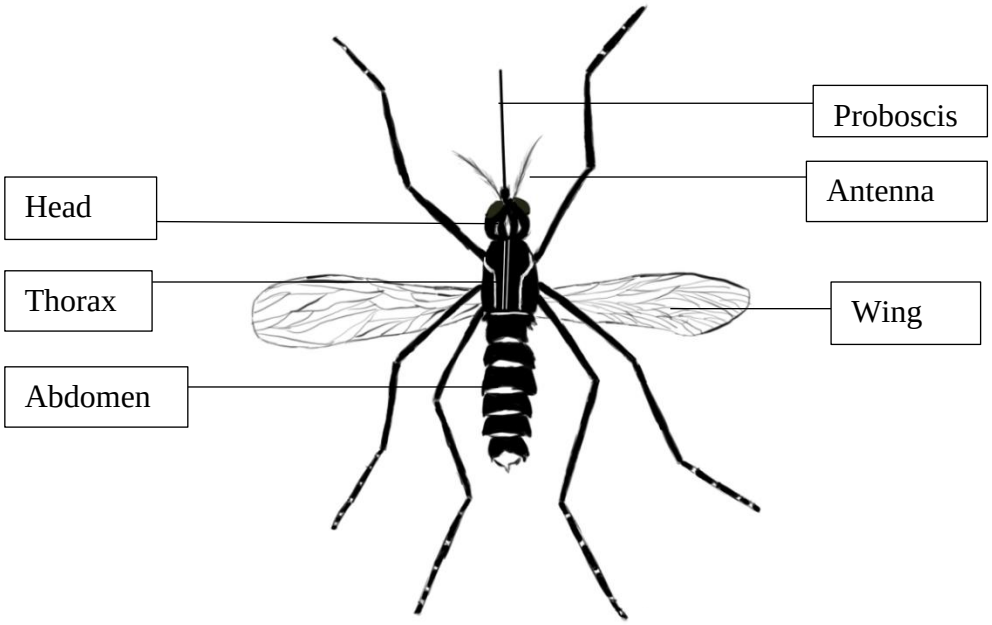

Figure 1.1: A labelled illustration of adult female *Ae. aegypti*.

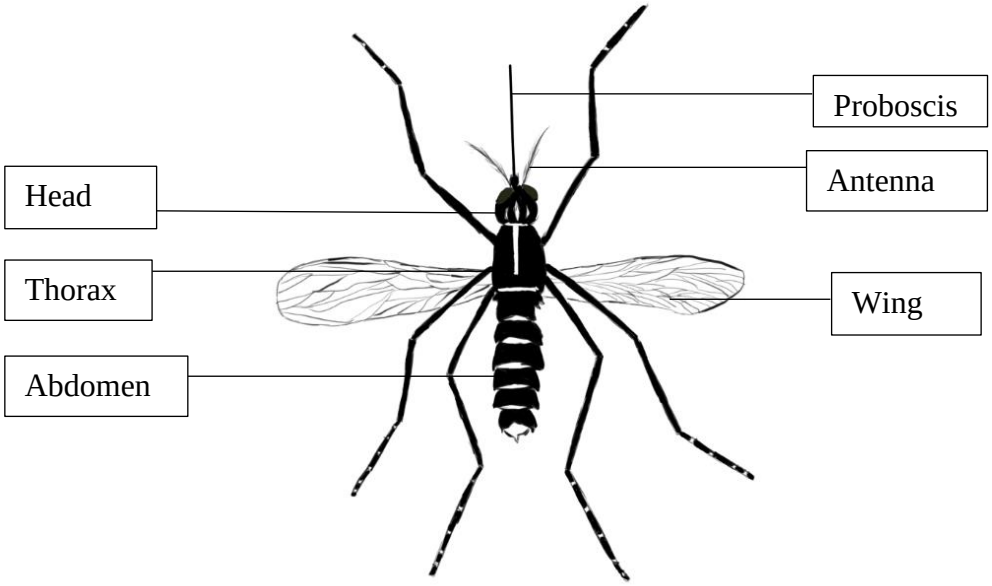

Figure 1.2: A labelled illustration of adult female *Aedes albopictus.*

A study by the Institute of Medical Research Malaysia (IMR) has demonstrated that a female *Aedes* mosquito is ready to lay eggs four to five days after copulation (Vythilingam et al., 1992). The *Aedes* females usually lay multiple batches of eggs separately at different sites to ensure high survival rates. The newly laid eggs are white which turns black after a few minutes. According to the Center for Disease Control (CDC) (2020), the eggs are sticky and would stick on a substrate especially the walls of the water container. Apart from only laying eggs in clear water, Mortimer (1998), has observed the ability of *Aedes* spp. eggs to thrive in chlorinated water and the eggs can withstand desiccation for more than a year. Vythilingam et al. (1992) and CDC (2020), stated that the time taken for the *Aedes* spp. egg to emerge into an adult while experiencing full metamorphosis is 7-10 days. The average lifespan of female adult *Ae. albopictus* and *Ae. aegypti* mosquitoes are 26 days and 34 days, respectively (Vythilingam et al., 1992). The larvae and pupae of the mosquitoes live fully in water

