



Faculty of Medicine and Health Sciences

**Detection of Dengue Virus Serotypes in Aedes Mosquito Population from
Sibu and Miri Divisions of Sarawak using Reverse Transcription
Polymerase Chain Reaction (RT-PCR)**

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Detection of Dengue Virus Serotypes in Aedes Mosquito Population from
Sibu and Miri Divisions of Sarawak using Reverse Transcription Polymerase
Chain Reaction (RT-PCR)

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DECLARATION

I hereby declare this particular thesis entitled 'Detection of Dengue Virus Serotype in Aedes Mosquito Population from Sibuan and Miri Divisions of Sarawak using reverse transcription polymerase chain reaction (RT-PCR)', is solely the result of my experimental research, effort and hard work. This thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Dengue has been a public health concern for many years in Malaysia. Having knowledge on the current circulating dengue serotype and population of vector mosquito is key in controlling outbreaks and future outbreak predictions. The current study reports the first study on detecting dengue virus serotypes in the *Aedes* mosquito population in Sibu and Miri divisions of Sarawak. Mosquito samples were collected at selected localities from September 2016 to December 2017. Localities were selected randomly mainly focussing on urban residential areas. The mosquitoes collected comprised of two types namely the field-caught adults and immatures collected from artificial and natural water containers. Collected mosquitoes were identified to species level and screened for presence of dengue virus and serotyped using conventional reverse transcription polymerase chain reaction (RT-PCR) and semi-nested PCR. Dengue virus serotype 2 (DENV-2) was identified in 3 pools of field-caught female *Aedes albopictus* adults that were collected from Jalan Tong Sang, Sibu, Sibu Lake Garden, and Taman Ceria, Permyjaya, Miri, respectively. DENV-2 was also detected in one pool of adult male *Aedes albopictus* that had emerged from immatures collected from Taman Ceria, Permyjaya, Miri. The findings in this study revealed that *Aedes albopictus* was the main species colonizing the study areas, and the current circulating dengue virus serotype is DENV-2. This study also reports the first natural evidence of transovarial transmission of dengue in the natural population of *Aedes albopictus* within the study area. This latest report may aid in providing information on establishing strategies in an effective vector control and, also to control any current and future outbreaks if necessary.

Keywords: *Aedes albopictus*, dengue virus, RT-PCR, dengue virus serotype 2, field-caught adult mosquitoes, immature mosquitoes, transovarial transmission.

Pengesanan Serotip Virus Denggi dalam Populasi Nyamuk *Aedes* dari Bahagian Sibuan dan Miri Sarawak menggunakan Reaksi Berantai Polimerase Transkripsi-Berbalik (RT-PCR)

ABSTRAK

Denggi telah menjadi kebimbangan kesihatan awam selama bertahun-tahun di Malaysia. Pengetahuan mengenai virus denggi (DENV) yang berkitar dan populasi nyamuk vektor adalah penting dalam mengawal wabak dan ramalan tentang wabak. Kajian ini melaporkan kajian pertama mengenai pengesanan serotip virus denggi dalam populasi nyamuk *Aedes* di bahagian Sibuan dan Miri di Sarawak. Sampel nyamuk dikumpulkan di lokasi terpilih dari September 2016 hingga Disember 2017. Lokaliti dipilih secara rawak terutamanya pada kawasan kediaman bandar. Nyamuk yang dikumpulkan terdiri daripada dua jenis iaitu nyamuk dewasa dan jentik-jentik yang dikutip dari bekas air buatan serta bekas air semula jadi dari kawasan persampelan. Nyamuk yang terkumpul telah dikenalpasti ke tahap spesies dan disaring untuk kehadiran virus denggi menggunakan RT-PCR konvensional. Denggi serotip 2 (DENV-2) telah dikenalpasti di dalam 3 kumpulan nyamuk *Ae. albopictus* betina dewasa yang ditangkap dari Jalan Tong Sang, Sibuan, Taman Tasik Sibuan, dan Taman Ceria, Permyjaya, Miri. DENV-2 juga dikesan dalam satu kumpulan nyamuk jantan *Ae. albopictus* yang membesar dari peringkat pramatang yang dikumpul dari Taman Ceria, Permyjaya, Miri. Penemuan dalam kajian ini mendedahkan bahawa *Ae. albopictus* adalah spesis utama nyamuk di kawasan kajian, dan serotip virus denggi semasa ialah DENV-2. Kajian ini juga membuktikan penyebaran virus denggi semula jadi secara transovari dalam populasi *Ae. albopictus* di kawasan kajian. Laporan

terkini ini boleh membantu dalam membentuk strategi dalam kawalan vektor yang efektif dalam mengawal wabak denggi semasa dan dimasa akan datang.

Kata kunci: *Ae. albopictus*, virus denggi, RT-PCR, virus denggi serotip 2, nyamuk dewasa tangkapan dari lapangan, nyamuk pramatang, penyebaran secara transovari

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LIST OF ABBREVIATIONS

%	percentage
°C	degree Celsius
μL	microliter
A.D	<i>Anno Domini</i>
<i>Ae.</i>	<i>Aedes</i>
AMCA	American Mosquito Control Association
bp	basepair
BSC	biosafety cabinet
CDC	Center for Disease Control
c-DNA	complementary deoxyribonucleic acid
C/prM	capsid and pre-membrane
DENV	dengue virus
DF	dengue fever
DHF	dengue haemorrhagic fever
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
DSS	dengue shock syndrome
ELISA	enzyme-linked immunosorbent assay
Ig	immunoglobulin
kb	kilobase
mg	milligram
MgCl ₂	magnesium chloride

min	minutes
MIR	minimum infection rate
M-MLV	Moloney Murine Leukemia Virus
mm	millimetre
nm	nanomolar
PAP	Peroxidase-antiperoxidase
PCR	polymerase chain reaction
RNA	ribonucleic acid
Rnasin	rnase inhibitor
rpm	revolutions per minute
RT	reverse transcription
<i>sp.</i>	species
TBE	tris-borate-edta
WHO	World Health Organization

CHAPTER 1

INTRODUCTION

1.1 Background

Dengue has been one of the most important public health concerns worldwide and has caused significant number of deaths every year. According to the World Health Organization (2017), approximately half of the global population are at risk of dengue infection in over 125 endemic countries. Dengue is an acute systemic form of a viral disease which has established itself both epidemically and endemically which can lead to a range of clinical manifestations from mild fevers to severely fatal cases (Bhatt et al., 2013). Dengue manifest in two forms: dengue fever (DF) and dengue haemorrhagic fever (DHF). The World Health Organization estimates that 50 to 100 million DF cases occur worldwide, including 500,000 DHF cases yearly. DF is characterized by an acute and sudden onset of symptoms including fever, body aches, and skin rashes (CDC, 2012). DHF/DSS is a much more severe and chronic form of dengue manifestation. It is characterized by onset of symptoms including severe pain, bleeding from nose and gums, and vomiting blood. (CDC, 2012). Dengue infection is caused by the dengue virus (DENV), a single-stranded RNA virus belonging to family and genus of *Flaviviridae* and *Flavivirus* respectively, similar to yellow fever virus, West Nile virus, chikungunya and Zika virus. There are four serotypes of DENV; DENV-1, -2, -3, and -4. All of the serotypes can be found in all dengue endemic and epidemic areas around the world. Malaysia has all four serotypes of DENV making it hyperendemic for dengue (Cheah et al., 2014; Mohd-Zaki et al., 2014).

DENV is a vector-borne virus. The main vector for DENV is the *Aedes* mosquitoes primarily

Ae. aegypti and *Ae. albopictus*. Both mosquitoes can be found worldwide in most tropical and sub-tropical areas. These mosquitoes transmit dengue from humans to humans through blood-feeding, therefore being the vector of the virus. This mode of transmission is called the horizontal transmission. Transmission of dengue can also occur in the mosquito population itself. This mode of transmission is called the vertical or transovarial transmission, where the virus is inherited by the progeny from the DENV infected female *Aedes* mosquito (Martins et al., 2012). Furthermore, vertical transmission of DENV does not only occur in mosquitoes but also in human beings. There were evidences of DENV being transmitted to infants from their mother during late stages of pregnancy (Chotigeat et al., 2000; Phongsamart et al., 2008; Yin et al., 2016).

1.2 Problem statement

Most of the published studies regarding the detection of DENV serotypes in *Aedes* mosquito populations were conducted in Peninsular Malaysia but to date, no such study had been published from the State of Sarawak. Furthermore, studies conducted in Sarawak mainly focuses on the distribution of *Aedes* mosquito population where some of them were conducted more than 30 years ago (Chang & Jute, 1982). Recently, an ovitrap survey study was also conducted in Sarawak, where only one species of *Aedes* mosquito was identified, which is *Aedes albopictus* (Lau et al., 2017). Studies regarding the circulation of DENV have been conducted but focuses only on clinical samples rather than on the entomological aspect (Holmes et al., 2009). Amidst the increase of dengue cases in Sarawak in recent

years, especially in Sibü and Miri, it is appropriate that this study be conducted. The outcome would greatly assist in establishing effective strategies in monitoring, controlling and preventing dengue outbreaks. It will not only benefit the community in Sarawak but may also be beneficial for the whole community in Malaysia.

1.3 Aim

This study aims to identify the current circulating dengue virus serotype in the natural population of *Aedes* mosquitoes in Sibü and Miri divisions of Sarawak.

1.4 Objectives

The objectives of this study are:

- i. To identify the species of *Aedes* mosquitoes in selected localities in Sibü and Miri divisions of Sarawak.
- ii. To detect the presence of DENV in the natural population of *Aedes* mosquitoes in the study areas.
- iii. To determine the circulating DENV serotypes in the study areas.
- iv. To determine the presence of transovarial transmission of DENV in *Aedes* mosquito population.

CHAPTER 2

LITERATURE REVIEW

2.1 Epidemiology of dengue

2.1.1 Global epidemiology of dengue

Dengue is one of the world's most infectious disease that affects approximately 400 million people worldwide, which the incidence has grown dramatically over the past decade causing half of the global population to be at risk in over 125 endemic countries (World Health Organization, 2017). Dengue is an acute systemic form of a viral disease which has established itself both epidemically and endemically leading to a range of clinical manifestations from mild fevers to severely fatal cases (Bhatt et al., 2013). The global epidemiology trends of dengue have been changing due to several factors. One of the factor is changes in demography such as population growth, economic trends in tropical countries and patterns of land usage. Another important factor is the rise in population size and density from rural to urban migration (Ferreira, 2012). This may be due to increased job opportunities making more people from rural areas moving into the urban area. Furthermore, modernisation of transportation has also contributed to changes in dengue epidemiology due to increased movement of human, disease vectors and pathogens. Constant development in public health policies and infrastructures also play a part in changing the trends of dengue epidemiology (Ferreira, 2012).

The first recorded incidence of a disease similar to dengue dated back 1800 years ago during the Chinese Chin Dynasty (265-420 A.D). The Chinese assumed that the disease was similar to the water poison disease associated with flying insects and water (Gubler, 1998). In addition, there were also reports on an illness outbreak in the French West Indies and Panama during the middle and end of the 17th century, which was likely to be dengue. Therefore, dengue could have a wide geographic distribution prior to the 18th century. Dengue only became a public health burden in Southeast Asia right after the Second World War during the late 1940s and early 1950s (Ooi & Gubler, 2009). Due to the ecological and demographic changes, it created a suitable environment and conditions for the transmission of this vector-borne disease. This was also heightened by the movement of equipment and people during the war, therefore increasing the transportation of the dengue virus vector primarily the *Aedes aegypti* mosquito.

According to the World Health Organization (WHO) (2017), only 9 countries were affected by dengue epidemics prior to the 1970s. Currently, the number of countries affected have increased significantly and at present more than 100 countries are affected by dengue epidemics (Cheah et al., 2014). These countries are mostly located in most of the globes major continents such as, South-East Asia, the Western Pacific, the Middle East, Africa, America, Europe, Australia and New Zealand (Wilder-Smith et al., 2010). Furthermore, people in the South-East Asia region and the Western Pacific region bear closely to 75% of the dengue global disease burden and the Latin America region in recent years showed an increase in amplitude and frequency in dengue epidemics (Ferreira, 2012). Figure 2.1 shows the current global dengue affected areas from the 1960's until today.

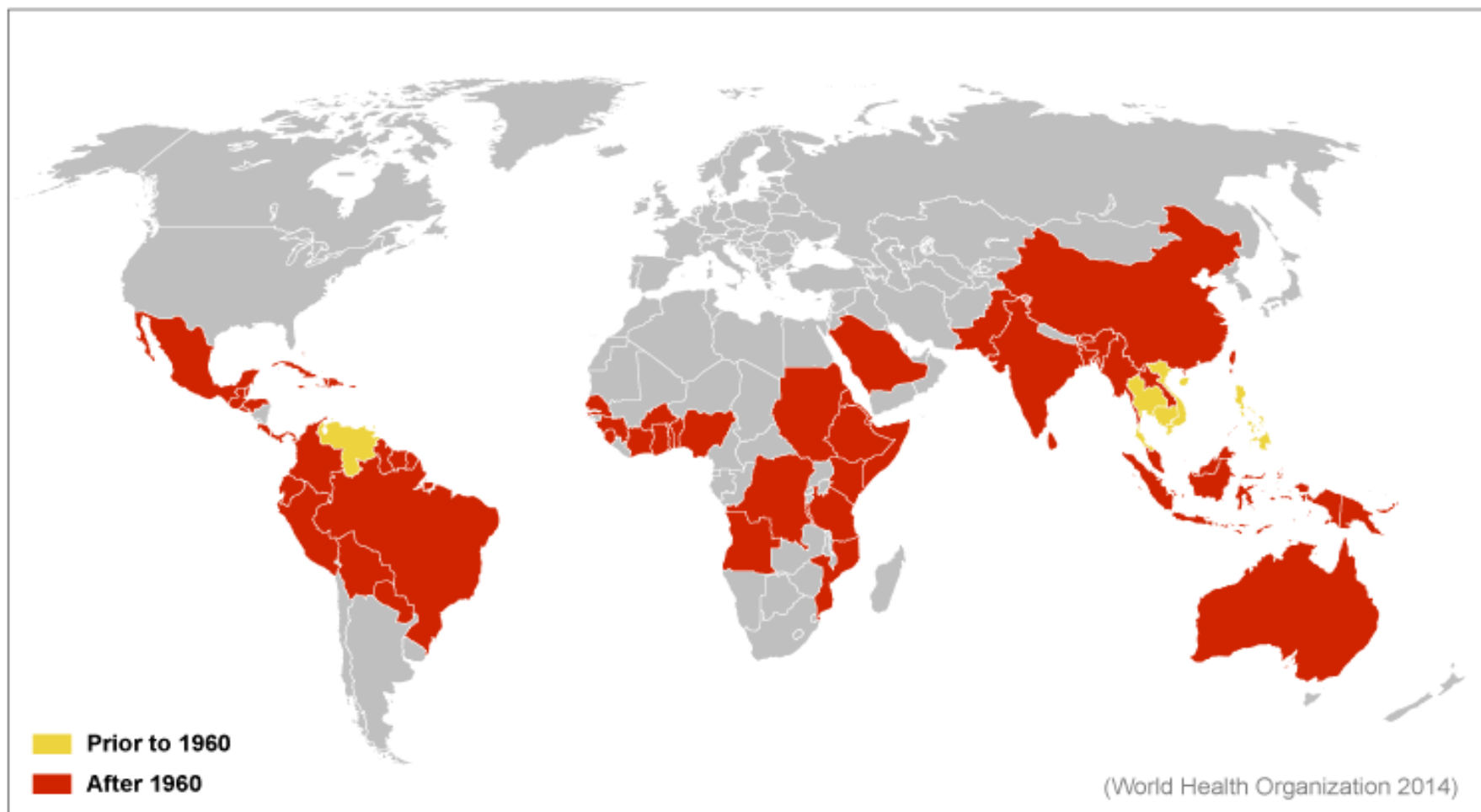


Figure 2.1: The geographic distribution of areas affected by dengue. Yellow zone indicates areas affected before the 1960's and the red zone denotes areas affected after the 1960's till present (Biomedical Primate Research Centre, n.d).

2.1.2 Epidemiology of dengue in Malaysia

Malaysia is one of Asia's developing country and has been rapidly developing ever since. This rapid urbanization has caused the ecology of dengue in Malaysia to be altered (Kwa, 2008). Malaysia consists of two divided regions: Peninsular/West Malaysia and East/Borneo Malaysia and has 13 states and 3 federal territories. The first evidence of dengue in Malaysia was reported in 1902. Researchers described a dengue outbreak in the island of Penang from late 1901 to early 1903 (Skae, 1902). Then in 1965, Malaysia, specifically in Georgetown, Penang, observed the first case of severe dengue infection (dengue haemorrhagic fever) (Rudnick et al., 1965). Since then dengue has become endemic and a staple public health issue in Malaysia. According to Rose Nani (2015), there are multiple factors that contributes to the dengue epidemics in Malaysia. These factors include climate change, rapid urbanization and industrialization, increased human movement, and poor environmental cleanliness among the community.

The incidence rates of dengue in Malaysia mostly show an upward trend from the year 2000 to 2017 with a rate of 31.6/100,000 population in the year 2000 to 258.9/100,000 population in 2017. However, there was severe drop in incidence rate from 2010 to 2012 with the lowest of 69.6/100,000 population in 2011. Nevertheless, the rate increased dramatically from the year 2013 to 2015. The highest ever incidence recorded in a decade was during 2015 with a rate of 396.4/100,000 population, which also marks the highest case and death record of 120, 836 and 336 respectively (Malaysian Remote Sensing Agency (MRSA), 2018). Figure 2.2 and Figure 2.3 shows the yearly incidence rate and total number of cases and deaths from the year 2000 to 2017 respectively. Table 2.1 shows

the current number of cases of dengue in Malaysia from end of 2017 to April 2018. Selangor recorded the highest number of dengue cases for that period of four months.