before emerging into adults. The larvae are very active and move in a wiggling movement and are known as “wigglers” in some regions (CDC, 2020).

A seasonal abundance study of *Aedes* spp. done by Rozilawati et al. (2007) demonstrated that rainfall assists with the outdoor breeding of the species due to the increase of breeding sites from the increase of water collecting surfaces. However, Foo et al. (1985) and Hornby et al. (1994) discovered that heavy rainfall and windy weather could negatively affect the number of eggs and larvae due to the risk of eggs and larvae being washed off from overflowing containers as well as causing a difficulty of flight of female *Aedes* spp. to reach the suitable breeding sites (cited by Rozilawati et al., 2007). Similarly, a slight increase in temperature could also increase the abundance of the mosquito populations by speeding up the metabolism of female *Aedes* spp. in digesting blood and inducing them to feed more. The heat in the water could also shorten the maturation duration of the larvae, although a continuous increase in temperature could dry out the water container and also be inconvenient to larvae growth because it has exceeded the optimum temperature. In conclusion, Rozilawati et al. (2007) stated that mosquitoes can complete a cycle in a week when rain is abundant, and the optimum temperature is achieved.

1.1.3 *Aedes* spp. as vectors of infectious diseases

The *Aedes* mosquitoes are known to be the main vectors of several important arboviral diseases including dengue, chikungunya, and Zika. Female mosquitoes are known to feed on the blood of vertebrates for the eggs’ development and are highly anthropophilic. It is adapted mostly to feed on the human host hence becoming an excellent vector of arboviral diseases. Dengue viruses are transmitted to humans through the bites of infective female *Aedes* mosquitoes that feed on infected blood.

The virus infects and replicates in the midgut epithelial cells. Then the replicated virus travels to the salivary glands via the haemolymph. Hosts are infected when the mosquito feeds with an infected salivary gland (Mukherjee et al., 2019). According to CDC (2020), the symptoms of dengue are headaches, fever, joint pain, rash, fatigue, loss of appetite, and low white blood count. Prolonged or untreated dengue may escalate and develop into fatal severe dengue (CDC, 2020).

Apart from dengue, *Aedes* spp. are also known to spread several arbovirus diseases making the species a medically important vector. According to Dick et al. (1952) and Macnamara (1954) Zika virus was found in 1947 and the first human infection case was described in 1954 (as cited by Agumadu & Ramphul, 2018). After its first major outbreak on the Island of Yap, Micronesia in 2007 (Duffy et al., 2009), there are reports of multiple outbreaks worldwide and the latest outbreak was in the United States of America reported between 2015-2016 (CDC, 2019). According to the CDC (2019), the symptoms of Zika include fever, joint pain, headache, redness of eyes or conjunctivitis, and rash. Therefore, Zika has a high risk of being misdiagnosed since they are similar to that of dengue and chikungunya. Misdiagnosis can cause complications in pregnant women as it will cause brain malformations including microcephaly (CDC, 2019). Based on the study by Hamel et al. (2015), Zika infection from vector to host is also similar to dengue. The infection is transmitted from one vertebrate to another vertebrate via *Aedes* spp. bites. Ten days after ingesting the infected blood from another host, the saliva of the vector becomes infected by the virus and poses a risk of spreading the disease to another host (Hamel et al., 2015). While Zika is not as deadly as dengue, preventive measures against the vector are important as it poses a risk to the overall quality of life of individuals and society (Agumadu & Rampul, 2018).

Chikungunya is another disease that is transmitted by *Aedes* spp. According to the World Health Organization (WHO) (2020), the disease was described for the first time in 1952 in Tanzania and the name was derived from a word in the local language that describes the appearance of someone suffering from joint pain, one of the main symptoms of the disease. Other symptoms of chikungunya include fever, fatigue, rash, headache, nausea, and muscle pain. Similar to dengue and Zika, chikungunya is also a disease that is often misdiagnosed which may harm the patients, especially among the elderly because it could also cause early death (WHO, 2020).

1.1.4 Dengue cases in Malaysia

As a significant public health problem in Malaysia, dengue prevalence monitoring has always been a top priority of the Ministry of Health (MOH). The statistics from the MOH (2015), had shown that the incidence was 396 cases per 100 000 population with Selangor having the highest number of cases. In the next year, there was a reduction in the reported dengue cases by 16.1% making the incidence cases to be 328 cases per 100 000 population. The state with the highest number of reported cases was also Selangor (MOH, 2016). Subsequently, in 2017, there was a 17.2% reduction of dengue cases compared to the previous year and the dengue outbreak localities also reduced from 698 localities in August to 314 localities by the end of the year (MOH, 2017). The number of reported cases from that year was 83 849 and compared with the previous years, which were 120 836 cases and 101 357 cases in 2015 and 2016, respectively, indicating improvements in the quality of health of Malaysians at that time. The number of dengue cases was further reduced in 2018 with 80615 reported dengue cases (MOH, 2018) making it 245.7 per 100 000 population. However, there was a 61.4% increase in dengue cases with 390 cases per 100 000

population (130 101 cases) with the highest number of cases (36% of total cases) is from Selangor and Kuala Lumpur (MOH, 2019).

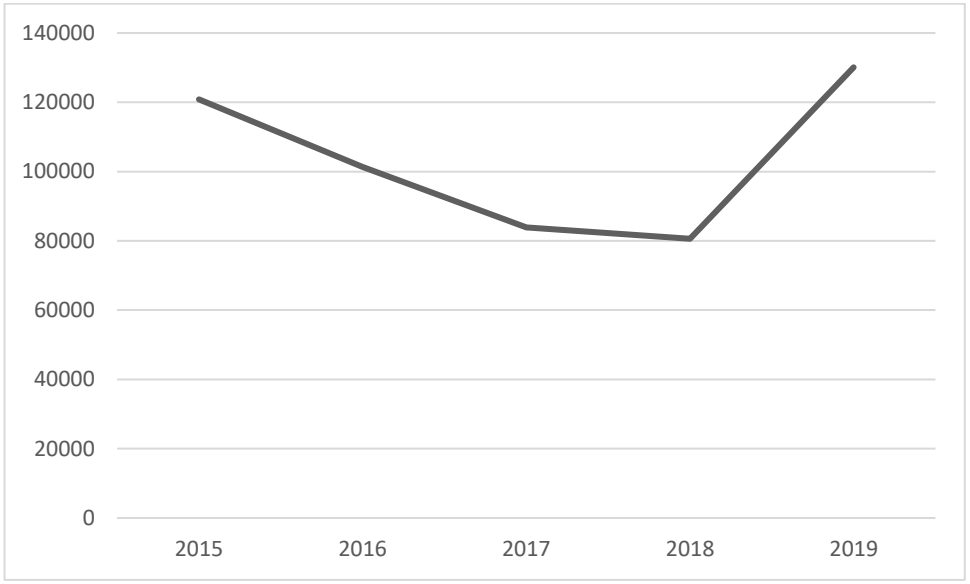

Figure 1.3: Reported dengue cases in Malaysia 2015-2019. (Figure adapted from the Annual Report of MOH, 2015; 2016; 2017; 2018; 2019)

From the year 2015, the number of deaths recorded was 336, which reduced to 237 deaths in the next year. The number of mortalities continued to drop from 177 in 2017 to 147 in 2018. However, the number rose to 182 deaths in 2019 (MOH, 2015; 2016; 2017; 2018; 2019).

1.1.5 *Aedes* mosquito control and preventions in Malaysia

Mosquito population control in Malaysia is achieved from a collective effort from various task forces that was formed in 2014 that includes the local authorities, Ministry of Housing and Local Government, Ministry of Education, Ministry of Higher Education, Ministry of Human Resources, Ministry of Internal Affairs, Ministry of Defence, Ministry of Works, and Ministry of Communication & Multimedia. MOH (2017) also reported that there were major cleaning and environmental management procedures that involved intense solid waste management by the local authority as well as cleaning campaigns in that year covering 241 locations.