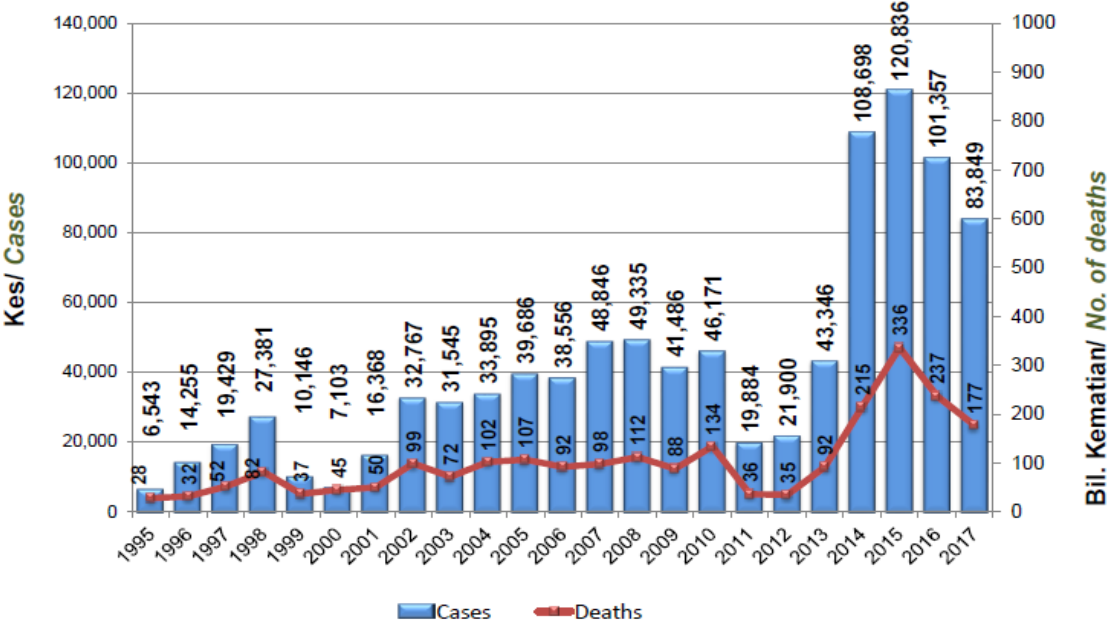


Figure 2.3: The number of dengue cases and deaths related to dengue in Malaysia throughout the year 1995 to 2017 (MRSA, 2018)

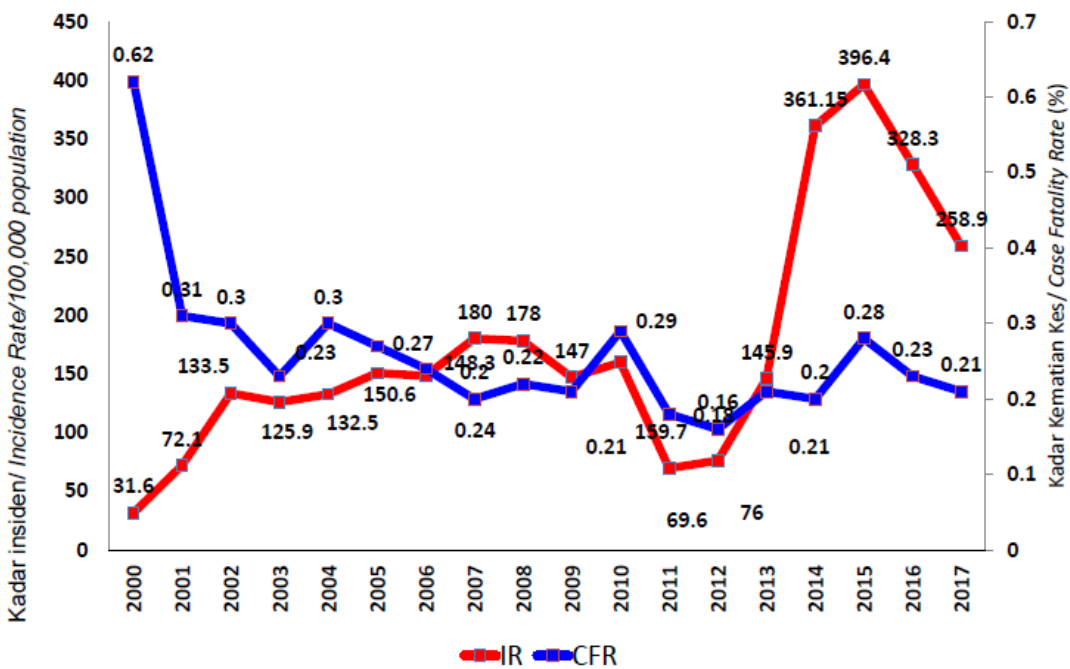


Figure 2.2: The incidence and case fatality rate of dengue in Malaysia from the year 2000 to 2017 (MRSA, 2018)

Table 2.1: The number of dengue cases in Malaysia up to 25th April 2018 (MRSA, 2018)

State	Daily cases as of 25 April 2018	Total accumulated cases from 31 December 2017 to 25 April 2018
Johor	17	1,761
Kedah	8	458
Kelantan	3	291
Malacca	0	161
Negeri Sembilan	4	375
Pahang	2	289
Perak	7	1,041
Perlis	0	81
Penang	12	1,213
Sabah	17	1,193
Sarawak	4	167
Selangor	106	10,242
Terengganu	1	58
FT Kuala Lumpur	19	1,271
FT Labuan	0	18
FT Putrajaya	1	49
Total	201	18,668

*FT = Federal territory

The state of Sarawak is also an endemic area for dengue in Malaysia. However, Sarawak does not contribute much to the number of cases in the country, but still shows a quite significant number of cases. For example, in 2009, the Sarawak State Health Department reported that there were 4,564 dengue cases with 7 deaths in total. Nevertheless, the number of cases showed a decreasing trend on the following years but showed a slight increase in 2015. A total of 1,120 cases were reported which was a two-fold increase when compared to the number of cases reported in the same period of 2014. Currently, as of 31st December 2017, only 167 cases were reported in Sarawak, which is not a grave concern when compared with the State of Selangor of 10, 242 cases of dengue within the past four months duration (Table 2.1).

2.2 Dengue virus (DENV)

2.2.1 Introduction to DENV and its serotypes

Dengue infection is caused by the dengue virus (DENV), which is a virus that belongs to the family *Flaviviridae*, genus *Flavivirus* (Martina et al., 2009). It is an enveloped single stranded positive-sense RNA virus that is made up of three structural proteins (capsid [C], membrane [M], and envelope [E]) and seven non-structural (NS) proteins (NS1, NS2, NS2B, NS3, NS4A, NS4B, and NS5) (Tuiskunen Bäck & Lundkvist, 2013). Figure 2.4 shows the basic structure of DENV.

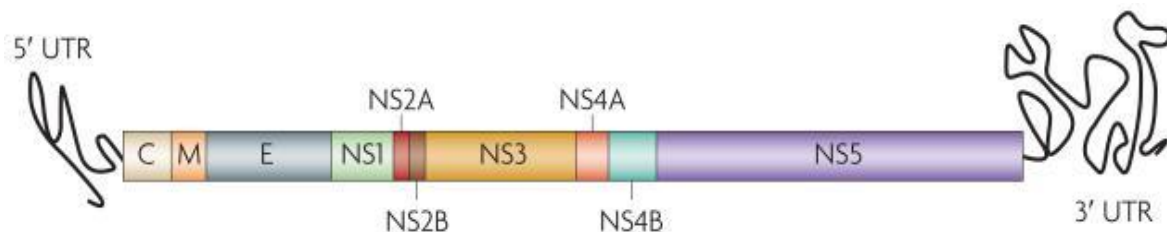


Figure 2.4: Basic structure of DENV consisting of the three structural proteins and seven non-structural proteins (Guzman et al., 2010).

The virus also has four serotypes, namely serotype 1 (DENV-1), serotype 2 (DENV-2), serotype 3 (DENV-3), and serotype 4 (DENV-4). DENV manifest in two ways: the classical dengue fever (DF) and the more severe dengue haemorrhagic fever (DHF) or dengue shock syndrome (DSS). Generally, DF is an acute illness which usually exhibit symptoms like high fever and at least two of the following symptoms: severe headache, pain behind the eyes, joint, muscle and bone pain, skin rash with red spots, and leukocytopenia (Centers for Disease Control and Prevention (CDC), 2012). On the other hand, the much more severe and chronic DHF/DSS exhibits extreme symptoms like severe

abdominal pain and constant vomiting, bleeding from nose or gums, vomiting blood, hyperventilation, black/tarry stools, drowsiness, lethargic, and a significant drop in body temperature (CDC, 2012). On the contrary, a person may also be asymptomatic to dengue infection (Guzman & Kouri, 2004).

DENV manifest the human body by targeting the immune cells. When a mosquito bites on a human, the virus is injected into the bloodstream where it infects the immature Langerhans cells (epidermal dendritic cells) and keratinocytes through cell-mediated endocytosis (Limon-Flores et al., 2005). These infected cells then migrate towards the lymph node, where the body recruit macrophages and monocytes, which in turn gets infected. Consequently, the virus multiplies, and the infection is amplified, where the virus is disseminated through the body's lymphatic system (Martina et al., 2009). This will then result in the primary infection of DENV and cause several other immune cells of the mononuclear lineage to be infected such as the blood-derived monocytes, myeloid dendritic cells, and splenic and liver macrophages (Blackley et al., 2007; Boonnak et al., 2008; Durbin et al., 2008).

During the secondary stage of infection, the body will mostly activate its adaptive immune response and produce immune cells like antibody-secreting B-cells and cytotoxic T-cells that specifically target the cells that are infected with the virus (Mukhopadhyay et al., 2005). The B-cells will secrete immunoglobulins (Ig), also known as antibodies, that will bind with the mature dengue virus. On the other hand, the cytotoxic T-cells will attack and kill any cells that has been infected with the virus. It is believed that the human body's adaptive immune response has been providing a person with long-term immunity against

dengue infection, however, it still requires the innate immune response to help neutralise the dengue virus in an infection (Nature, 2014). Furthermore, a person may also be susceptible to secondary infection of DENV. A person may gain long term immunity against one serotype after an initial infection, but may not gain immunity against the other serotypes (Guzman & Kouri, 2204). That person can get a much more severe infection when infected with a different DENV serotype than the previous infection. The actual pathogenesis of dengue virus in a secondary infected person is still not clearly understood (Cheah et al., 2014).

2.2.2 Distribution of DENV serotypes

The very first isolation of DENV serotype was done back in 1943 and 1945. It was DENV-1 that was first reported in areas of French Polynesia and Japan during 1943. Then DENV-1 was also reported in Hawaii during 1944 and 1945 (Messina et al., 2014). DENV-2 saw a different history where it was first reported in Papua New Guinea and Indonesia in 1944, then followed by the Philippines during 1954 and 1956. Many Asian countries such as Malaysia, Thailand, China, India, Sri Lanka, and Singapore have reported consecutive occurrence of DENV-2 during the early 1960's until the 1980's (Messina et al., 2014). Furthermore, DENV-3 and DENV-4 was not reported until 1953 in the Philippines and Thailand, in which Thailand reported many cases of DENV-3 throughout a 37-year span from 1973 to 2010. Since the first reports, all the DENV serotypes have spread across the world into all major continents including Asia, Africa, America and even Australia and the Western Pacific, and are found to be co-circulating among each other (Guzman et al., 2010;

Messina et al., 2014). Figure 2.5 shows the distribution and co-circulation of DENV serotypes across the globe along a 34-year period from the 1970's to 2004.

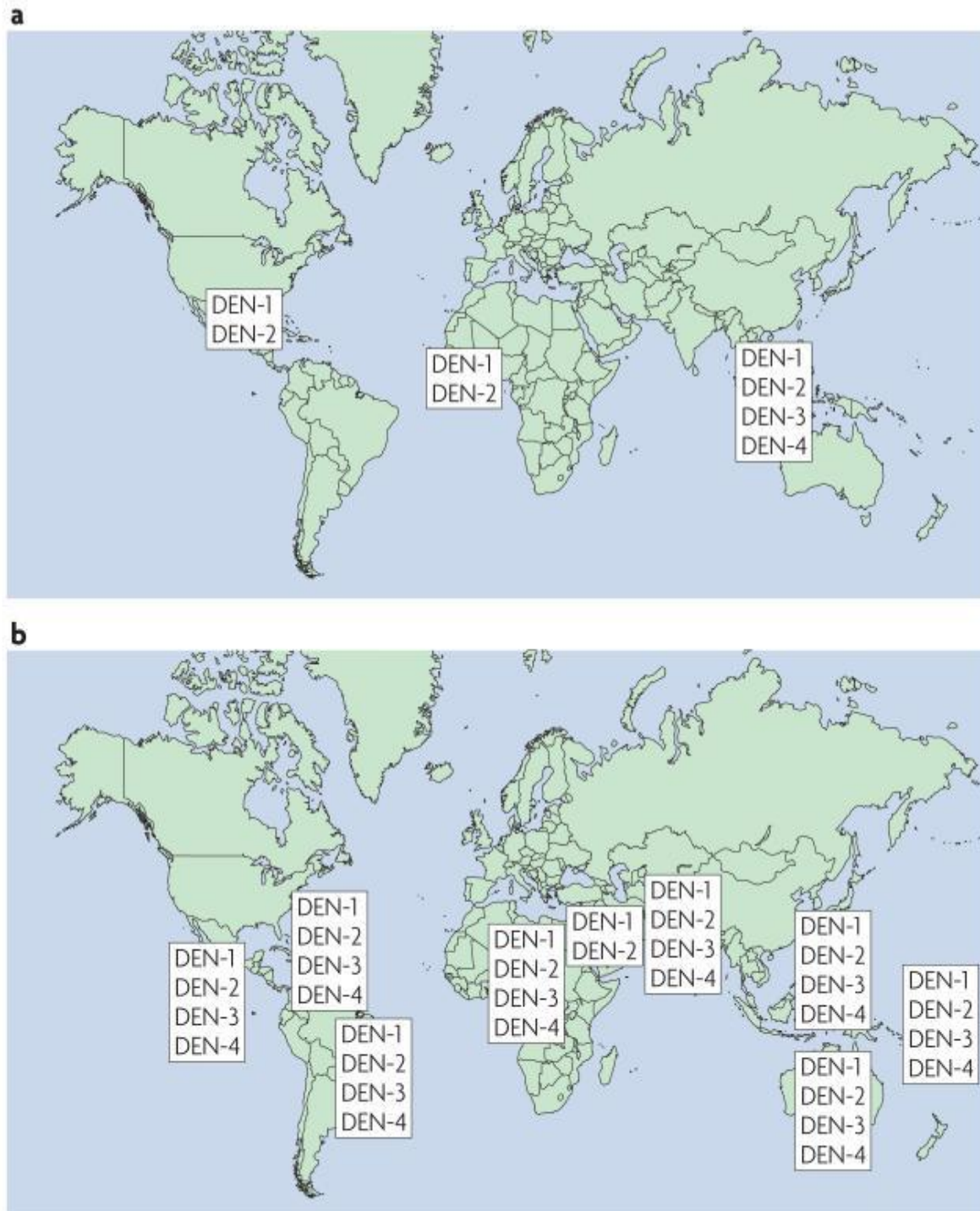


Figure 2.5: Map showing the distribution and co-circulation of dengue virus across the globe. Map A represents the distribution of DENV serotypes during the 1970's, whereas map B represents the distribution of DENV serotypes since 2004 (Guzman et al., 2010)

Malaysia is a ‘hyperendemic’ country for dengue. That means that all the DENV serotypes can be isolated at any point of time, due to their simultaneous presence (Cheah et al., 2014). According to the national data, the dominant circulating DENV serotype in Malaysia was constantly changing during the year 2000 to 2011. DENV-2 was predominant in 2000 and also in 2006 to 2009. DENV-3 was predominant for two years from 2001 to 2002. The following year until 2005 saw a shift in serotype predominance where DENV-1 was the predominant serotype. However, after 3 years (2006 to 2009) being replaced by DENV-2, DENV-1 became the predominant serotype again from 2010 to 2011 (Mohd-Zaki et al., 2014). DENV-4 was once predominant during the late 1960’s, but less than 5% was isolated after the period, although there was a slight increase in 2001 (Abu Bakar et al., 2002). DENV-4 also accounted for only less than 20% of circulating serotype during the year 2000 to 2011. Furthermore, the circulating dengue serotype showed a heterogenous distribution in Malaysia (Mohd-Zaki et al., 2014). For example, DENV-3 was predominant in the states of Kelantan, Negeri Sembilan, and Malacca, whilst DENV-2 was predominant in Johor, Sabah, and Terengganu. DENV-1 was much more predominant in Kedah, Perlis and Selangor, and DENV-4 was only predominant in Pahang and Penang. On the contrary, in the Borneo state of Sarawak, three DENV serotypes were found to be co-circulating, namely DENV-2, DENV-3, and DENV-4 from the year 1997 to 2002, however, DENV-1 was absent (Holmes et al., 2009). Moreover, the DENV-2 and DENV-4 isolated from this period were closely linked to the ones circulating in different areas in South East Asia (Holmes et al., 2009; Cheah et al., 2014). Unfortunately, there is no new data regarding the circulating DENV serotype in Sarawak so, the current situation on the circulating DENV serotype could not be determined. Figure 2.6 shows the distribution of the circulating DENV serotypes in Malaysia.

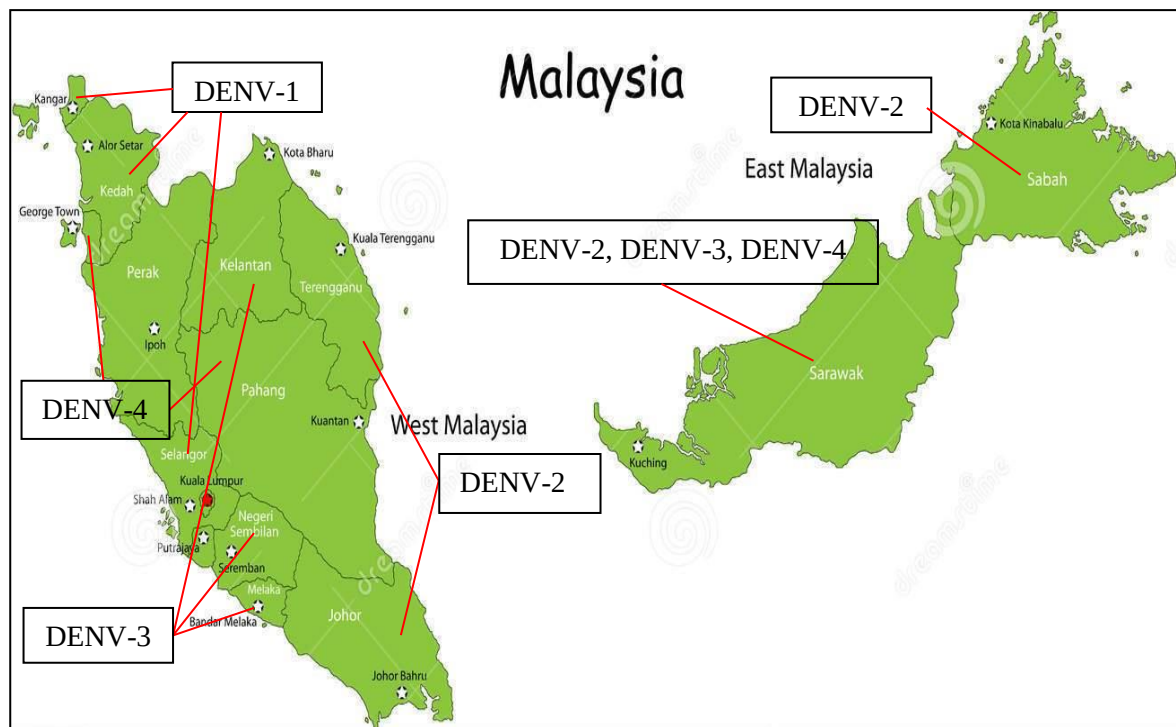


Figure 2.6: Map showing the distribution of the circulating DENV serotypes across Malaysia (Mohd-Zaki et al., 2014; Holmes et al., 2009; Ahmad Nizal et al., 2012; Pang and Loh, 2016).