Another method of control that was established in Malaysia is COMBI, which stands for Communication for Behavioural Impact. The concept of COMBI makes use of the social mobilization and communication strategy to instil healthy behaviour changes within the communities (Gombak District Health Office, GDHO, 2020). The goal of the COMBI program is to empower communities to change present behaviour to a new behaviour to reduce the incidence of infectious diseases and to encourage and enhance inter-agency collaboration to prevent and control the incidence of infectious diseases. COMBI project targets communities with several criteria, which consist of communities from areas that volunteered for the program, the area should have a high entomology count, and the area should not have incident cases, repeated outbreaks or uncontrollable epidemics. The program is implemented by creating awareness in the communities towards health problems and preventive measures to avoid them. The strategies of the COMBI implementation include the advocacy of the administration and public relations organizations, such as forming a local special committee from the

already available COMBI committee, which handles meetings and discussions regarding the behavioural change plans, community mobilization, advertising, interpersonal communication, and setting up promotion service centres (GDHO, 2020). According to Baharudin (2017), there are three aspects to focus on in this COMBI program with regards to dengue control, which are knowledge, attitude, and practices. The communities should know and learn about dengue fever especially the biology of the vector and its breeding habits as the cause of the disease, as well as law enforcement related to the control and prevention of dengue fever so that proper prevention practices can be carried out within the communities. In short, by having knowledge about the causes and methods of prevention of the disease, there will be a change in attitude and opinions towards the habits that have been established that are harmful and help to spread the diseases. As a result, necessary actions are introduced within members of the communities in helping to prevent the spreadable diseases (Baharudin, 2017).

There are many mosquito adulticides used in dengue vector control in Malaysia, which are either used as active ingredients in household insecticides or issued by the MOH during occasional fogging activities. The insecticides are known to be deltamethrin, permethrin, alpha-cypermethrin, cyfluthrin, lambda-cyhalothrin, malathion, resigen, and sumithion (Wan et al., 2010; Loke et al., 2012; Ishak et al., 2015; MOH, 2017; 2020). Pyrethroid insecticides such as alpha-cypermethrin and deltamethrin have residual effects up to 4-5 months (Lukwa et al., 2012; Uragayala et al., 2015) and lambda-cyhalothrin has a residual effect of 6 months (Lukwa et al., 2012).

1.2 Types of insecticides

There are a few groups of insecticides, each has different active ingredients that make up its chemical composition and mechanism. They are known as carbamates, neonicotinoids, organochlorides, organophosphates, and pyrethroids (Environmental Protection Agency, EPA, 2019).

.

1.2.1 Pyrethrin or pyrethroids

Pyrethrin insecticides are originated from a pyrethrum powder, a species of chrysanthemum flower from the Dalmatia region, *Chrysanthemum cinerariaefolium* (Katsuda, 2012). In nature, pyrethrins are a self-defence mechanism of the plant against herbivores. Synthetic pyrethrins, pyrethroids are made because natural pyrethrins are sensitive to sunlight and oxygen, making the components degrade easily (Matsuda, 2011). According to Clark and Symington (2012), the function of at least one type of voltage-gated sodium channel (VGSC) can be disrupted by any pyrethroids. Pyrethroids are safe for humans and have less toxicity to other mammals but high insecticidal activity due to poor dermal absorption and high lipophilicity making it difficult to be absorbed orally due to aqueous suspension. The knockdown effect such as rapid paralysis is known to be caused by pyrethroids due to prolonged activation of VGSC that leads to blocking of the conduction of action potential (Clark & Symington, 2012). Sonderlund (2020) stated that the amino acids polymorphisms of the VGSC in organisms determined the sensitivity and the resistance of the channels to pyrethroids in mammals and insects, respectively.

Pyrethroids can be classified into two types, which is type I pyrethroids and type II pyrethroids, according to the presence of α-cyano group in their chemical structure (Nasuti et al., 2003). According to Khambay and Jewess (2005), the type of

pyrethroids differ in poisoning symptoms and preferences in binding to channels of different orientations. Type I pyrethroid prefers binding to closed channels and has a rapid symptoms onset at low levels. This type of pyrethroid produces results of low killing with high recovery rate compared to type II, which has high killing and low recovery rates despite having slow symptoms onset. Type II pyrethroids also prefer to bind to open sodium channels (Khambay & Jewess, 2005) Some examples of pyrethroid insecticides commonly used are permethrin and lambda-cyhalothrin.

1.2.3(a) Permethrin

Permethrin is also known as the common name for the compound 3-phenoxyl (1RS)–cis,trans–3-(2,2-dichlorovinyl)-2,2-dimethylcyclopropanecarboxylate. It is a contact insecticide widely used against insects in the Order Lepidoptera, Coleoptera, and Diptera (Swaine & Tandy, 1984; Naumann, 1990). It is a type I pyrethroids due to the lack of α-cyano group in its composition. Permethrin would cause repetitive discharges of axons in the nerves and before knockdown, hyperactivity is often observed in the insects (Khambay and Jewess, 2005). Permethrin does not harm mammals due to low toxicity, however, slight eye and skin irritant is expected when in contact with the insecticides. Permethrin is heat-stable although some degradation due to light exposure is observed. Permethrin often comes in emulsifiable concentrates, wettable powders, fogging solutions, dust and ultralow-volume (ULV) formulation (Swaine & Tandy, 1984).