2.2.3 Laboratory diagnosis of DENV infection

An accurate and efficient method to detect DENV is very crucial in diagnosing patients with dengue infection, so that clinicians can employ the correct treatment and care. Throughout the years, there are various techniques that have been established for the detection of DENV, which is not only for use in clinical laboratories but also used by researches for surveillance and studies regarding DENV and its vector. These techniques include direct virus isolation, NS1 antigen detection, serological assays and usage of molecular techniques such as reverse transcription polymerase chain reaction (RT-PCR) for detection of viral genome (Guzman and Kouri, 2004). In past years, virus isolation has been routinely used for the diagnosis of dengue infection (Gubler, 1998). This method particularly involves the use of clinical samples such as the serum, plasma or organ tissue

samples such as liver and spleen. This method has also been implemented in detecting DENV in the vector mosquitoes (Samuel and Tyagi, 2006). One of the most common method is using a cell-culture method by employing the use of C6/36 *Aedes albopictus* Skuse cell lines and followed by identification using immunofluorescence assay with serotype-specific monoclonal antibodies (Samuel and Tyagi, 2006). However, this method has its drawbacks as it requires large number of infected mosquitoes and time consuming, making it less suitable for rapid detection of DENV.

Another common method used at present in most clinical laboratories is the dengue NS1 antigen detection. It is a method used for the early diagnosis of dengue infection and mostly employed during the early stages of dengue infection in patients (Young et al., 2000). The dengue NS1 is highly conserved glycoprotein, produced in both membrane-associated and secretion forms, and is highly abundant in serum samples of dengue patients (Fauziah et al., 2011). Targeting of the dengue NS1 antigen mostly employ the use of serological assays such as the enzyme-linked immunosorbent assay (ELISA), where it is available as commercialized kits. This method was proven to be successful in the laboratory with dengue infected *Aedes* mosquitoes, and field-caught *Aedes* mosquitoes. It is a sensitive, rapid, and convenient test for DENV surveillance in the vector mosquito population (Samuel and Tyagi, 2006; Voge et al., 2013).

The use of molecular methods such as the polymerase chain reaction (PCR) has gain popularity throughout the years. This method can be utilized in clinical samples as well as in vector mosquito samples. RT-PCR is one of the methods commonly used as it is much more efficient, highly sensitive, precise and specific, however the method can be subjected

to some drawbacks such as costly and prone to contaminations (Samuel & Tyagi, 2006). There are many methods using RT-PCR available nowadays (De Paula et al., 2002; Reynes et al., 2003; Pinheiro et al., 2005; Saxena et al., 2008) but the protocol developed by Lanciotti et al., (1992) was the one that pioneered the use of PCR in detection of DENV. Furthermore, this method was also proven successful in many studies of DENV using mosquito larvae samples (Rohani et al., 1997; Rohani et al., 2008). Another more current molecular method developed for detection, quantitation, and serotyping of DENV is using real time RT-PCR. The method was also demonstrated to successfully detect DENV in mosquitoes inoculated with the virus (Gurukumar et al., 2009). Currently, many studies have use the one-step real time RT-PCR or the one-step multiplex real-time RT-PCR for the detection and serotyping of DENV (Waggoner et al., 2013; Alm et al., 2015; Mun et al., 2018). The one-step real-time RT-PCR involves the simultaneous reaction of RT and PCR in a single sample tube focusing on a single target gene and the results can be determined at real time. On the other hand, a one-step multiplex real-time RT-PCR employs the same techniques but amplifies multiple target genes in a single reaction. Both these methods provide accurate representation of target copy and it is a simple and rapid method for high-throughput screening situations.

2.2.4 Modes and cycles of DENV transmission

2.2.4.1 Horizontal transmission of DENV

Viruses from the *Flaviviridae* family such as DENV, yellow fever virus, West Nile virus, Japanese encephalitis virus and Zika virus are arthropod borne viruses, therefore they are transmitted mostly by mosquitoes (CDC, 2014). Among these viruses, DENV is by far the

most important arthropod borne viruses worldwide. The modes and cycle of DENV transmission are described in two ways namely the horizontal transmission and the vertical transmission.

The horizontal transmission of DENV is the principal mode of transmission in infecting humans. All serotypes of DENV utilizes *Aedes* mosquitoes as the principal vector for transmission. It is believed that DENV came into contact with humans through the sylvatic cycle of the virus (Weaver & Vasilakis, 2009). It is hypothesised that the endemic DENV evolved from the sylvatic strain of the virus originating from Africa and Asia, which utilises non-human primates as host and forest-dwelling *Aedes* mosquitoes as vectors (Gubler, 1997). The sylvatic strain of DENV is also presumed to be an ancestral strain. Ancestral relates to non-human transmission, and an efficient inter-human transmission requires a large population number with a minimum of 10, 000 to 1 million people, which did not exist until 4000 years ago during the dawn of urban civilizations (Gubler, 1997). Furthermore, this hypothesis was further supported by various phylogenetic studies regarding the sylvatic strain of DENV including in Malaysia. Studies conducted in the 1960's and 1970's isolated DENV-1, -2, and -4 of sylvatic origins. These serotypes were genetically distinct from endemic isolates collected from humans and urban vectors (Weaver & Vasilakis, 2009). Due to rapid urbanization, the contact between humans and non-human primates are closer. This increases the chances of sylvatic DENV infected *Aedes* mosquitoes to cause dengue infection in humans (Durbin et al., 2013).

Horizontal transmission of DENV involves the transfer of DENV from human to human via a vector. This occurs when a DENV-infected *Aedes* mosquito bites or blood-feed on a

person. Once entered, the virus will undergo an incubation period of 5-7 days before the person starts to manifest multiple symptoms of dengue fever (Tomashek et al., 2017). During this period, a non-infected Aedes mosquito may come in contact and feeds on the infected person and gets infected. Therefore, it is then capable of transmitting the virus to other non-infected humans and the cycle continues. Figure 2.7 shows a typical DENV transmission cycle through vector mosquitoes.

2.2.4.2 Transovarial transmission of DENV

Transovarial transmission or vertical transmission of DENV involves the transfer of the virus from a gravid female mosquito to offspring or progeny in Aedes mosquitoes (Martins et al., 2012). When DENV enters the mosquito through blood-feeding, it will infect and replicate in multiple areas within the body of the mosquito which includes the midgut, ovaries, and neural tissues (Akbar et al., 2008). As the ovaries are infected, the dengue virus also infects the eggs. Therefore, this causes the virus to be transferred to the immature mosquitoes as they emerge into adult mosquitoes. The natural vertical transmission of DENV in Aedes mosquitoes have been reported numerous time since the last three decades (Khin & Than, 1983; Rosen et al., 1983; Hull et al., 1984). Many studies have been done later throughout the years on the natural population of *Ae. aegypti* and *Ae. albopictus* mosquitoes, where immature forms (eggs, larvae, and pupae) of these mosquitoes are collected from the field (Hull et al., 1984; Akbar et al., 2008; Martins et al., 2012; Cruz et al., 2015; Gutiérrez-Bugallo et al., 2017). The immatures would then be raised until adults before being screened for DENV. Besides this, other studies have also proven the ability of DENV to be vertically transmitted in laboratory-infected Aedes mosquitoes. In many cases, *Ae. aegypti* or *Ae. albopictus* mosquitoes would be artificially

infected either through intrathoracic inoculation, oral inoculation, or parental inoculation (Mitchell & Miller, 1990; Mourya et al., 2001; Joshi et al., 2002; Castro et al., 2004; Rohani et al., 2008). The infected mosquito would then be allowed to mate and blood feed to successfully breed and lay eggs. The progeny was later screened for DENV either in the immature form (larvae) or raised to adults. Furthermore, vertical transmission of DENV acts as a virus maintenance mechanism for all the dengue serotypes to persist in the absence of a human host or any favourable condition in the natural environment (Hartanti et al., 2010). In addition, DENV is also capable of persisting in successive generations of mosquitoes and able to last up to five generations, as proven in several studies conducted under laboratory conditions (Joshi et al., 2002; Rohani et al., 2008). This proves that vector mosquitoes play an important role in the maintenance of virus in the natural environment, and mosquitoes act as a reservoir of DENV.

Besides vertical transmission in mosquitoes, DENV was also proven to be vertically transmitted in human beings. Studies conducted on pregnant mothers have observed that the infant could contract the virus from their mother having dengue fever during final stages of pregnancy (Chye et al., 1997; Chotigeat et al., 2000; Yin et al., 2016). Researchers were able to isolate DENV from the infant's blood together with IgM antibodies specific to DENV. Furthermore, researchers also identified a possibility of DENV to be vertically transmitted through breast feeding (Barthel et al., 2013). DENV was isolated from breast milk of a pregnant women having dengue fever after giving birth. However, further work needs to be done to prove the possibility of transmitting DENV through breast milk.

2.2.4.3 Sexual transmission and uncommon transmission of DENV

According to a study conducted in the late 1980s, DENV could be transmitted through copulation of male and female *Aedes* mosquito (Leon, 1987). Laboratory infected male *Ae. albopictus* was observed to sexually infect female *Ae. albopictus* with all serotypes of DENV. The transmission was further enhanced if the females were blood-fed 2 to 7 days prior to mating and the infected females would then transfer the virus to their progeny (Leon, 1987). There are also reports on other modes of dengue transmission which does not involve the vector mosquito. These modes of transmission include transfusion-related transmission, transplantation-related transmission, and needle-stick related transmission (Wiwanitkit, 2010). Transfusion-related transmission obviously involves blood transfusion. Since dengue is considered and classified as a blood pathogen, infection of blood recipient can be expected (Tambyah et al., 2008; Seed et al., 2009).

Transplantation related transmission on the other hand involves DENV being transmitted during organ transplantations. There were reports of renal and bone marrow transplant recipients infected with DENV after transplantations (Tan et al., 2005; Rigau-Pérez and Laufer, 2006). DENV is not classified as one of the infectious agents required for screening prior to organ transplant, therefore the risk of DENV transmission is highly possible (Wiwanitkit, 2010). Another uncommon mode of DENV transmission is through needle-stick injury. This is one of the most common accidents in routine medical practices. Several infectious diseases such as HIV and Hepatitis B have been known to be transmitted through needle-stick-related injury (Wiwanitkit, 2010). DENV have also been reported to be transmitted through needle-stick-related injuries, however only a few incidents were reported (Nemes et al., 2004; Wagner et al., 2004).

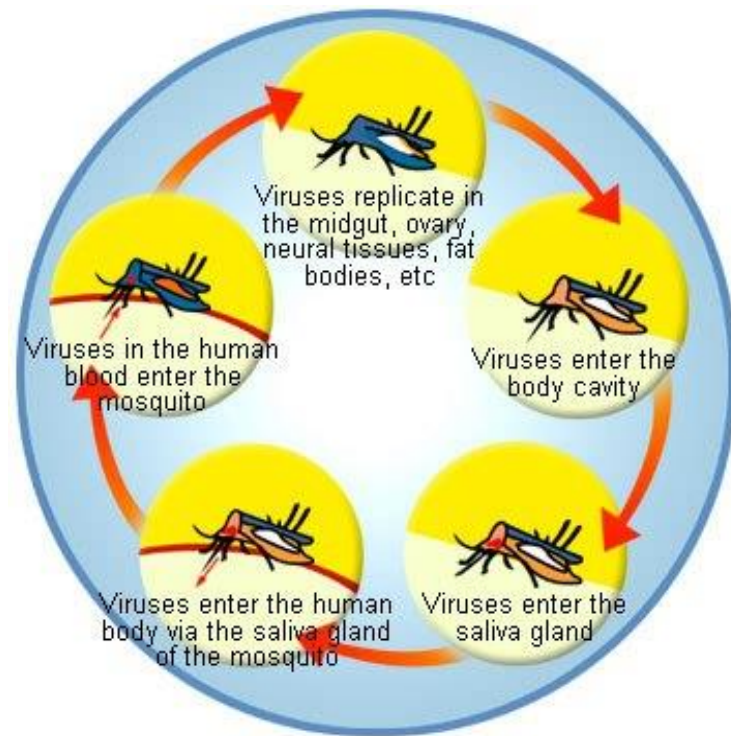


Figure 2.7: The replication and horizontal transmission cycle of DENV (Mahidol University, n.d)

2.2.4.4. Estimation of DENV infection rate in *Aedes* mosquitoes

The most common tool used for DENV infection rate in mosquito is the minimum infection rate (MIR). MIR is defined as the ratio of the number of positive pools to the total number of mosquitoes and are usually represented as the number of infected mosquitoes per 1,000 tested (Gu et al., 2003). It is an assumption that only one infected individual is present in a positive pool. However, this may be invalid if the infection rates are high or large pool sizes. MIR can be calculated using the formula where the number of female/male mosquitoes in a positive pool divided by the number of female/male mosquitoes tested multiplied by 1000 (Kow et al., 2001; Vikram et al., 2015). A study conducted by Vikram et al., (2015) also stated that areas with mosquitoes indicating a high

MIR value have a higher risk of dengue transmission and are more prone to a dengue outbreak.

$$\text{MIR} = \frac{\text{DENV Positive Female/Male Mosquito Pool}}{\text{Total Number of Female/Male Mosquito}} \times 1000$$

Figure 2.8: Formulae for MIR calculation (Vikram et al., 2015).

2.3 Vector of DENV

As mentioned previously, dengue is an arthropod-borne or vector-borne disease. Dengue infects its host mostly through mosquitoes, primarily *Aedes* mosquitoes. Mosquitoes are widely known to vector various types of diseases and the most common are yellow fever, West Nile fever, chikungunya, Zika, Japanese encephalitis, and malaria. For many years, mosquito have been a public health enemy due to their ability to transmit infectious diseases.

2.3.1 Introduction to mosquito

Mosquitoes are insects classified under the order of Diptera, which consists of flies and flying insects (Harbach, 2007). It is then further grouped in a family of Culicidae which mostly consist of mosquitoes. According to the American Mosquito Control Association (AMCA) (2014), there are roughly more than 3000 species of mosquitoes recorded worldwide. All mosquitoes are mostly morphologically similar to each other regardless the species, where there consists of three major parts: the head, thorax, and abdomen. On its head, mosquitoes are equipped with two compound eyes, a long proboscis and a pair of antennae. The antennae function as chemical sensors. In addition, mosquitoes have a pair

of wings and six functional legs attached to its thorax (AMCA, 2014). The mosquito goes through four separate stages in its life cycle consisting of the egg, larvae, pupa and adult, where each stage can be recognized easily through its special appearance.

The life cycle of mosquito starts with the eggs. Eggs are laid one at a time or attached together in 'raft' form, often floating on the water surface. Certain genus of mosquitoes such as *Anopheles*, *Ochlerotatus*, and *Aedes* lay their eggs singly rather than attached together (AMCA, 2014). Furthermore, mosquitoes from the genus *Culex*, *Culiseta*, and *Anopheles* species lay their eggs on the surface of the water, whereas mosquitoes of the *Aedes* and *Ochlerotatus* species lay their eggs on damp surfaces close to the surface of water. Most eggs hatch into larva after 48 hours and live most of its life in water. During the larval stage, mosquitoes normally undergoes four stages of moulting process, where they grow larger after each moult (AMCA, 2014). Each stage of moulting is categorized as instars. Mosquito larva rely on siphon tube to breathe and normally hang upside down from the water surface. Some species of mosquito do not have siphon tubes for breathing but breathe through openings on their bodies and lay parallel to the water surface (AMCA, 2014). Moreover, mosquitoes of *Coquillettidia* and *Mansonia* species attach themselves to roots of aquatic plants to obtain oxygen. Mosquito larva also feed on microorganisms or organic matter. After the fourth instar, the mosquito larvae will evolve into a pupa.

The pupa stage of a mosquito is the resting stage. The pupa does not feed anymore but are still mobile (AMCA, 2014). This is the final immature stage of the mosquito before becoming an adult. The pupal stage normally last for two days, then will fully developed into an adult mosquito. The pupal skin will split open and the adult mosquito emerges

(AMCA, 2014). However, the newly emerged adult does not fly straight away. It will rest on the surface of the water for a short while to allow for its body and wings to dry and the body parts to harden. The adult mosquito only blood feeds and mate a few days after emerging. The adult stage of mosquitoes last depending on the environmental temperature and species characteristics (AMCA, 2014).

2.3.2 *Ae. aegypti* and *Ae. albopictus*

Ae. aegypti and *Ae. albopictus* are the principal vector for DENV. These two mosquitoes also vector other viruses like yellow fever, West Nile virus, chikungunya, and Zika (Morrison et al., 2008). *Ae. aegypti* is a dark brown-coloured mosquito. It has a pair of submedian-longitudinal white stripes on the scutum part of the thorax (Rueda, 2004) (Figure 2.8). The male and female differ in size and the antennae. Female mosquitoes are usually larger than the males. The antennae of males have denser hairs than that of the females (Figure 2.9). *Ae. aegypti* are day biters and can be found mostly in human dwelling area either outdoors or indoors. However, *Ae. aegypti* prefers to blood feed on humans indoors (CDC, 2016a). Furthermore, *Ae. aegypti* breeds in natural or artificial water containers, and in water containing decaying organic materials preferably dark-coloured containers in shady places (CDC, 2016a). *Ae. aegypti* is widely distributed worldwide and is believed to have originated from Africa (Powell & Tabachnick, 2013). Since then, this species has spread throughout the globe covering tropical and sub-tropical regions including Malaysia. Figure 2.10 shows the global distribution of *Ae. aegypti*. The increase in transportation, global trade, human travels and rapid urbanization have caused the distribution of *Ae. aegypti* to be accelerated (Kraemer et al., 2015).

Ae. albopictus is a well-known vector for DENV in Asia. *Ae. albopictus* have mostly the same characteristics with *Ae. aegypti*, that is black in colour and has banded legs. However *Ae. albopictus* have a distinct difference located on the scutum of the thorax. A single narrow median-longitudinal white stripe can be seen on the scutum of *Ae. albopictus* (Figure 2.8) (Rueda, 2004). The male and females are morphologically similar to *Ae. aegypti* (Figure 2.9). *Ae. albopictus* are strongly attracted to humans but will feed on other mammals as well as birds (CDC, 2016b). They mostly feed during the day in outdoor and indoor locations but are mostly found outdoors. *Ae. albopictus* prefers to lay their eggs in natural water containers such as tree holes, rock holes, and hollow bamboo stumps while *Ae. aegypti* prefers laying eggs in artificial water containers such as tyres, flower pots, buckets, and tin cans (CDC, 2016b). *Ae. albopictus* is an indigenous species of Asia, hence it is named the Asian Tiger mosquito (Bonizzoni et al., 2013). For the past 30-40 years, *Ae. albopictus* have colonized every continent in the world except Antarctica (Benedict et al., 2007). Figure 2.11 shows the global distribution of *Ae. albopictus* for the past six decades.

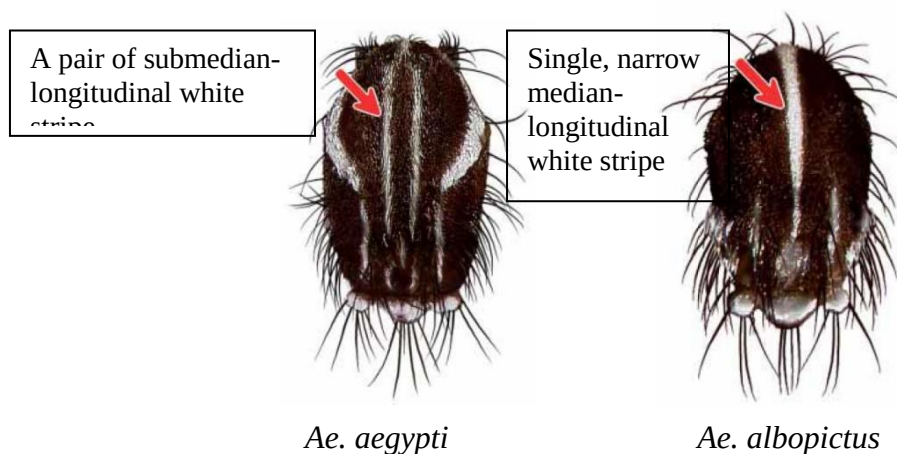


Figure 2.9: Scutum part of the thorax. Left: A pair of submedian-longitudinal white stripes of *Ae. aegypti*; Right: A single, narrow median-longitudinal white stripe of *Ae. albopictus* (Rueda, 2004).

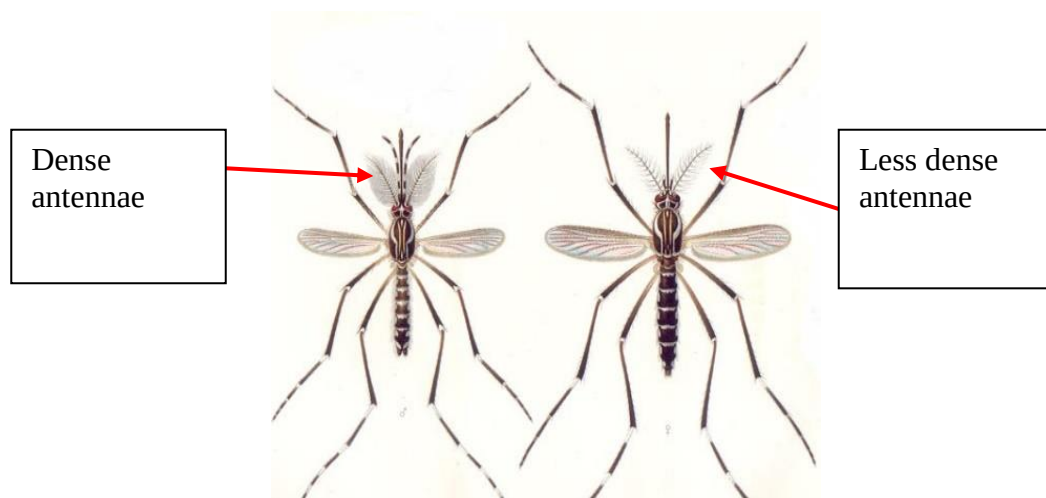


Figure 2.10: Female and male *Aedes* mosquitoes. The males have denser antennae than the females (Wikipedia, 2018)

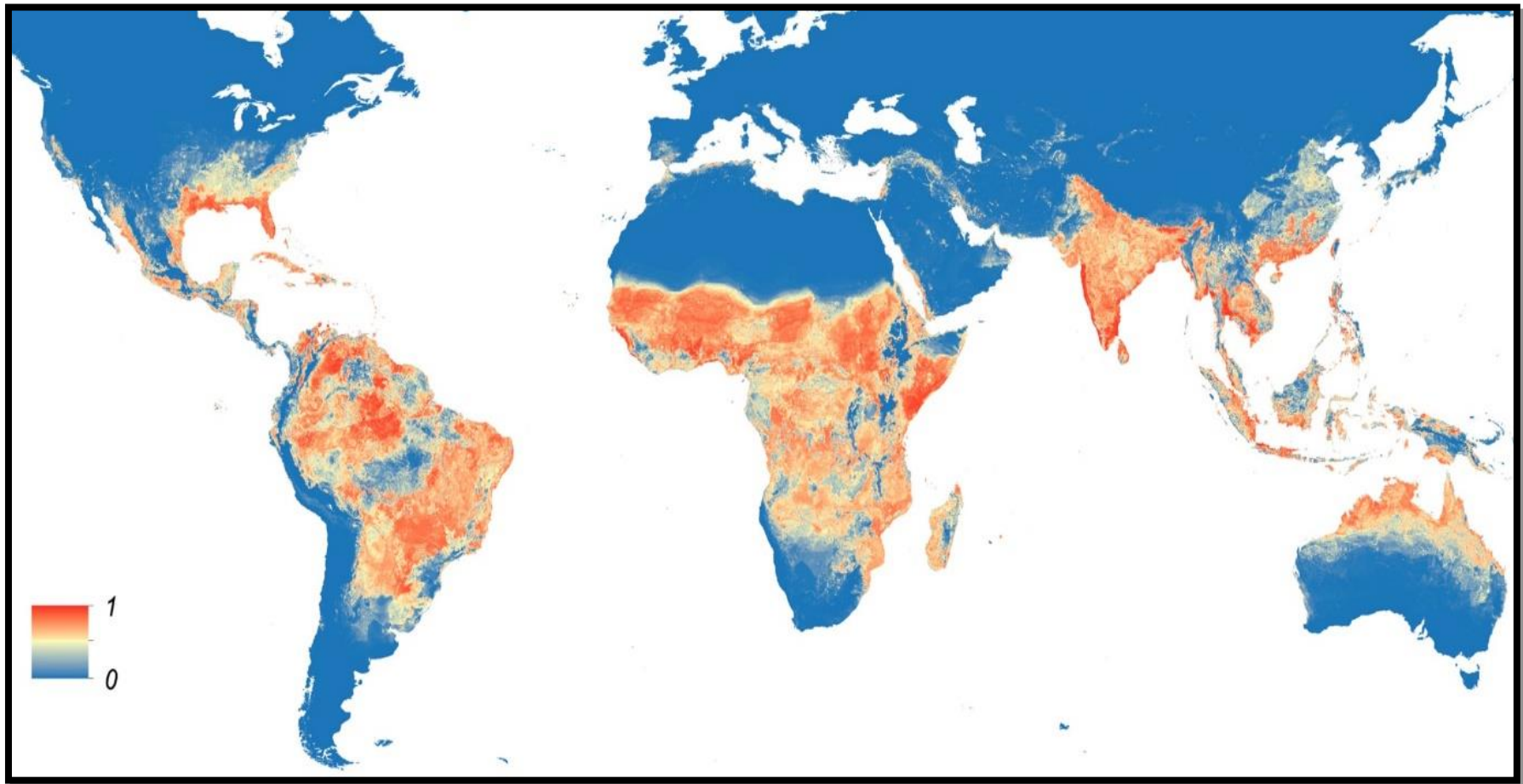


Figure 2.11: Map showing the global distribution of *Aedes aegypti*. Red-coloured area indicates areas having the highest density of *Ae. aegypti* population (Kraemer et al., 2015).

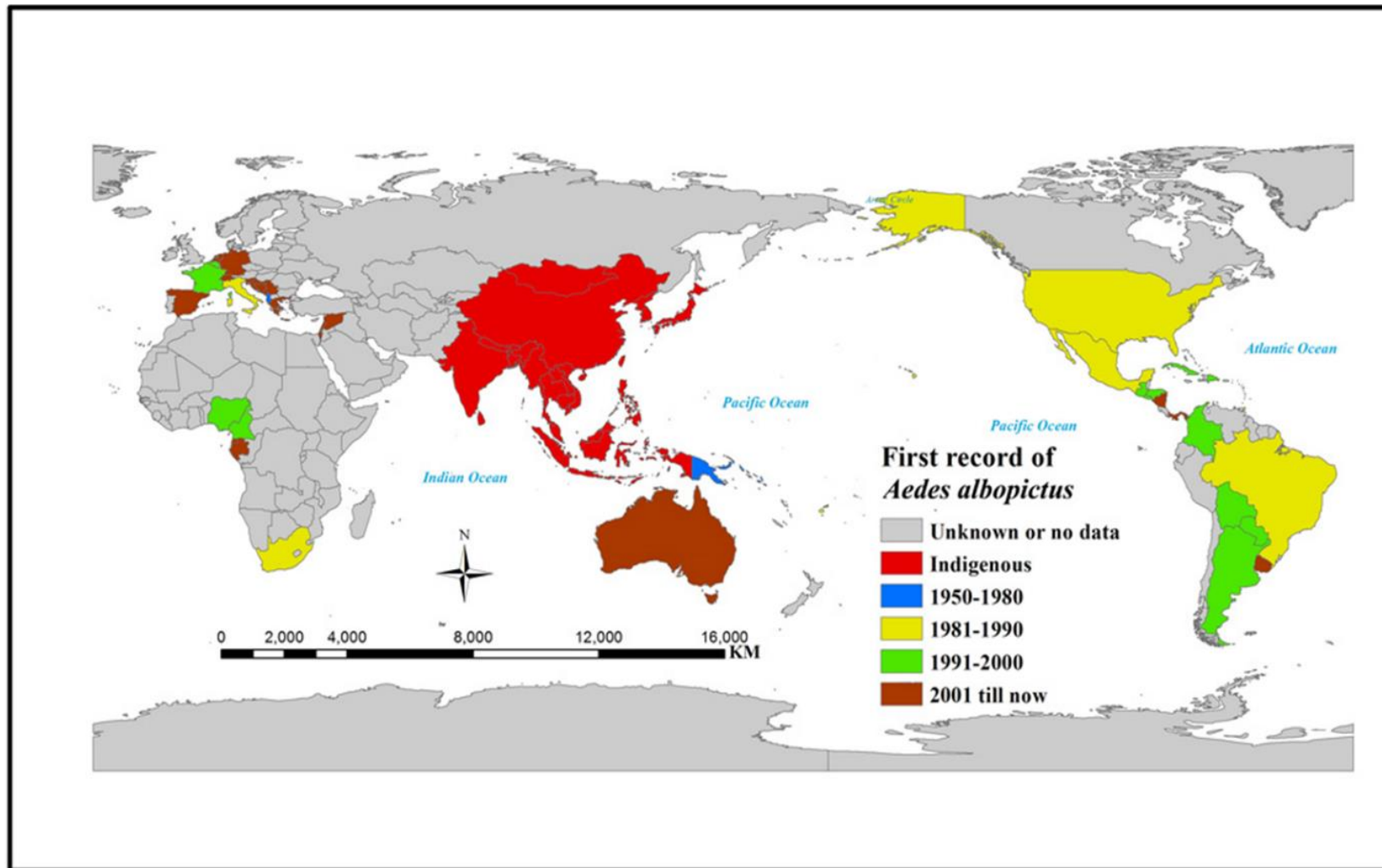


Figure 2.12: Map showing the global distribution of *Aedes albopictus* (Bonizzoni et al., 2013).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study areas

The study areas were located within the Sibu and Miri Division of Sarawak. Sibu and Miri was chosen as the study areas due to their history of increasing number of dengue cases in the state of Sarawak during the year 2014 to early 2015 (Sarawak State Health Department, 2014; Sarawak State Health Department, 2015). The localities were chosen randomly covering urban and sub-urban areas around residential, commercial and, industrial areas. These areas were chosen due to the higher density of human population. This makes it highly possible for efficient dengue transmission. These areas also have the potential of providing good breeding ground for *Aedes* mosquitoes.

Ten locations (Figure 3.1) were covered in Sibu division focusing on residential, commercial and a few industrial areas. These locations include Jalan Sungai Antu, Jalan Maludan, Rejang Park, Jalan Merdeka, Jalan Tiong Hua, Jalan Ulu Oya, Jalan Tong Sang, Jalan Teku, Jalan Salim, and Sibu Lake Garden (Appendix B: Figure B4 to B13). These locations, except Jalan Sungai Antu and Sibu Lake Garden, are mostly urban residential areas consisting of housing estates. The houses are primarily single story and double story terrace style brick houses. The houses are arranged close to each other, therefore the dengue transmission among the residence are easier, making it a potential dengue hotspot area. Sampling in Jalan Sungai Antu focuses on light industrial areas such as around food processing factories, automotive workshops, and small furniture factories, which were

mostly surrounded with discarded items such as artificial containers and used tyres, a good place for mosquito breeding. Sibu Lake Garden is a recreational park situated roughly 9 km from the town centre and next to the Sibu General Hospital. The park was also surrounded by residential areas consisting of terrace houses with a dense population. Therefore, this area has a high potential of mosquito to human transmission of dengue virus.

The type of localities selected in Miri for mosquito sampling were similar to the one in Sibu.

Nine locations (Figure 3.2) were covered namely, Bandar Baru Permyjaya, Taman Ceria, Permyjaya, Taman Merbau, Jalan Dato Permaisuri, Taman Wah Shin, Jalan Sekolah Anchi, Jalan Desa Senadin, Jalan Shangrila, and Kampung Pujut Tanjung Batu (Appendix B: Figure B14 to B22). Sampling in these localities were focused on urban residential areas consisting of housing estates and commercial shop lots, except for Kampung Pujut Tanjung Batu. The housing estates and commercial shop lots were mostly made up of brick houses and shop buildings. On the other hand, Kampung Pujut Kampung Batu was a Malay village located approximately 2 kilometres from the town centre comprising of singly built brick and wooden houses. Sampling in this locality were focused on areas surrounding the houses such as around the backyard and front lawn. Table 3.1 shows the general description of the selected study area.



Figure 3.1: Location map showing the ten localities selected for sampling in Sibu, Sarawak (Photo courtesy of Google maps).

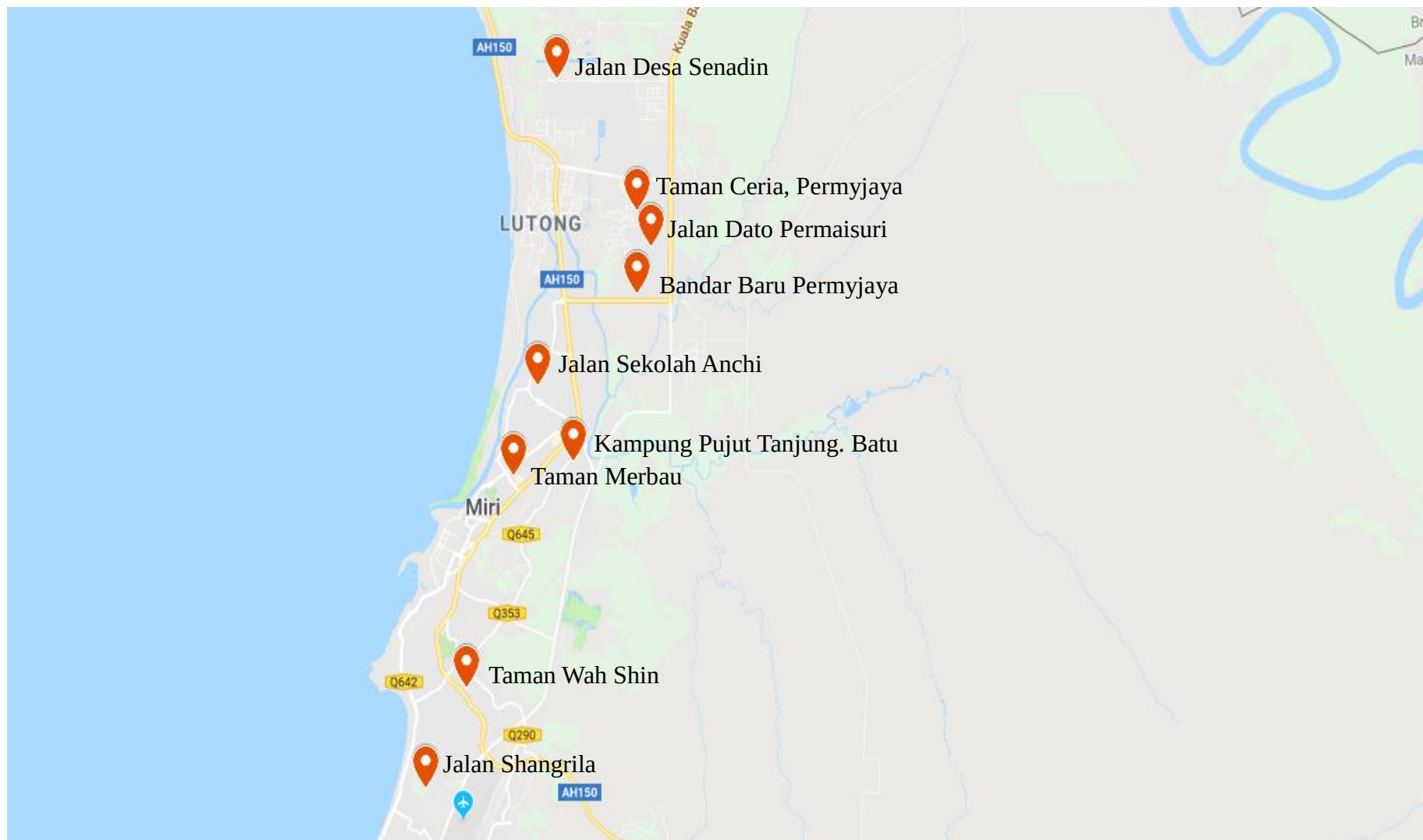


Figure 3.2: Location map showing the nine localities selected for sampling in Miri, Sarawak (Photo courtesy of Google maps).

Table 3.1: Geographical location and type of localities selected for mosquito sampling in Sibu and Miri, Sarawak.

Division	Location	Geographical location	Type of locality
Sibu	Jalan Sungai Antu	2°19'17.47"N, 111°49'55.63"E	Light industrial area.
	Jalan Maludan	2°15'51.70"N, 111°51'24.80"E	Residential areas.
	Rejang Park/Taman Rejang	2°18'31.5"N, 111°50'18.2"E	Residential areas.
	Jalan Merdeka	2°18'17.90"N, 111°50'29.56"E	residential areas.
	Jalan Tiong Hua	2°17'52.92"N, 111°50'32.16"E	Residential and commercial areas.
	Jalan Ulu Oya	2°17'28.10"N, 111°54'5.14"E	Residential areas.
	Jalan Tong Sang	2°17'39.77"N, 111°50'24.83"E	Residential and commercial areas
	Jalan Teku	2°20'1.46"N, 111°50'13.63"E	Residential areas
	Jalan Salim	2°15'44.42"N, 111°51'57.06"E	Residential areas
	Sibu Lake Garden	2°18'11.12"N, 111°53'35.79"E	Residential areas.
Miri	Bandar baru Permyjaya	4°27'0.97"N, 114° 1'58.33"E	Commercial areas.
	Taman Ceria, Permyjaya	4°28'12.70"N, 114° 1'58.68"E	Residential areas.
	Jalan Dato Permaisuri	4°27'41.08"N, 114° 2'12.19"E	Residential areas.
	Taman Merbau	4°24'24.62"N, 113°59'58.34"E	Residential areas.
	Taman Wah Shin	4°21'21.92"N, 113°59'13.06"E	Residential and commercial areas.
	Jalan Sekolah Anchi	4°25'42.20"N, 114° 0'22.07"E	Residential areas
	Jalan Desa Senadin	4°30'5.18"N, 114° 0'40.62"E	Residential areas
	Jalan Shangrila	4°19'55.30"N, 113°58'33.86"E	Residential areas
	Kampung Pujut Tanjung Batu	4°24'36.93"N, 114° 0'57.06"E	Residential areas

3.2 Mosquito sample collection

Mosquito sample collection were performed from September 2016 to December 2017. Two types of sample were collected, the first samples were adult male and female *Aedes* mosquitoes. Secondly, were the immature form (larvae and pupae) of the mosquitoes. Both type of sample was collected in the same area for each locality.