1.2.3(b) Lambda-cyhalothrin

Lambda-cyhalothrin is a synthetic pyrethroid insecticide with the molecular formula $C_{25}H_{19}ClF_3NO_3$. Like other pyrethroid type insecticides, the compound attacks the nervous system of insects and can cause paralysis on top of possessing the ability to repel insects (Agency for Toxic and Disease Registry, ATSDR, 2003). It is a type II pyrethroid that contains α-cyano group in its composition (Khambay and Jewess, 2005). When in contact with mammals, spasms and salivation can be observed (ATSDR, 2003). The chemical would bind preferentially to open channels and disrupts the synapse conduction (Khambay & Jewess, 2005).

1.3 Insecticide resistance in mosquitoes

According to Martins and Valle (2012), when an organism population develops the ability to survive a lethal dosage of a given compound that is known to negatively impact several individuals that are vulnerable to the compound, the population is known to have developed a resistance against the compound. Resistance is usually based on the genetic variability of an organism (Martins & Valle, 2012). Lethal doses of insecticides could induce resistance in the insect once the insect has been exposed to the insecticide for a certain period of time. As stated by Roush and Daly (1990) and also by Taylor and Feyereisen (1996) with basing it on the biochemical or physiological properties of the insects such as either mechanism of decreased response or mechanisms of decreased exposure, the insect could adapt to survive the lethal dose of insecticides. From a study by Kasai et al. (2014), there are three main mechanisms causing resistance to insecticides; the first one is when the sensitivity of the target site of the insect is reduced, secondly, an occurrence of alteration of the insecticides

cuticles causing difficulty in penetration of the insecticides into the systems of the insects, and thirdly is the increase of detoxification enzyme level or activity. As for the cuticle alterations, Martins and Valle (2012) presumed that it is not caused by high levels of resistance, rather a synergistic interaction with other mechanisms.

1.3.1 Metabolic resistance

Metabolic resistance is defined as the prevention of insecticides to the target site in the nervous system with the ability of the detoxifying enzymes to metabolizes the insecticides. It occurs when there is gene amplification or expression activation thus increases the number of molecules available and the alteration or mutations of the gene that codes for enzyme coding to increase the efficiency of the metabolization. Hemingway and Ranson (2000) and Montella et al. (2007) identified that there are three important enzyme superfamilies involved in metabolic resistance, which are esterases, glutathion-s-transferases and multi-function oxidases P450 (cited by Martins and Valle, 2012). Such alterations and mutations could cause different enzymes to produce the same metabolites and therefore lead to cross-resistance among different classes of insecticides (Ranson et al., 2011). Unfortunately, only a few specific metabolic pathways involved or the location of the resistance in the insect are reported despite its molecular study has been carried out extensively. Especially in *Ae. aegypti* populations, the metabolic resistance can still vary during various phases in a day. In a study by Prapanthadara et al. (2002), there is DDT resistance in *Ae. aegypti* due to the increased DDTase activity and cytochrome P450 content, and it is assumed that the permethrin resistance in one of the strains involved a non-metabolic knockdown resistance *(kdr)* mechanism. The study also discovered that there is no evidence of cross-resistance of DDT to pyrethroids, although, the mechanism of pyrethroid

resistance based on *kdr* is involved in the increase of DDT resistance (Prapanthadara et al., 2002).

1.3.2 Behaviour resistance

There is also the case of behavioural resistance where Sparks et al. (1989) described the insects instinctively avoid the insecticide, which presumably stemmed from genetic inheritance. According to Ranson et al. (2011), if the selection of insecticide is based on physiological features, behavioural changes would negatively impact an insecticide's efficiency because there is little to no contact from the insects to insecticides.

1.3.3 Knockdown resistance (*kdr*)

In the case of pyrethroid and DDT resistance, the commonly understood mechanism is the VGSC mutations caused by amino acid substitution (Kasai et al., 2014). The mechanism is known as the knockdown resistance (Soderlund & Knipple, 2003). Furthermore, metabolism mediated insecticide resistance is also considered as the key mechanism of insecticide resistance in insects (Hemingway et al., 2004; Marcombe et al., 2009). The mechanism for this is called the target site or phenotypic resistance, which happens when there are modifications on the insecticide binding site to reduce and inhibit the insecticidal effects on the insect. According to Martin and Valle (2012), the target sites are identified to be the enzyme acetylcholinesterase, gama-aminobutyric acid, nicotinic acetylcholine receptors, and VGSC.