3.2.1 Collection, and preservation of adult *Aedes* mosquitoes

The male and female adult mosquitoes were caught using a simple technique which is the human landing catch (HLC). The HLC technique requires a group of few people (two or more) to conduct. The mosquito was captured when it lands on a part of the body, mainly arms and legs. Once it has landed, it was given 2-5 seconds to settle down. Before it blood feeds, the mosquito was captured using a tube or a hand-held aspirator. Once the sample number was sufficient for each locality, the mosquitoes were knocked out by placing them in a freezer or ice for a few minutes. Then, the adult mosquitoes were transferred to a 2.0 ml cryotube and placed in a portable mini liquid nitrogen tank before being brought back to the lab. Once in the laboratory, the collected mosquitoes were identified using pictorial keys (Rueda, 2004). Then, the mosquitoes were pooled together in a tube of 15-20 mosquitoes according to species, sex, and location and kept in a -80°C freezer until further processing.

3.2.2 Collection, rearing, and preservation of immature *Aedes* mosquitoes.

Immature *Aedes* mosquitoes were collected from discarded waste containers such as food cans, paint buckets, vehicle tyres, disposable food containers etc. All larvae and pupae were collected using disposable pipettes and placed in 250 ml round containers with ventilated water-tight lids (Appendix B: Figure B3). All containers were labelled accordingly with the name of locality, type of container collected from, and date of collection. The pH and temperature of the water were also recorded (Appendix A: Table A1 and Table A2). The samples were brought back to the laboratory and the larvae and pupae were separated into different containers. All larvae were fed using fish food or dried beef liver. The pupae were kept in bottles with water until they emerged into adults. Emerged adults were harvested and identified at species level. After identification, the emerged adults were pooled together in a tube of 15-20 mosquitoes according to species, sex and location and kept in a -80°C freezer until further processing.

3.3 Viral RNA extraction

Viral RNA extraction was performed using Qiagen's RNeasy Mini RNA extraction kit (Catalogue no: 74104) containing Buffer RLT, Buffer RW1, Buffer RPE, RNase-free water, and RNeasy spin column attached with 2.0 ml collection tube. The extraction procedure was all based on the manufacturer's protocol but with a slight modification. Extraction protocol was performed on ice to protect all RNA from degradation. Each 1.5 ml tube of pooled mosquito sample (15-20 mosquitoes) was added with 350 µL of Buffer RPE (lysis buffer). The sample was then thoroughly homogenised using a sterile plastic homogeniser (OPTIMA Biomasher II). Once uniformly homogenised, the samples were

centrifuged for 3 minutes at 12,600 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5427R) set at 0°C. The remaining lysate was transferred to a new 1.5 ml tube before adding 700 µL of 70% ethanol and thoroughly mixed by pipetting.

700 µL of the lysate was later transferred to a RNeasy spin column attached to a 2.0 ml collection tube and centrifuged for 15 seconds at 12,600 rpm. The filtrate from the centrifugation was discarded and the 2.0 ml collection tube was reused for the next step. 350 µL of Buffer RW1 was added as a wash buffer to the column and centrifuged similarly. The filtrate was discarded, and the tube reused. Next, 500 µL of Buffer RPE was added to the column and centrifuged for 15 seconds at 12,600 rpm. After centrifugation, the filtrate was discarded and another 500 µL of Buffer RPE was added to the column to dry the column membrane. The column was centrifuged again for 2 minutes at 12,600 rpm.

Once the column was completely dried, the filtrate was discarded along with the collection tube. A new 1.5 ml collection tube was attached to the column. 80 µL of RNase-free water was added to the column, precisely on the membrane. The column was then centrifuged for a minute at 12,600 rpm to elute the RNA from the membrane. The eluted RNA was kept in a tube labelled accordingly and kept in a -80°C freezer before the next step of analysis.

3.4 Reverse transcription-polymerase chain reaction (RT-PCR)

The RT-PCR analysis method in this study was based on protocols described by Reynes et al. (2003), Ayers et al. (2006), and Saxena et al. (2008). The detection of DENV involved two separate steps which were the reverse transcription of the viral RNA to produce the first strand cDNA and two steps of semi-nested PCR for virus detection and serotyping. The PCR primers used in this study were based on primers developed by Lanciotti et al. (1992) and Saxena et al. (2008), as shown in Table 3.2. These primers are widely used in DENV related studies, which includes two consensus primers that targets the capsid and pre-membrane (C/prM) genes (D1 and D2) and four serotype specific primers (TS1, TS2, TS3, and TS4) (Sarkar et al., 2012; Organji et al., 2017; Vicente-Santos et al., 2017).

Table 3.2: Sequences of the consensus primers (D1 and D2) and the dengue-serotype specific primers (TS1, TS2, TS3, and TS4) (Lanciotti et al. (1992), and Saxena et al. (2008))

Primer	Sequence	Expected size of DNA product (bp)
D1	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3'	511
D2	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	511
TS1	5'-CGTCTCAGTGATCCGGGGG-3'	482(D1 and TS1)
TS2	5'-CGCCACAAGGGCCATGAACAG-3'	119(D1 and TS2)
TS3	5'-TAACATCATCATGAGACAGAGC-3'	290(D1 and TS3)
TS4	5'-CTCTGTTGTCTTAAACAAGAGA-3'	392(D1 and TS4)

3.4.1 Reverse transcription (RT) of DENV RNA for synthesis of first strand c-DNA

Since the DENV is a single-stranded RNA virus, it is unable to be amplified. Therefore, the RNA must be converted to a double-stranded DNA to be amplified, which is through reverse transcription. A master mix was prepared for the RT reaction by using a complete RT kit (Promega GoScript® Reverse Transcription System). The master mix was prepared inside a biosafety cabinet (BSC). Before starting, the BSC was sterilised by wiping the sidewalls and bench surface down with 70% alcohol and turning on the UV lights for 15 minutes to avoid any cross-contamination. All the RT reagents were kept on ice, as they are temperature sensitive, until they were thawed and ready for use. A 1.5 ml microcentrifuge tube for the master mix and 0.2 ml PCR tubes were labelled accordingly to the number of samples being tested. The amount of reagents required for each sample is summarised in Table 3.3. The amount of master mix prepared was based on the number of samples being tested. For example, if 10 samples were to be tested, the master mix was prepared for 11 samples.

Table 3.3: The content of the master mix for each tube per RT reaction.

Reagent	Volume per reaction (µL)	Final concentration
Nuclease-free water	4.4	-
5X RT buffer	3	1X
dNTP mix (10mM each dNTP)	0.2	500µM (125µM each)
D2 primer (10µM)	0.4	0.25µM
Rnase inhibitor (RNasin)	1	20 unit
Final total	9	

Once all the reagents were added, the mixtures were mixed thoroughly by pipetting and centrifuged briefly for 2-5 seconds to remove any unwanted air bubbles. Five μL of the extracted RNA were added to each 0.2 mL PCR tube, followed by 9 μL of the master mix, and 1 μL of Moloney Murine Leukemia Virus (M-MLV) Reverse Transcriptase enzyme. The enzyme was added last to avoid any premature degradation. The tubes were centrifuged briefly to remove air bubbles and for the reagents to mix well. Then, the tubes were transferred to a thermal cycler (Applied Biosystems® Veriti® Thermal Cycler) set to a specified programme as shown in Table 3.4.

Table 3.4: The programme settings on the thermal cycler for the RT reaction.

Reaction step	Time (min)	Temperature (°C)
Heating	1	42.0
Reverse transcription	60	42.0
Hold	∞	4.0

Once the reaction was completed, the samples were taken out and place in ice immediately. The samples were then analysed again using the semi-nested PCR to detect any presence of DENV or kept in a -20°C freezer.

3.4.2 PCR: First round of semi-nested PCR to detect presence of DENV in samples.

The first round of PCR amplifies the DENV c-DNA. The preparation of the PCR master mix is shown in Table 3.5. The preparation was done in a BSC sterilized with 70% alcohol by wiping down the sidewalls and bench surface and turning on the UV light for 15 minutes. Before starting, the reagents and samples were thawed and place on ice

immediately. 0.2 mL PCR tubes for the samples were labelled accordingly including a 1.5 mL microcentrifuge tube for the master mix.

Table 3.5: The content of the master mix for each tube per PCR reaction.

Reagent	Volume per reaction (μL)	Final concentration
Nuclease-free water	26.6	-
MgCl₂ (25mM)	5	2.5mM
dNTP mix (10mM each dNTP)	1	0.2mM each
D1 primer	1	0.2mM
D2 primer	1	0.2mM
5X Taq Green Buffer	10	1X
Final total	44.6	

The mixture was thoroughly mixed and centrifuged briefly to remove any air bubbles. 5 μL of the RT product were then added to the PCR tubes, followed by 44.6 μL of the master mix. Lastly, 0.4 μL of the Taq DNA polymerase (Promega GoTaq® Flexi DNA Polymerase 500 units) was added to the mixture. The tubes were then briefly centrifuged to remove any air bubbles formation. A DENV positive control and negative control was also included in the test. The tubes were subsequently transferred to a thermal cycler (Applied Biosystems® Veriti® Thermal Cycler) set to a specified programme as shown in Table 3.6.

Table 3.6: The programme settings on the thermal cycler for the first round semi-nested PCR.

Reaction step	Time (min)	Temperature (°C)	Cycles
Heating	1	50	-
Initial denaturation	5	95	-
Denaturation	1	94	35
Annealing	1	55	
Extension	1	72	
Final extension	10	72	-
Hold	∞	4	-

Once the reaction has been completed, the sample tubes were taken out of the thermal cycler. They were either kept in a -20°C freezer or immediately visualized through an agarose gel electrophoresis.

3.4.3 Agarose gel electrophoresis

The amplification of the DENV c-DNA was visualised through agarose gel electrophoresis. The gel was prepared by mixing 1.6g of agarose powder (Vivantis Agarose Catalogue No: PC0701-1KG) with 100 mL of 0.5X Tris-Borate-EDTA or TBE buffer, to make a 1.6% gel concentration. The mixture was heated thoroughly for 1 minute and 40 seconds in a microwave oven. The melted gel was allowed to cool slightly and 10 µL of SYBR® Safe DNA gel stain (Invitrogen Lot 1879547) was added. Then the mixture was transferred to a casting tray (with comb attached) and left to hardened for 30 minutes. Once hardened, the gel was placed in a horizontal electrophoresis tank, using 0.5X TBE as the running buffer. A 100 bp DNA ladder (Promega BenchTop 100bp DNA ladder) was added to the first well, the DENV positive control on the second well, the negative control on the third well, and the samples on the rest of the wells. The gel electrophoresis was

conducted for an hour at 100 volts. When the electrophoresis has been completed, the gel was visualized using a Syngene UV transilluminator under dark conditions in a dark room. The bands appearing in the gels analysed was recorded and the image printed to be kept for future use.

3.4.4 PCR: Second round of semi-nested PCR for DENV molecular typing.

Samples positive for the first round of PCR will be subjected to another round of PCR. This part uses DENV serotype-specific primers (TS1, TS2, TS3, and TS4) to identify the specific DENV serotype in the samples. Similar to the previous PCR step, a master mix was first set up with the contents as shown in Table 3.7. All 0.2 mL PCR tubes were labelled accordingly including a 1.5 mL microcentrifuge tube for the master mix. In addition, all frozen reagents and samples were thawed first-hand and placed immediately on ice before starting.

Table 3.7: The content of the master mix for each tube per PCR reaction.

Reagent	Volume per reaction (µL)	Final concentration
Nuclease-free water	23.6	-
MgCl ₂ (25mM)	5	2.5mM
dNTP mix (10mM each dNTP)	1	0.2mM each
D1 Primer (10 µM)	1	0.2mM
TS1 Primer (10 µM)	1	0.2mM
TS2 Primer (10 µM)	1	0.2mM
TS3 Primer (10 µM)	1	0.2mM
TS4 Primer (10 µM)	1	0.2mM
5X Taq Green Buffer	10	1X

Final total	40.6
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The amount of master mix prepared was based on the number of samples being tested. Five μL of the PCR product was added to each corresponding PCR tubes, followed by 40.6 μL of the master mix. Lastly, 0.4 μL of the Taq DNA polymerase was added to the mixture. Once done, the tubes were vortexed briefly and centrifuged for 2-5 seconds to remove any air bubbles. The tubes were later transferred to a thermal cycler set to a specified programme as shown in Table 3.8.

Table 3.8: The programme settings on the thermal cycler for the second round semi-nested PCR.

Reaction step	Time (min)	Temperature ($^{\circ}\text{C}$)	Cycles
Heating	1	50	-
Initial denaturation	5	94	-
Denaturation	1	94	25
Annealing	1	55	
Extension	1	72	
Final extension	10	72	-
Hold	∞	4	-

Once the reaction has been completed, the sample tubes were taken out of the thermal cycler. They were either kept in a -20°C freezer or immediately visualized through an agarose gel electrophoresis, as described previously in Section 3.5.3.

3.5 Data analysis

Positive samples were analysed for its minimum infection rate (MIR). Only positive pools of female or male *Aedes* mosquitoes, both field-captured and emerges, were calculated for

their MIR. The MIR was estimated from the number of female/male mosquitoes in a positive pool divided by the number of female/male mosquitoes tested multiplied by 1000.

$$\text{MIR} = \frac{\text{DENV Positive Female/Male Mosquito Pool}}{\text{Total Number of Female/Male Mosquito}} \times 1000$$

Furthermore, DENV positive samples were also confirmed through DNA sequencing. The samples were sent out to a commercial laboratory (Apical Scientific Sdn. Bhd.) for gene sequencing. The retrieved results were checked for its authenticity using National Centre for Biotechnology Information's (NCBI) nucleotide BLAST programme function and comparing it with sequences available in the GenBank database. Multiple alignment and phylogenetic analysis was also performed on positive samples.

CHAPTER 4

RESULTS

4.1 DENV in field-caught *Aedes* mosquitoes from Sibuland Miri.

4.1.1 Field-caught female adult *Aedes* mosquitoes.

A total of 517 female *Aedes* mosquitoes were captured in 10 localities across Sibuland division and a total of 660 female *Aedes* mosquitoes were caught in 9 localities in Miri division. Furthermore, all mosquitoes captured were identified as *Ae. albopictus* and there was no *Ae. aegypti* or any other *Aedes* mosquitoes identified. All the captured adult female *Ae. albopictus* were screened for DENV using conventional RT-PCR. The mosquitoes were tested in pools of 15-20 mosquitoes per sample tube.

A total of 25 pools of adult mosquitoes from Sibuland were screened for DENV (Table 4.1). Out of the 25 pools analysed, only 2 pools were positive for DENV, which is one pool (n=20) from Jalan Tong Sang and one pool (n= 20) from Sibuland Lake Garden, as indicated by a 511 bp DNA band in the gel electrophoresis images (Figure 4.1 and Figure 4.2). The MIR was calculated for both samples and indicated an MIR of 11.8 and 12.5 for Jalan Tong Sang and Sibuland Lake Garden respectively. As for Miri, 37 (n= 660) pools of adult mosquitoes (Table 4.2) were screened for DENV but only one pool (n=20) from Taman Ceria, Permyjaya was positive for DENV, indicated by a clear 511 bp DENV c-DNA band in the gel electrophoresis image (Figure 4.3). The MIR was estimated for the DENV positive sample and indicated a value of 12.5. All the DENV positive samples were then serotyped using a second round of semi-nested PCR with serotype specific primers. The

results obtained showed that all the positive samples consisted of DENV serotype 2 (DENV-2), as indicated by a 199bp DNA band in the gel electrophoresis images (Figure 4.4 and Figure 4.5).

Only two pools from Sibul and one pool from Miri could be observed with DENV-2. The 2 pools from Sibul consisted of one pool (23.5%) from Jalan Tong Sang and one pool (25.0%) from Sibul Lake Garden, and for Miri, one pool (25.0%) from Taman Ceria, Permyjaya. The MIR for these positive samples were estimated at 11.8 and 12.5 from the two pools from Sibul and 12.5 from the one positive pool from Miri.

Table 4.1: The percentage, presence, and MIR of DENV-2 in pools of field-caught female *Ae. albopictus* from 10 localities in Sibul.

Location	No. of pools	No. of female mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Jalan Sungai Antu	2	43	<i>Ae. albopictus</i>	0	-	-
Jalan Maludan	2	38	<i>Ae. albopictus</i>	0	-	-
Rejang Park	1	23	<i>Ae. albopictus</i>	0	-	-
Jalan Merdeka	5	99	<i>Ae. albopictus</i>	0	-	-
Jalan Tiong Hua	3	68	<i>Ae. albopictus</i>	0	-	-
Jalan Ulu Oya	1	21	<i>Ae. albopictus</i>	0	-	-
Jalan Tong Sang	4	85	<i>Ae. albopictus</i>	23.5	DENV-2	11.8
Jalan Teku	2	36	<i>Ae. albopictus</i>	0	-	-
Jalan Salim	1	24	<i>Ae. albopictus</i>	0	-	-
Sibul Lake Garden	4	80	<i>Ae. albopictus</i>	25.0	DENV-2	12.5
Total	25	517				

Table 4.2: The percentage, presence, and MIR of DENV-2 in pools of field-caught female *Ae. albopictus* from 9 localities in Miri.

Location	No. of pools	No. of female mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Bandar Baru Permyjaya	3	52	<i>Ae. albopictus</i>	0	-	-
Taman Ceria, Permyjaya	4	80	<i>Ae. albopictus</i>	20.0	DENV-2	12.5
Taman Merbau	1	19	<i>Ae. albopictus</i>	0	-	-
Jalan Dato Permaisuri	7	131	<i>Ae. albopictus</i>	0	-	-
Taman Wah Shin	9	159	<i>Ae. albopictus</i>	0	-	-
Kampung Pujut	2	27	<i>Ae. albopictus</i>	0	-	-
Tanjung Batu	1	20	<i>Ae. albopictus</i>	0	-	-
Jalan Sekolah Anchi	2	35	<i>Ae. albopictus</i>	0	-	-
Jalan Desa Senadin	8	137	<i>Ae. albopictus</i>	0	-	-
Jalan Shangrila	8	137	<i>Ae. albopictus</i>	0	-	-
Total	37	660				

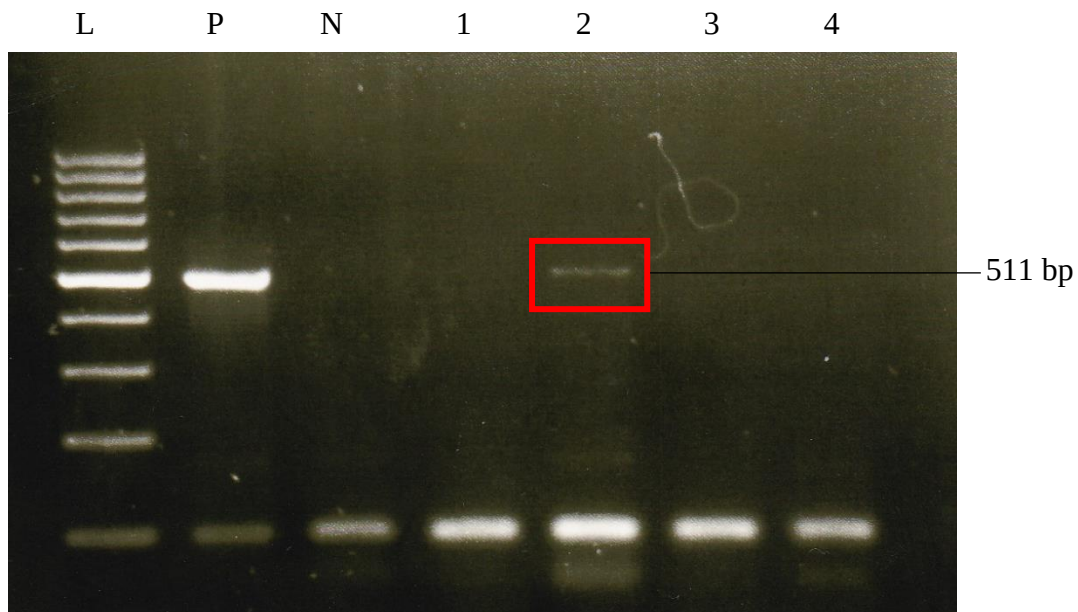


Figure 4.1: Gel electrophoresis image of female *Ae. albopictus* pools from Jalan Tong Sang, Sibuluan division. One pool shows positive result indicated by a 511bp c-DNA band of DENV. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 2: Positive sample showing 511bp DENV c-DNA band; Lane 1, 3, and 4: Negative samples)

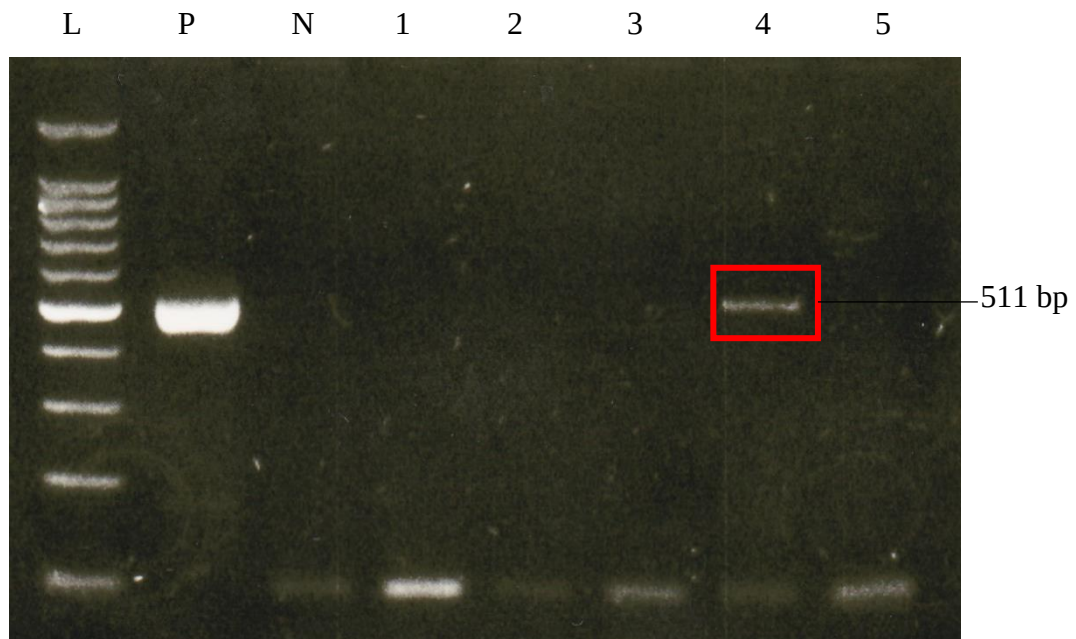


Figure 4.2: Gel electrophoresis image of female *Ae. albopictus* pools from Sibu Lake Garden, Sibul division. One pool shows positive result indicated by a 511bp c-DNA band of DENV. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N; Negative control; Lane 4: Positive sample showing 511bp DENV c-DNA band; Lane 1, 2, 3, and 5: Negative samples)

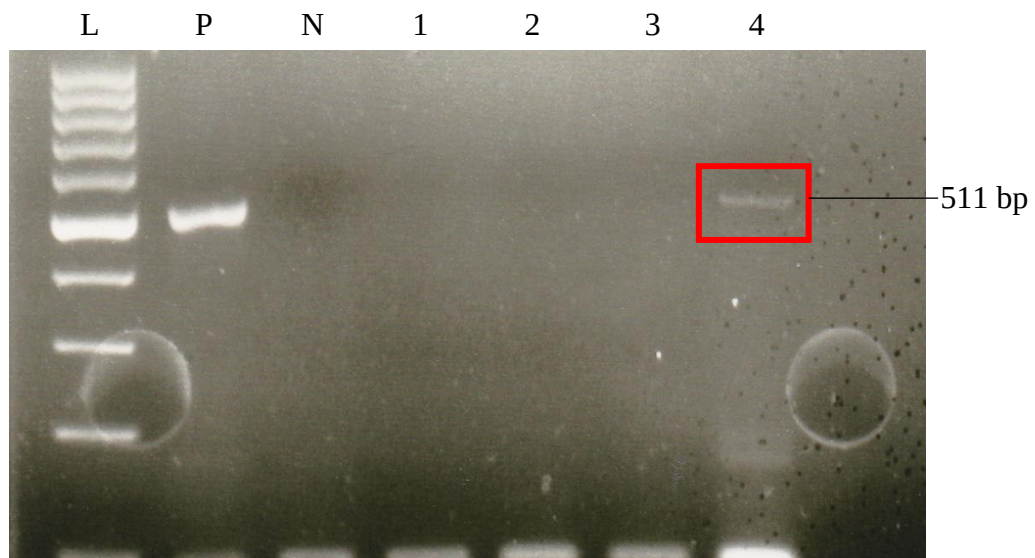


Figure 4.3: Gel electrophoresis image of female *Ae. albopictus* pools from Taman Ceria Permyjaya, Miri division. One pool shows positive result indicated by a 511bp c-DNA band of DENV. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N; Negative control; Lane 4: Positive sample showing 511bp DENV c-DNA band; Lane 1, 2, and 3: Negative samples)

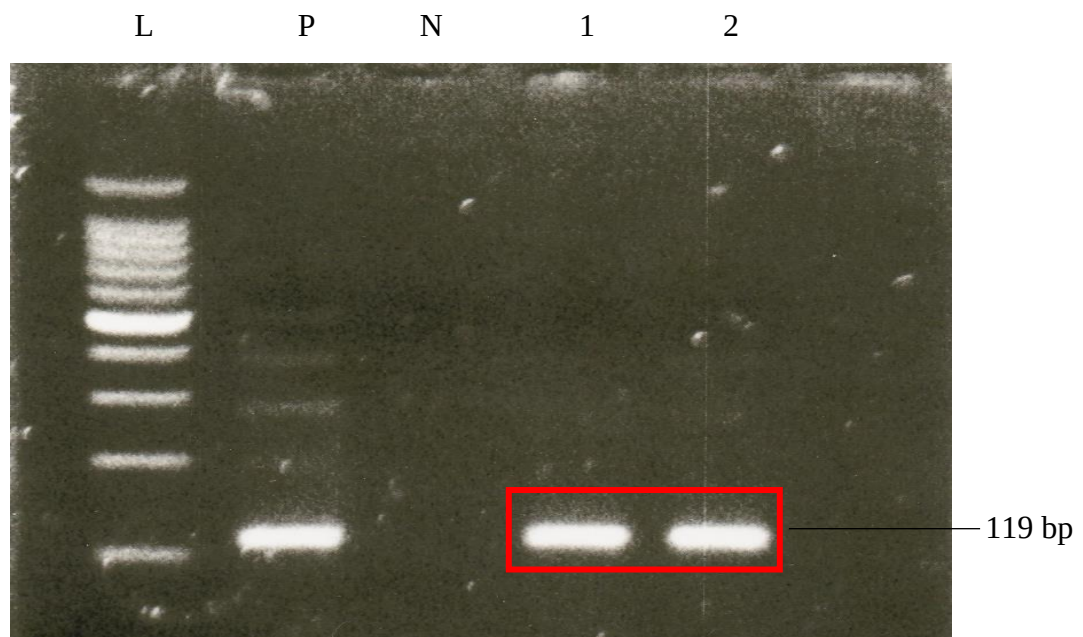


Figure 4.4: Gel electrophoresis image showing the serotyped DENV positive mosquito pools from Jalan Tong Sang and Sibu Lake Garden respectively. Both pools were positive for DENV-2, as indicated by the 119bp DENV c-DNA band. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1, and 2: Positive samples showing 119bp DENV c-DNA band.)

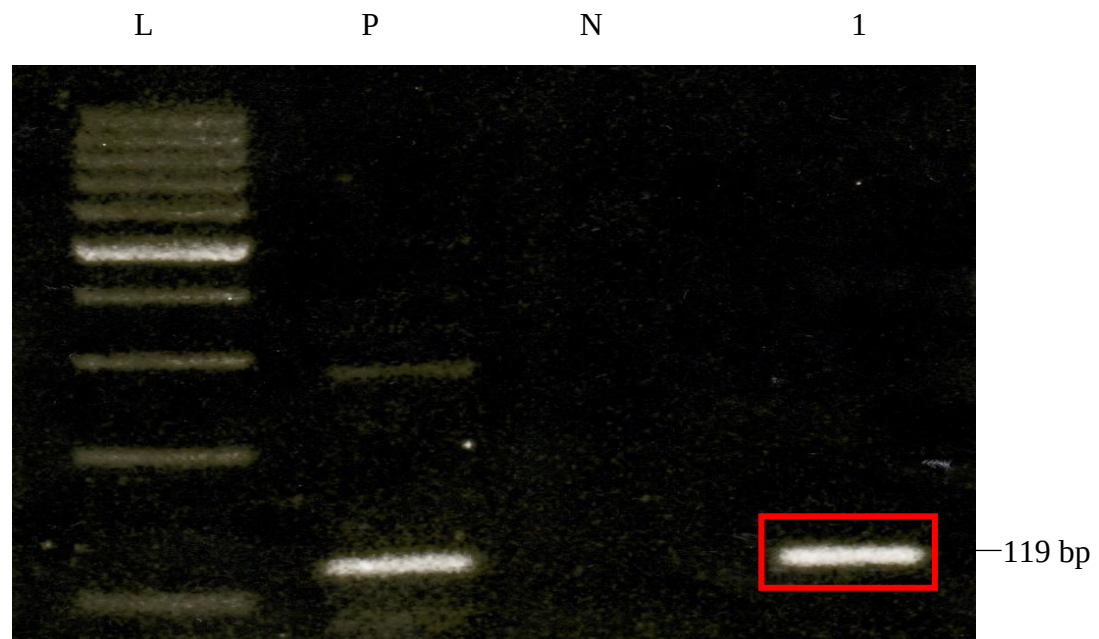


Figure 4.5: Gel electrophoresis image showing the serotyped DENV positive mosquito pool from Taman Ceria Permyjaya, Miri. The pool was positive for DENV-2, as indicated by the 119bp DENV c-DNA band. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1: Positive sample showing 119bp DENV c-DNA band.)

4.1.2 Field-caught male *Aedes* mosquitoes

A total of 254 male *Aedes* mosquitoes were caught in 10 localities across Sibu division and a total of 303 male *Aedes* mosquitoes were caught in 9 localities in Miri division. All mosquitoes were identified as *Ae. albopictus*. No *Ae. aegypti* or any other *Aedes* species mosquitoes were caught. Male mosquitoes were also screened for DENV using RT-PCR to identify any possibility of transovarial transmission, as male mosquitoes contract the virus through this mode of transmission. The mosquitoes were tested in pools of 15-20 whole bodied mosquitoes comprising of 11 and 15 pools in total from Sibu and Miri divisions respectively (Table 4.3 and Table 4.4). There was no presence of DENV in all the male *Ae. albopictus* tested, as no 511 bp band appeared in the gel electrophoresis image, hence the MIR could not be calculated.

Table 4.3: Absence of DENV in pools of field-caught male *Ae. albopictus* from 10 localities in Sibu

Location	No. of pools	No. of male mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Jalan Sungai Antu	1	16	<i>Ae. albopictus</i>	0	-	-
Jalan Maludan	1	19	<i>Ae. albopictus</i>	0	-	-
Rejang Park	0	4	<i>Ae. albopictus</i>	0	-	-
Jalan Merdeka	2	45	<i>Ae. albopictus</i>	0	-	-
Jalan Tiong Hua	0	12	<i>Ae. albopictus</i>	0	-	-
Jalan Ulu Oya	0	11	<i>Ae. albopictus</i>	0	-	-
Jalan Tong Sang	4	70	<i>Ae. albopictus</i>	0	-	-
Jalan Teku	0	10	<i>Ae. albopictus</i>	0	-	-
Jalan Salim	0	10	<i>Ae. albopictus</i>	0	-	-
Sibu Lake Garden	3	57	<i>Ae. albopictus</i>	0	-	-
Total	11	254				

Table 4.4: Absence of DENV in pools of field-caught male *Ae. albopictus* from 9 localities in Miri.

Location	No. of pools	No. of male mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Bandar Baru	1	17	<i>Ae. albopictus</i>	0	-	-
Permyjaya						
Taman Ceria,	1	25	<i>Ae. albopictus</i>	0	-	-
Permyjaya						
Taman Merbau	2	37	<i>Ae. albopictus</i>	0	-	-
Jalan Dato	0	6	<i>Ae. albopictus</i>	0	-	-
Permaisuri						
Taman Wah Shin	4	80	<i>Ae. albopictus</i>	0	-	-
Kampung Pujut	1	20	<i>Ae. albopictus</i>	0	-	-
Tanjung Batu						
Jalan Sekolah	0	6	<i>Ae. albopictus</i>	0	-	-
Anchi						
Jalan Desa	2	32	<i>Ae. albopictus</i>	0	-	-
Senadin						
Jalan Shangrila	4	80	<i>Ae. albopictus</i>	0	-	-
Total	15	303				

4.2. Transovarial DENV in field-collected immature of *Aedes* mosquitoes

4.2.1 Laboratory bred adult female mosquitoes emerged from immatures

Immature mosquitoes were collected from either natural or artificial water containers in similar localities to the field-caught adults for Sibu and Miri divisions. The *Aedes* mosquito larvae and pupae were collected from various type of containers such as car tyres, plastic containers, waste plastic bags, discarded food containers, plastic bottles, flower pots, paint buckets, food cans, and coconut shells (Appendix A: Table A1 and Table A2). The pH and temperature of the water was also recorded. The immature mosquitoes preferably thrive in water with pH ranging from 6.6 to 8.6 with temperature range of 25.0°C to 30.0°C. Collectively, a total of 627 and 812 adult female mosquitoes emerged

from immature mosquitoes collected from Sibu and Miri respectively (Table 4.5 and Table 4.6). Furthermore, all the mosquitoes were identified as *Ae. albopictus* and no *Ae. aegypti* or any other *Aedes* species mosquitoes were found. None of the mosquito pools were positive for DENV as there was no appearance of a 511 bp DNA band in the gel electrophoresis image. Therefore, the MIR could not be calculated.

Table 4.5: Absence of DENV in pools of laboratory-bred female *Ae. albopictus* from 10 localities in Sibu.

Location	No. of pools	No. of emerged female mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Jalan Sungai Antu	2	43	<i>Ae. albopictus</i>	0	-	-
Jalan Maludan	1	20	<i>Ae. albopictus</i>	0	-	-
Rejang Park	2	40	<i>Ae. albopictus</i>	0	-	-
Jalan Merdeka	2	45	<i>Ae. albopictus</i>	0	-	-
Jalan Tiong Hua	5	100	<i>Ae. albopictus</i>	0	-	-
Jalan Ulu Oya	2	63	<i>Ae. albopictus</i>	0	-	-
Jalan Tong Sang	4	100	<i>Ae. albopictus</i>	0	-	-
Jalan Teku	4	73	<i>Ae. albopictus</i>	0	-	-
Jalan Salim	4	86	<i>Ae. albopictus</i>	0	-	-
Sibu Lake Garden	3	57	<i>Ae. albopictus</i>	0	-	-
Total	29	627				

Table 4.6: Absence of DENV in pools of laboratory-bred female *Ae. albopictus* from 9 localities in Miri.

Location	No. of pools	No. of emerged female mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Bandar Baru	15	272	<i>Ae. albopictus</i>	0	-	-
Permyjaya						
Taman Ceria, Permyjaya	1	21	<i>Ae. albopictus</i>	0	-	-
Taman Merbau	6	120	<i>Ae. albopictus</i>	0	-	-
Jalan Dato Permaisuri	7	135	<i>Ae. albopictus</i>	0	-	-
Taman Wah Shin	4	80	<i>Ae. albopictus</i>	0	-	-
Kampung Pujut Tanjung Batu	4	74	<i>Ae. albopictus</i>	0	-	-
Jalan Sekolah Anchi	2	32	<i>Ae. albopictus</i>	0	-	-
Jalan Desa Senadin	3	52	<i>Ae. albopictus</i>	0	-	-
Jalan Shangrila	5	102	<i>Ae. albopictus</i>	0	-	-
Total	47	888				

4.2.2 Laboratory bred adult male mosquitoes emerged from immatures

A total of 675 and 820 immature mosquitoes were successfully bred to adult males from both Sibul and Miri respectively (Table 4.7 and Table 4.8). All the male mosquitoes were identified as *Ae. albopictus*, and no *Ae. aegypti* or any other *Aedes* mosquitoes were identified. All the emerged male mosquitoes were screened for transovarial DENV using RT-PCR. A total of 32 pools from Sibul and a total of 41 pools from Miri were screened all together. All the 32 pools of male mosquitoes from Sibul tested using RT-PCR were

negative for the presence of DENV as the 511 bp DNA band was absent in the gel electrophoresis image. A total of 41 pools of male mosquitoes from Miri were tested similarly but only one pool (n=21) from Taman Ceria, Permyjaya, Miri, was positive for DENV with a clear 511 bp DNA band in the gel electrophoresis image (Figure 4.6). The positive sample was subsequently serotyped and was positive for DENV-2. Figure 4.7 shows a clear 119 bp DENV-2 DNA band in the gel electrophoresis image. The positive pool was calculated for its MIR and the value was at 10.0.