1.4 VGSC and *kdr* mutations

VGSC is one of the active components in the cell membrane that is responsible for selective permeability of a substance into and out of the cell, specifically known to generate an action potential in a nervous system of an organism (Catterall, 2000; Randall et al., 2001; Alberts et al., 2002). VGSC functions with the depolarization of the membrane creating a cell action potential with a more positively charged internal side, activating and opening the channel to the stimulus that causes the depolarization. This causes an influx of sodium ions (Na^+) to the cell further making the depolarization of the membrane more intense. As the membrane reaches the equilibrium potential of Na^+, the channel is deactivated. Within a few milliseconds before closing the channel, a conformation to halt the ion influx into the cell is assumed. The channel is closed because of the repolarization of the membrane. Randall et al. (2001) and Catterall (2003) stated that the process of opening and closing of VGSC is simultaneous with other channels such as the voltage-gated calcium channels (VGCC) and the voltage-gated potassium channels (VGPC) that restore the original electric potential of the cells (cited by Martins and Valle, 2012). A disruption of this process causes nervous damages to the organisms, possibly paralysis and even death, which is the mechanism of some insecticides.

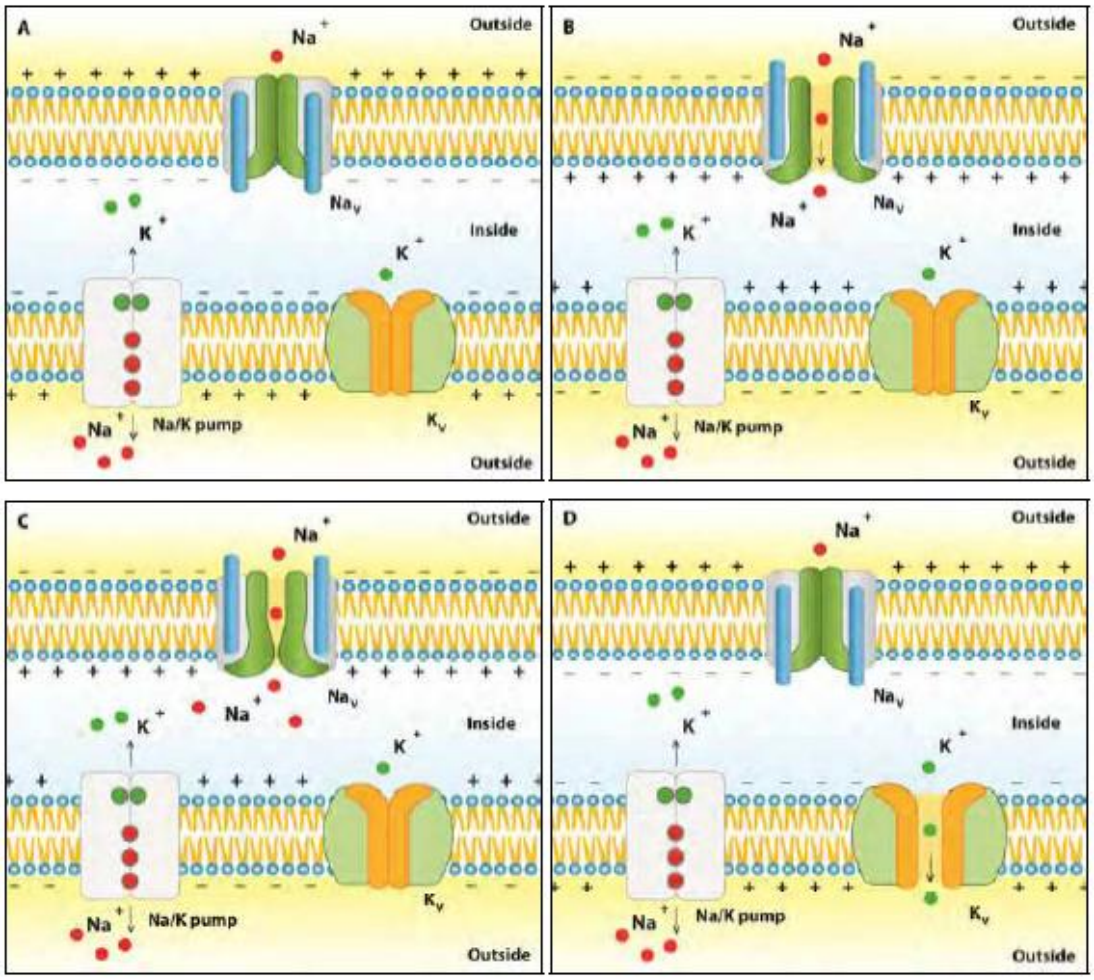

Figure 1.4: Action potential mechanism of VGSC

(A) The membrane is in a resting potential stage with the VGSC closed. (B) An influx of positive charge in the cell due to the sodium ions and continues to increase until equilibrium potential is reached, the channel is then deactivated but the channel has not yet closed. (C) The potassium ions exit the cell through VGPC, therefore shows that the channels work simultaneously in (D) where the VGSC is now closed, preventing sodium ions from entering while VGPC opens to let the potassium out, making the polars inside and outside the cell to return to resting potential. (Figure adopted from Martins and Valle, 2012)

Knockdown resistance *(kdr)* was found in the early 1950s where a resistant strain of houseflies was observed soon after the introduction of the insecticide DDT. According to Busvine (1951), Harrison (1951), and Milani (1954), instead of suffering from paralysis and death like the rest of the insects towards DDT, the resistant house flies were only impeded by a temporary paralysis and recovered completely (cited by Martins and Valle, 2012). Since then, *kdr* was frequently found in insects against DDT even after the introduction of pyrethroids which Hemingway and Ranson (2000), assumed to be caused by previous DDT pressure and cross-resistance between DDT and pyrethroids. The first *kdr* mutation founded to express the *kdr* trait in insects was a substitution of leucine to phenylalanine (L1014F) in the IIS6 segment of the VGSC of a housefly. Later, the homologous site 1014 was explored for various amino acid substitution in various insects with *kdr* traits (Martins and Valle, 2012). However, unlike other insects and even different species of mosquitoes, *Ae. aegypti* mosquitoes, the *kdr* mutation is not found at the widely identified homologous site 1014, instead, there were two homologous sites; substitution of valine to glycine at the 1016 site (V1016G) in the IIS6 segment and substitution of phenylalanine to cysteine in the 1534 site (F1534C) of the IIIS6 segment (Brengues et al., 2003; Saavedra-Rodriguez et al., 2007; Martins et al., 2009; Harris et al., 2010).