Table 4.7: Absence of DENV in pools of laboratory-bred male *Ae. albopictus* from 10 localities in Sibü.

Location	No. of pools	No. of emerged male mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Jalan Sungai Antu	2	50	<i>Ae. albopictus</i>	0	-	-
Jalan Maludan	2	35	<i>Ae. albopictus</i>	0	-	-
Rejang Park	2	40	<i>Ae. albopictus</i>	0	-	-
Jalan Merdeka	3	54	<i>Ae. albopictus</i>	0	-	-
Jalan Tiong Hua	5	110	<i>Ae. albopictus</i>	0	-	-
Jalan Ulu Oya	2	70	<i>Ae. albopictus</i>	0	-	-
Jalan Tong Sang	5	95	<i>Ae. albopictus</i>	0	-	-
Jalan Teku	4	86	<i>Ae. albopictus</i>	0	-	-
Jalan Salim	4	73	<i>Ae. albopictus</i>	0	-	-
Sibü Lake Garden	3	62	<i>Ae. albopictus</i>	0	-	-
Total	32	675				

Table 4.8: Percentage of positive pools, presence, and MIR of DENV-2 from laboratory-bred male *Ae. albopictus* from 9 localities in Miri.

Location	No. of pools	No. of emerged female mosquitoes	Species identified	Percentage of positive pools (%)	Presence of DENV	MIR
Bandar Baru Permyjaya	12	231	<i>Ae. albopictus</i>	0	-	-
Taman Ceria, Permyjaya	1	21	<i>Ae. albopictus</i>	100.0	DENV-2	47.6
Taman Merbau	6	115	<i>Ae. albopictus</i>	0	-	-
Jalan Dato Permaisuri	6	120	<i>Ae. albopictus</i>	0	-	-
Taman Wah Shin	4	75	<i>Ae. albopictus</i>	0	-	-
Kampung Pujut Tanjung Batu	2	49	<i>Ae. albopictus</i>	0	-	-
Jalan Sekolah Anchi	1	20	<i>Ae. albopictus</i>	0	-	-
Jalan Desa Senadin	2	43	<i>Ae. albopictus</i>	0	-	-
Jalan Shangrila	5	106	<i>Ae. albopictus</i>	0	-	-
Total	39	780				

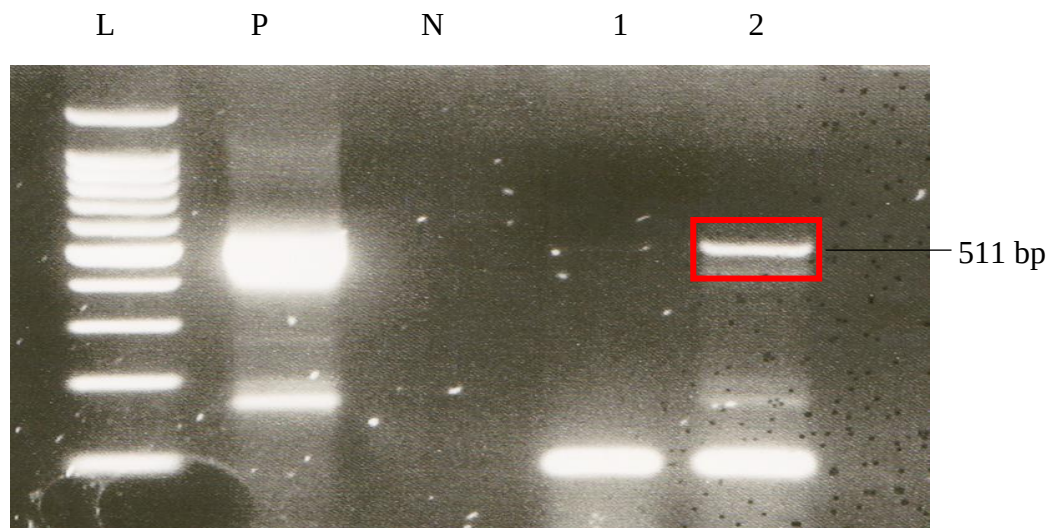


Figure 4.6: Gel electrophoresis image of DENV positive male adult *Ae. albopictus* pools emerged from immatures from Taman Ceria, Permyjaya, Miri. One pool shows positive result indicated by a 511bp c-DNA band of DENV. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N; Negative control; Lane 2: Positive sample showing 511bp DENV c-DNA band; Lane 1: Negative sample.

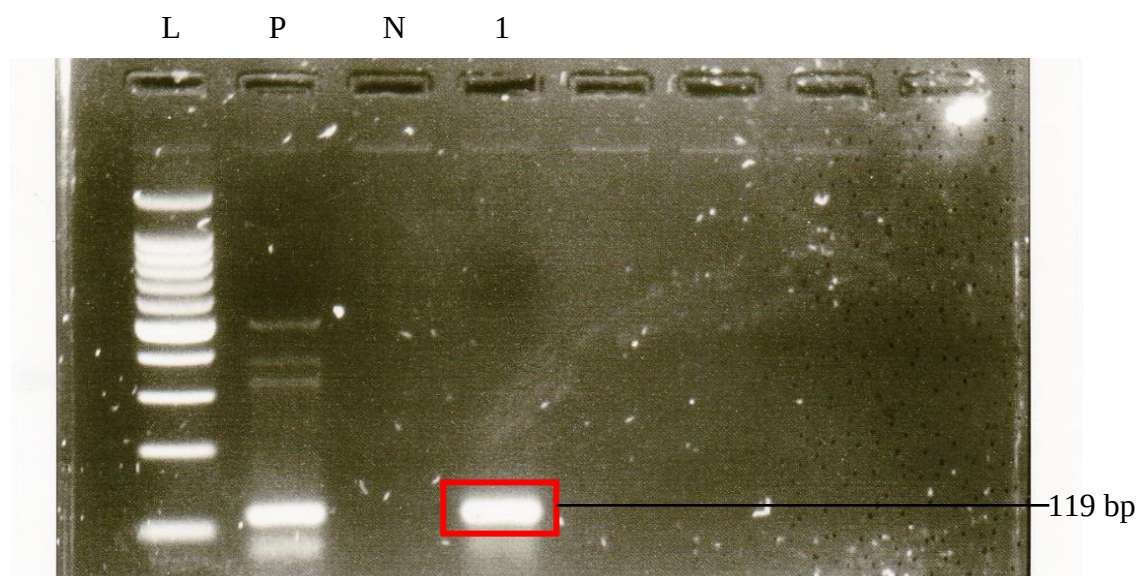


Figure 4.7: Gel electrophoresis image showing the serotyped DENV positive adult male *Ae. albopictus* emerged from immatures mosquito pool from Taman Ceria Permyjaya, Miri. The pool was positive for DENV-2, as indicated by the 119bp DENV c-DNA band. (Lane L: 100bp DNA ladder; Lane P: Positive control; Lane N: Negative control; Lane 1: Positive sample showing 119bp DENV c-DNA band.)

4.3 Gene sequencing and phylogenetic analysis of DENV-2 from Sibul and Miri

4.3.1 Gene sequence of DENV-2

DENV positive samples were assigned with each individual code and sequenced individually. The samples consisted of three pools of field-caught female adult *Ae. albopictus* from Jalan Tong Sang (JTSSBUAFD2), Sibul, Sibul Lake Garden (SLGSBUAFD2) and Taman Ceria, Permyjaya, Miri (TCPMYYAFD2) respectively, and one pool of adult male *Ae. albopictus* emerged from immatures from Taman Ceria, Permyjaya, Miri (TCPMYYIMD2). The DENV-2 sequence from this study were checked using NCBI Nucleotide BLAST function and aligned with 36 similar reference sequences from GenBank using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) software (Edgar, 2004). Figure 4.8 shows the aligned sequences.

JTSSBUAFD2(Sample)	CGGA-G-TGATGTACACAGCTGAC-AAGAGA-TCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCGATGTTTT-----
SLGSBUAFD2(Sample)	CGGAAG-TGATGTACACAGCTGAC-AAGAGA-TCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCGACAAAA-----
TCPMYAFD2(Sample)	AGGCGGGTGATGTA-ACAGCTGAC-AAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCGA-----
TCPMYIMD2(Sample)	CGGCGGATGATGT-CACAGCTGAC-AAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCGA-----
FM210217/Vietnam/2008	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
Z74048/Vietnam/1996	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY586693/Thailand/1995	-----ACAGCTGACGAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY586692/Thailand/1997	-----ACAGCTGACGAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY586691/Thailand/1998	-----ACAGCTGACGAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY586688/Thailand/1998	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY586686/Thailand/2000	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KU094070/China/2015	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
GQ199901/Papua New Guinea/2008	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
GQ199900/Papua New Guinea/2008	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
GQ199899/Papua New Guinea/2008	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KM204118/Papua New Guinea/1944	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
FJ390389/Papua New Guinea/1944	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
AF038403/Papua New Guinea/1944	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF704358/Cuba/1981	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
JF730055/USA/2009	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461767/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461766/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461765/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461764/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461762/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461761/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KY461768/Costa Rica/2013	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
JQ710659/Brazil/2001	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF704357/Cuba/1981	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF704356/Cuba/1981	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF704355/Cuba/1981	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGCGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF955388/Nicaragua/2001	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF955373/Puerto Rico/1997	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF955363/Puerto Rico/1997	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAAGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
KF955360/Puerto Rico/1997	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
JN796245/USA/2008	-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
JF730054/USA/2009	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
FJ906969/Asian/2009	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
FJ906968/Asian/2009	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----
FJ906966/Asian/2009	TGTACA-----ACAGCTGACAAAGAGATTCTCACTTGGAAATGCTGCAGGGACGAGGACCATTAAAACTGTTTCATGGCCCTTGTGGCG-----

4.3.2. Phylogenetic analysis

To identify the origin of the DENV-2 isolates in this study, phylogenetic analysis was performed using the Molecular Evolutionary Genetics Analysis (MEGA v10.0.5) software (Kumar et al., 2018). The analysis was performed on the sample sequences and 36 reference sequences from GenBank. Phylogenetic tree was assembled using the maximum parsimony method with subtree-pruning-regrafting (SPR) and 100 bootstrap replications. Figure 4.9 shows the maximum parsimony tree. Information on genotype of all sequences were obtained from the NIAID Virus Pathogen Database and Analysis Resource (ViPR) website (www.viprbrc.org) (Pickett et al., 2012). The three DENV-2 isolates from Jalan Tong Sang, Sibuluh (JTSSBUAFD2) and Taman Ceria Permyjaya, Miri (TCPMYYAFD2, TCPMYYIMD2) cluster together in the Asian genotype, the same genotype with DENV strains reported from Vietnam, Thailand, and Papua New Guinea. Furthermore, the three isolates are closely related to strains isolated from Vietnam (Z74048, FM210217) and the DENV-2 strain from Jalan Tong Sang and two strains from Miri clustered into one group indicating similarities. The DENV-2 isolate from Sibuluh Lake Garden, Sibuluh (SJGSBUAFD2), however, was grouped together with a DENV-2 strain isolated from Nicaragua of the Asian/American genotype.

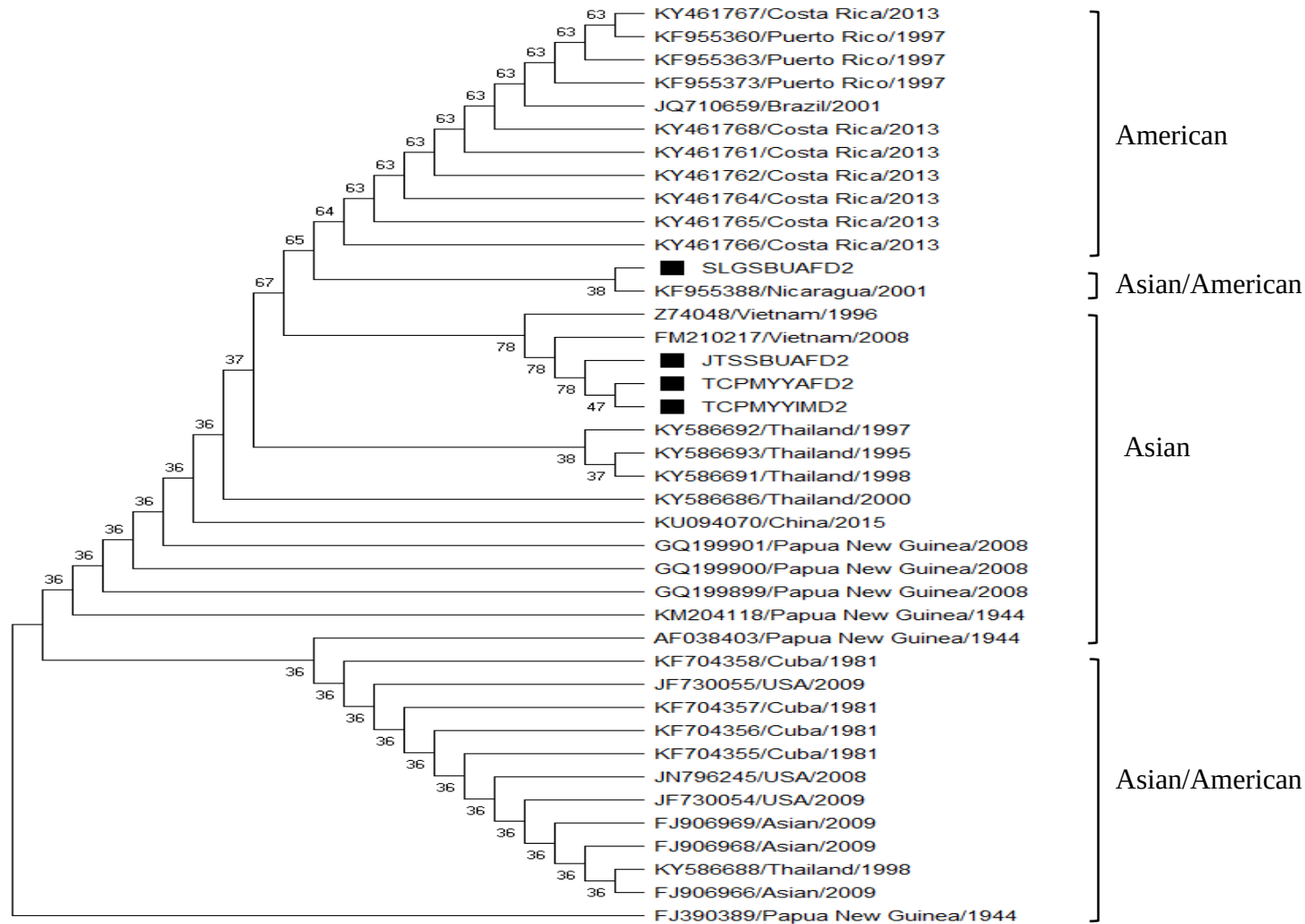


Figure 4.9: Maximum parsimony tree of 40 DENV-2 C/prM gene sequences (4 from this study, 36 from GenBank). Black rectangles indicate the isolates from this study (2 from Sibiu, 2 from Miri). Bootstrap values are indicated at the respective nodes. The sequences were labelled according to the accession number/country/year of detection.

CHAPTER 5

DISCUSSION

5.1 *Aedes albopictus* in all selected localities in Sibü and Miri divisions of Sarawak.

One of the main purposes of this study is to identify the major circulating *Aedes* mosquitoes related to dengue within the selected locality of the study area. The locality selected mainly comprised of urban and suburban residential areas and was conducted outdoors close to human dwelling areas. The collection of mosquito samples, both field-caught adults and collected immatures from natural breeding sites, was conducted from September 2016 to December 2017. From the results obtained, only one species was identified in all the samples collected, that is *Ae. albopictus*. An ovitrap surveillance study conducted by Lau et al. (2017), presented a similar result where only *Ae. albopictus* were found in higher densities in urban and suburban residential areas and no *Ae. aegypti* was detected. Studies conducted three decades ago also indicated that *Ae. albopictus* were widespread across the state of Sarawak and presented in higher density than *Ae. aegypti* (Chang & Jute, 1982). Another study conducted by Chang and Jute (1994) also observed that *Ae. albopictus* is much more abundant than *Ae. aegypti*. This may be due to origin of the species itself where *Ae. albopictus* is an indigenous species of Asia, including Malaysia (Bonizzoni et al., 2013), which explains why we only found *Ae. albopictus* in this study. In addition, *Ae. aegypti* preferably breed and feed indoors (Entomology and Nematology Department, UF, 2008). Therefore, it is likely possible to encounter *Ae. aegypti* if indoor sampling was also conducted in this study. With the information available, multiple strategies can be developed to reduce the population of *Ae. albopictus* in Sarawak. These

strategies may include identifying favourable oviposition sites, community-based control programmes, and education of prevention strategies. Biological control nowadays are some of the current vector control strategies. For example, the Institute of Medical Research, Malaysia developed a method in reducing the population of *Ae. aegypti* by infecting female and male mosquitoes with different types of *Wolbachia* bacteria strains that can impair viral development and reduce mosquito life span (Noor Afizah & Lee, 2013). However, this vector control method has not yet been performed on the *Ae. albopictus* population in Malaysia, especially in Sarawak. A study performed in the USA have reported that there is a reduction in the population number of female *Ae. albopictus* after the release of *Wolbachia*-infected male *Ae. albopictus* in the natural environment (Mains et al., 2016). Other biological control can also be developed to specifically target the population of *Ae. albopictus* in Sarawak such as the usage of plant extracts with larvicidal or insecticidal effects.

5.2. DENV in *Ae. albopictus* population of Sibü and Miri divisions of Sarawak.

5.2.1. Field-caught adult female and male *Ae. albopictus*.

One of the objective of this study is to identify the circulating DENV and its serotype in the natural population of *Ae. albopictus* in Sibü and Miri divisions of Sarawak. Out of the 62 pools of female *Ae. albopictus* tested, only 3 pools of mosquito were positive for DENV-2. These pools comprised of one pool (23.5%) from Jalan Tong Sang, Sibü, one pool (25.0%) from Sibü Lake Garden, and one pool (25.0%) from Taman Ceria, Permyjaya, Miri. All the positive pools contained the similar numbers of individual mosquitoes, so the percentage of positive pools are quite similar. The percentage can be

higher or lower depending on the number of pools positive for DENV. A study conducted by Urdaneta et al. (2005) only showed 5.2% of positive mosquito pools out of 154 pools of mosquito collected. Study conducted by Pinheiro et al. (2005) also obtained a low percentage of positive pools where only 17.1% of the mosquito pools were tested positive for DENV-3.

There is no evidence of other studies in terms of entomological aspects in Sarawak to be compared with this study, whether DENV-2 is the dominant serotype circulating in the *Aedes* mosquito population. However, the detection of DENV in adult mosquitoes is possible and has been done as far as two decades ago. A study conducted by Chow et al. (1998) in Singapore showed that all the DENV serotypes could also be isolated from both mosquito vectors, *Ae. albopictus* and *Ae. aegypti*, tested in pools. Studies conducted by Urdaneta et al. (2005) also detected multiple dengue virus serotypes such as DENV-1, DENV-2, and DENV-3 in field-caught mosquitoes. Besides that, DENV can also be detected in single individual mosquitoes, as proven by Chung and Pang (2002), from mosquito population in Singapore.