1.5 The study of insecticide resistance and susceptibility in Malaysia

Vontas et al. (2012) compiled the overall resistance status of *Ae. albopictus* and *Ae. aegypti*. Up to the year 2012, it was found that *Aedes albopictus* adults are not resistant to the pyrethroids tested; deltamethrin and permethrin as well as propoxur and malathion, which are a carbamate and organophosphate, respectively. Vontas et al. (2012) speculated that the *Aedes* species that live outdoors are not frequently exposed to the insecticides, resulting in more insecticide susceptibility. As for *Aedes aegypti,* there is an increase in insecticide resistance and molecular research had been done to understand the cause such as mutations in VGSC genes such as V1016G and F1534C, and the genes that encode cytochrome P450s that plays an important role in electron transfer chain in an organism. Based on the two molecular investigations, adult *Ae. aegypti* shows resistance to pyrethroids due to the ability of the mosquitoes to metabolise and detoxify pyrethroids developed as the result of the mutations. Davies et al. (2007) stated that cross-resistance from the mutations from the VGSC target site also contributed to the DDT resistance (cited in Vontas et al., 2012). There is also an expression of several detoxification genes, the glutathione transferases in the DDT resistant adult *Ae. aegypti.*

The most used insecticides in Malaysia to control adult mosquitoes in Malaysia are malathion and permethrin. According to the study by Chan et al. (2011), the *Ae. albopictus* population in two dengue hotspots in Penang were tested against pyrethroid insecticides: permethrin and deltamethrin. The methods of testing used were WHO test kit bioassay and topical application. With both methods, the *Ae. albopictus* in each population showed little to no resistance to the pyrethroids and deltamethrin was found to be more lethal than permethrin by having a higher knockdown number and mortality rate with a lower dose (Chan et al., 2011).

A study done by Ishak et al. (2015), on *Ae. aegypti* and *Ae. albopictus* resistance in Peninsular Malaysia demonstrated several findings. The populations used in this study were collected from four states, Penang, Kelantan, Kuala Lumpur, and Johor. From the study, all populations are resistant to both type I and type II pyrethroids used as well as DDT. Ishak et al. (2015) also studied the pattern of resistance between the species. There are differences observed in the susceptibility of *Aedes* spp. to pyrethroids because *Ae. albopictus* were recorded to be fully susceptible when compared with *Ae. aegypti*. However, the populations in the urban area are more resistant due to the frequent exposure to pyrethroids. In contrast, both species show high resistance towards DDT.