A similar study conducted by Nur Ain Mardiah (2018) in Kuching, Sarawak also detected presence of DENV tested in single individual mosquitoes. Furthermore, the MIR for all the positive pools were also estimated for all the positive pools. The MIR value for each positive pool were 11.8, 12.5, and 12.5 for Jalan Tong Sang, Sibul, Sibul Lake Garden, and Taman Ceria, Permyjaya respectively. It can be postulated that Sibul Lake Garden, Sibul and Taman Ceria, Permyjaya, Miri has high potential of dengue transmission among the population compared to the other areas due to its higher MIR value. According to Vikram

et al., (2015) the higher the MIR, the higher the risk of dengue transmission. Areas of high MIR values in the *Aedes* mosquito population are also prone to dengue outbreaks. For example, a study conducted by Peña-Garcia et al. (2016) in Colombia reported high MIR values of up to 73.53, which is high when compared with the MIR values in this study. Male adult *Ae. albopictus* caught in this study were not detected with any presence of DENV. However, other studies have reported that all DENV serotypes can be found in male mosquitoes which is an indication that the virus is being transmitted vertically from gravid female mosquito to its progeny (Kow et al., 2001). This transmission is also termed as transovarial transmission.

5.2.2 Transovarial transmission of DENV in field-collected *Ae. albopictus* immatures

Since the purpose of this study is to detect DENV serotypes in the *Aedes* mosquito population in the study area, it is important not to miss out on the transovarial transmission of DENV in the population. All immatures collected from natural or artificial containers were collected from September 2016 to December 2017 in all localities from Sibu and Miri divisions respectively. The immatures were then bred until adults prior to laboratory testing. From the results obtained, only one pool of male *Ae. albopictus* was positive from DENV-2 which was from Taman Ceria Permyjaya, Miri. This confirms that transovarial transmission of DENV does occur at least in this area. Studies regarding transovarial transmission of DENV have been done for many years by researchers globally and can be dated back as far as three decades ago (Jousset, 1981; Khin & Than, 1983; Hull et al., 1984). Jousset (1981) demonstrated transovarial transmission of DENV-2 in *Ae. aegypti*

raised from eggs of gravid *Ae. aegypti* females infected through intrathoracic inoculation. Furthermore, Khin and Than (1983) isolated DENV-2 from male *Ae. aegypti* mosquitoes collected from the nature as larvae and raised to adults in the laboratory, showing quite the similarity to this study. Hull et al. (1984) were also able to report findings of transovarial transmission in natural populations of *Ae. aegypti* mosquitoes conducted in Trinidad. They isolated DENV-4 from *Ae. aegypti* adults raised from eggs collected from nature.

Majority of studies regarding transovarial transmission of DENV in *Aedes* mosquito population are conducted in South America and Asia where these areas are endemic for dengue (Ferreira-de-Lima & Lima-Camara, 2018). To the best of our knowledge, this might be the first study to identify transovarial infection of DENV in the *Ae. albopictus* population in Miri division, Sarawak. There are other studies conducted regarding this matter in Malaysia, but majority of these studies were conducted in other states within Peninsular Malaysia. Findings in this study was somewhat similar to a study conducted by Rohani et al. (1997) in several states across Peninsular Malaysia. They were able to identify dengue virus in adult *Ae. albopictus* emerged from immatures using the PAP staining and RT-PCR method. However, the serotypes of DENV were not mentioned.

Furthermore, the transovarial transmission of DENV could be maintained in successive generations of *Aedes aegypti* mosquitoes for up to five generations, as proved by a few studies in laboratory infected female *Ae. aegypti* (Joshi et al., 2002; Rohani et al., 2008). Moreover, a study conducted in the late 1980's observed that in laboratory conditions, DENV could also be transmitted transovarially at a higher rate in other species of orally

infected *Aedes* mosquito such as *Ae. mediovittatus*, a forest and peridomestic mosquito found in the Caribbeans (Freier & Rosen, 1988).

5.3 Circulation of DENV-2 in population of *Ae. albopictus* of Sibul and Miri divisions of Sarawak

The findings of this study were authenticated through gene sequencing and compared with available data from the GenBank, in which all were confirmed of DENV-2. Phylogenetic analysis was also performed on all the four isolates from this study. Two isolates from Sibul and Miri respectively originated from the Asian genotype. This outcome can be compared with studies conducted by Holmes et al. (2009). They identified DENV-2 isolates from Sibul was from the Cosmopolitan genotype which includes South East Asia. However, the other DENV-2 isolates from Sibul (Sibul Lake Garden) were identified to be from the Asian/American genotype. There are no current studies in Sarawak to be compared with this outcome. This could indicate possibilities of importation of other DENV strains into Sarawak.

During the entire period of this study, DENV-2 could be the current circulating DENV serotype in the *Ae. albopictus* population in the selected localities. DENV-2 was detected in the field-caught adult females and male adults emerged from immatures in the laboratory. However, there are no published data to identify the current circulating DENV serotype in the study area, so findings in this study could not be compared with current settings. Studies on human serum samples have been performed in Sarawak where DENV-2 were mostly reported in clinical isolates from Sibul but none from Miri (Holmes et al., 2009). The findings were reported back in 2002, therefore the circulating DENV serotype

may differ from today. Thus, the correlation between the positive DENV-2 mosquito samples and the current circulating DENV serotypes cannot be determined. Furthermore, studies conducted in Peninsular Malaysia specifically in the state of Selangor and the east coast region reported multiple predominant serotypes of DENV ranging from DENV-1 to DENV-3 isolated from human serum samples from dengue infection patients (Vinomarlini et al., 2011; Ab-Fatah et al., 2015). A similar study conducted in Kuala Lumpur, Malaysia and its adjacent areas with the state of Selangor indicated that DENV-4 was the predominant serotype during 2011 (Chew et al., 2012). Prior to this study, the positive female mosquitoes might be horizontally infected with DENV or could obtained the virus through transovarial infection. Horizontal infection of DENV is primary mechanism for virus maintenance and happens when a mosquito is orally infected with DENV when it feeds on viremic blood of an infected human being (Mulyatno et al., 2012). Likewise, the DENV-2 positive males discovered in this study were infected with DENV through transovarial transmission of the virus.

Furthermore, there is also a possibility that the field-caught adult mosquitoes originated from a different locality. According to the World Health Organization (2017), an average adult *Aedes* mosquito can fly up to 400 meters from where they initially emerge. In addition, with increasing development in transports, infected mosquitoes can also travel from different states or country. The only study performed regarding the circulation of DENV serotypes was reported back in 2009. Holmes et al. (2009), managed to identify co-circulation of multiple DENV serotypes in Sarawak which includes DENV-2, DENV-3, and DENV-4. However, these data were based on clinical isolates of infected patients, not from the entomological aspect

CHAPTER 6

CONCLUSION

6.1 Conclusion

This study demonstrated the presence of DENV-2 in the natural population of *Aedes* mosquitoes in the selected localities within Sibul and Miri. DENV-2 was detected in three pools of field-caught adult *Ae. albopictus* collected from Jalan Tong Sang, Sibul (23.5%), Sibul Lake Garden, Sibul (25.0%) and Taman Ceria, Permyjaya, Miri (25.0%) respectively, when screened using RT-PCR. The MIR was also estimated for each of the positive pool and the value were 1.60, 3.03, and 2.50 for Jalan Tong Sang, Sibul Lake Garden, and Taman Ceria, Permyjaya respectively. Furthermore, transovarial transmission of DENV-2 was also reported in this study in a pool of male emerged from immatures collected from the field in Taman Ceria, Permyjaya, Miri. This could be the first report of transovarial transmission of DENV in Miri. However, all other samples including field-caught male and female from other localities, adult females emerged from immatures, and adult males emerged from immatures from other localities were negative for DENV. Moreover, this study also identified that *Ae. albopictus* was the major population of *Aedes* mosquito in the selected localities within Sibul and Miri. Thus, the finding in this study suggest that DENV-2 could be the current circulating DENV serotype and *Ae. albopictus* is the major species for vectoring DENV in Sibul and Miri, along with the possibility of transovarial transmission of DENV in the study areas. However, it is hypothesized that *Ae. aegypti* is also present in the study areas and all four DENV serotypes can be isolated from the mosquito population in the study areas.

6.2 Recommendations

This study indicated that the major *Aedes* mosquito species was *Ae. albopictus* collected from outdoor sampling. Further work should be put into doing indoor trapping of mosquitoes in order to capture other *Aedes* mosquitoes such as *Ae. aegypti*, as they prefer to breed and feed indoors. DENV-2 was also detected in all positive pools of *Ae. albopictus*, which could indicate the current circulating DENV serotype in the selected locality. Conducting sample collections in more localities could increase the possibility of detecting all four DENV serotypes in the natural population of *Aedes* mosquitoes in Sibu and Miri divisions of Sarawak.

6.3 Limitations of study

One of the limitations in this study is the mosquito collection/trapping method. The collection was mostly conducted outdoor and in urban areas. Several other studies suggest that indoor trapping should also be done for a complete surveillance, however authority clearance was needed to conduct indoor trapping in the study area, thus it should be considered in the future. Expansion of sampling locality should also be considered to obtain a more representative result of the study area. Furthermore, factors such as environmental humidity, temperature, and rainfall should also be considered as these factors may affect the outcome of this study. All these factors should be taken collectively and into consideration to obtain a more reliable and non-bias result.

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APPENDICES

Appendix A

Location	Type of container	pH	Temperature (°C)
Jalan Sungai Antu	Car tyre	7.5	25.0
Jalan Maludan	Car tyre	8.0	30.0
	Discarded ceramic bowl	8.2	30.0
Rejang Park	Car tyre	8.4	26.0
Jalan Merdeka	Car tyre	7.5	26.0
	Plastic food container	7.4	25.5
	Paint bucket	8.0	25.0
Jalan Tiong Hua	Plastic food container	7.7	25.5
	Motorcycle tyre	7.4	26.0
	Glass jar	7.9	25.0
Jalan Ulu Oya	Car tyre	7.1	27.0
	Plastic flower pot	7.5	25.0
	Plastic food container	7.2	28.0
Jalan Tong Sang	Car tyre	7.5	25.0
	Paint bucket	8.0	25.0
Jalan Teku	Car tyre	7.5	26.0
	Plastic food container	7.4	26.0
	Polystyrene food container	7.5	25.5
Jalan Salim	Car tyre	8.0	26.0
Sibu Lake Garden	Plastic bottle	7.1	26.7
	Car tyre	6.6	26.7

Table A1: Types of containers, and pH and temperature of water the immature mosquitoes were collected from in Sibu.

Location	Type of container	pH	Temperature (°C)
Taman Merbau	Old paint bucket	7.8	25.0
Bandar Baru Permyjaya	Discarded food can	7.1	25.0
	Car tyre	7.8	25.0
	Coconut shell	6.6	25.0
	Waste plastic bag	8.6	25.0
Kampung Pujut Tg. Batu	Car tyre	7.6	25.0
	Polystyrene food container	7.7	25.0
Jalan Dato Permaisuri	Discarded food can	7.4	25.0
	Car tyre	8.1	25.5
Jalan Sekolah Anchi	Car tyre	8.4	25.5
Taman Wah Shin	Abandoned car	7.8	25.0
	Car tyre	7.8	25.0
	Plastic food container	8.0	25.0
Taman Ceria, Permyjaya	Discarded food can	7.5	26.0
Jalan Desa Senadin	Car tyre	7.2	25.5
Jalan Shangrila	Plastic tray	7.16	26.0
	Plastic bottle	7.5	26.0

Table A2: Types of containers, and pH and temperature of water the immature mosquitoes were collected from in Miri.

Appendix B

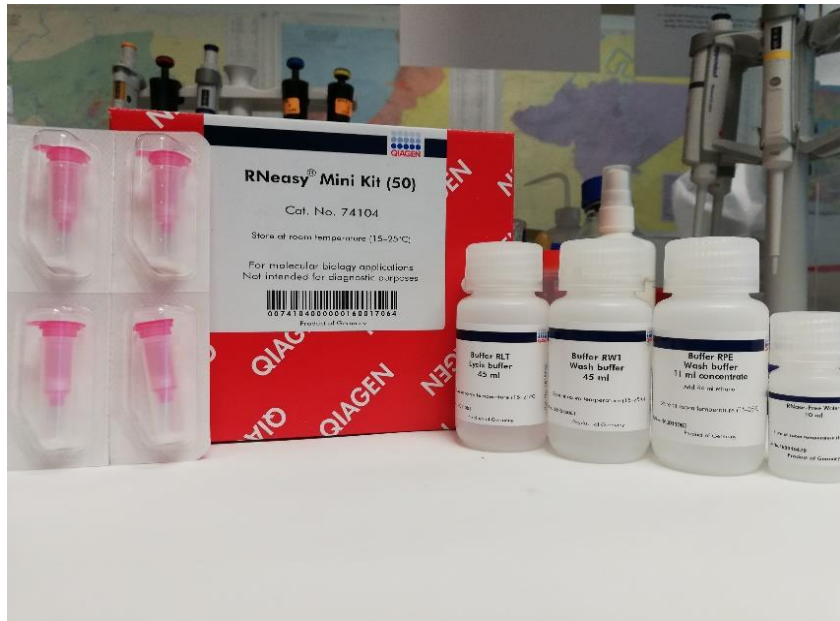


Figure B1: The Qiagen RNeasy Mini kit used to extract the viral RNA from the mosquito sample.

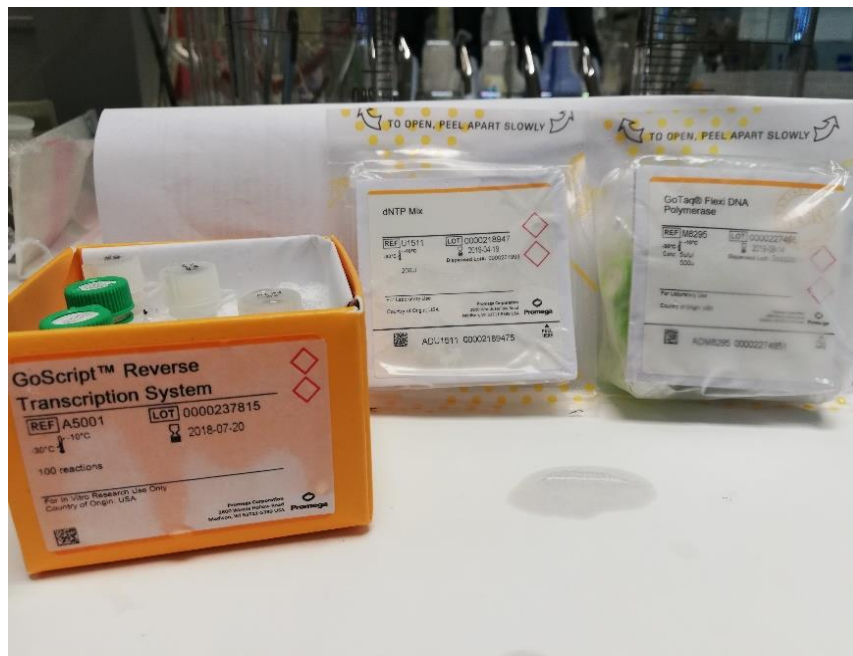


Figure B2: The reagents used to conduct the RT-PCR.



Figure B3: Containers used to transport the immature mosquitoes collected from the field back to the laboratory.



Figure B4: Sampling site at a light industrial area in Jalan Sungai Antu, Sib.



Figure B5: Sampling site at a residential area in Jalan Maludan, Sibü.



Figure B6: Sampling site at a residential area in Taman Rejang, Sibü.



Figure B7: Sampling site at a residential area in Jalan Merdeka, Sibü.



Figure B8: Sampling site at a residential area in Jalan Tiong Hua, Sibü.



Figure B9: Sampling site at a residential area in Jalan Tong Sang, Sibü.



Figure B10: Sampling site at a residential area in Jalan Teku, Sibü.



Figure B11: Sampling site at a residential area in Jalan Salim, Sibul.



Figure B12: Sampling site at a recreational park area in Sibul Lake Garden, Sibul.



Figure B13: Sampling site at a residential area in Jalan Ulu Oya, Sibul

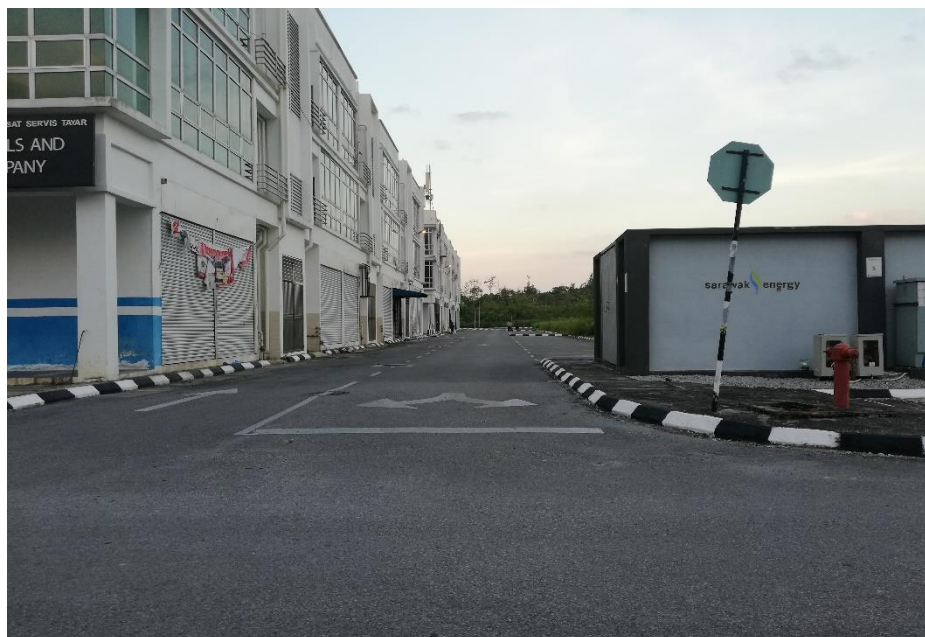


Figure B14: Sampling site at commercial area with shophouses in Bandar Baru Permyjaya, Miri.



Figure B15: Sampling site at a residential area in Taman Ceria, Permyjaya, Miri.



Figure B16: Sampling site at a residential area in Jalan Dato Permaisuri, Miri.



Figure B17: Sampling site at a residential area in Taman Merbau, Miri.



Figure B18: Sampling site at a residential area in Taman Wah Shin, Miri.



Figure B19: Sampling site at a residential area in Jalan Sekolah Anchi, Miri.



Figure B20: Sampling site at a residential area in Jalan Desa Senadin, Miri.



Figure B21: Sampling site at a residential area in Jalan Shangrila, Miri.



Figure B22: Sampling site at a Malay village area in Kampung Pujut Tanjung Batu, Miri.



Figure B23: Some of the containers where the immature mosquitoes were collected from.



Figure B24: Collection of adult mosquitoes using the HLC method.