To extend the research on susceptibility status of *Aedes* spp. beyond mortality rates, Ishak et al. (2015) also studied the correlation of genotypic and the phenotypic expression of the resistance towards pyrethroids and found out that except for some locations where the samples were taken, the mutation in F1534C genes heavily contributed to the resistance to both type I and type II pyrethroids in Penang but not in other regions. Also, a mutation in the V1016G gene had no significant correlation with insecticide resistance. However, a haplotype association study was conducted, and it was found that *kdr* mutations in V1016G, specifically 1016G allele showed additive properties that increased the resistance of the mosquitoes that already possessed the mutated 1534C allele (Ishak et al., 2015)

1.6 Rationale of the study (Research question)

Ae. aegypti and *Ae. albopictus are* the vectors for various tropical diseases including dengue, chikungunya, and Zika. One of the methods to curb the virus from spreading through mosquitoes is through the usage of insecticides. However, prolonged insecticide usage will affect the susceptibility of the *Aedes* spp. mosquitoes towards the insecticides, especially in dengue-endemic areas where fogging is frequently carried out. Thus, susceptibility and resistance status are needed to be checked more often especially in the hotspot dengue areas and the areas that received regular fogging treatment.

There are numerous studies of susceptibility and resistance status of *Aedes* spp. population that was done in dengue hotspot areas in Malaysia. However, it is also important to study the resistance and susceptibility statuses of the population within specific areas in order to control the vectors locally.

The study was conducted in Taman Harmoni, Panji in Kota Bharu because there is a concern regarding the frequency of fogging and ULV treatment prior to the study in the area. Fogging and ULV treatments are often done when there is a recent dengue case reported. It is important to know the composition of the *Aedes* spp. in that area as well as the susceptibility and resistance status of the population to control the spread of dengue among the residents in that area. Apart from fogging treatment, the residents were also observed to use household insecticides that had pyrethroids as active ingredients, which could affect the controlling methods for the area due to the resistance developed in the species.

1.7 Objectives of the study

1.7.1 General objective

To study the susceptibility and resistance status in *Aedes* spp. to pyrethroid group insecticides in Kota Bharu, Kelantan.

1.7.2 Specific objectives

1. To determine the *Aedes* spp. composition in Panji and Kubang Kerian, Kota Bharu.
2. To evaluate the susceptibility and resistance phenotype of *Aedes* spp. mosquitoes against pyrethroids.
3. To evaluate the susceptibility and resistance genotype of *Aedes* spp. mosquitoes against pyrethroids on a molecular level.
4. To compare the susceptibility and resistance status of *Aedes* spp. mosquitoes against pyrethroids in Panji and Kubang Kerian, Kota Bharu.

CHAPTER 2

MATERIALS AND METHODS

2.1 Materials

2.1.1 Mosquito samples

Mosquitoes used in this study were the F1 generation of field-caught strains of *Aedes* spp. The eggs and larvae collected from sampling sites were reared in the insectarium until the adult stage. Female mosquitoes, aged 4-5 days old, fed only with 10% sucrose solution were used in this study.

2.1.2 Reagents and chemicals

All reagents and chemicals used in this study were electrophoresis or molecular biology grade as listed in Appendix A.

2.1.3 Laboratory equipment and consumables

Laboratory equipment and consumables used in this study are listed in Appendix B and C, respectively.

2.1.4 Kits

INNUPrep DNA Mini Kit, Kaneka Easy DNA, and Nucleospin Tissue were used for DNA extraction. MyTaq Mix (Bioline, UK) was used for PCR amplification.

2.1.4(a) INNUPrep DNA mini kit

The extraction kit from Analytik Jena (Germany) consisted of lysis solution TLS, binding solution TBS, lyophilized proteinase K, concentrated washing solution HS, concentrated washing solution MS, elution buffer, spin filter, receiver tubes and elution tubes.

Proteinase K solution was prepared by diluting and mixing lyophilized proteinase K well in 1.5 mL deionized distilled water in the respective tubes. Washing solutions were prepared by diluting the concentrated HS and MS with 15 mL and 35 mL of absolute ethanol, respectively and then mixed thoroughly.

2.1.4(b) Easy DNA extraction kit

The Easy DNA extraction kit was from Kaneka (Japan). The kit consisted of a vial of 2 mL of alkaline solution A and a vial of 0.28 mL of solution B.