



Faculty of Medicine and Health Sciences

Vector Status and Transovarial Transmission of Chikungunya Virus (CHIKV) and Dengue Virus (DENV) in Aedes mosquitoes in Kampung Pulau Salak and Kampung Tanjong Bako, Sarawak

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Vector Status and Transovarial Transmission of Chikungunya Virus (CHIKV)
and Dengue Virus (DENV) in Aedes mosquitoes in Kampung Pulau Salak
and Kampung Tanjong Bako, Sarawak

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DECLARATION

I declare that the work in this thesis was carried out in accordance with the regulations of Universiti Malaysia Sarawak. Except where due acknowledgements have been made, the work is that of the author alone. The thesis has not been accepted for any degree and is not concurrently submitted in candidature of any other degree.

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ABSTRACT

Chikungunya virus (CHIKV) and dengue virus (DENV) are vector-borne viruses which can be transmitted through vector mosquitoes, *Ae. aegypti* and *Ae. albopictus*. Pulau Salak in Sarawak is one of those areas affected by chikungunya outbreak in 2009. Thus, this study was conducted to determine the vector status and transovarial transmission of CHIKV in *Aedes* mosquitoes on Kampung Pulau Salak, Kuching, Sarawak, since it is one of the areas which was affected due to the outbreak of CHIKV. The process of detection of DENV was carried out on the mosquitoes as well. The research was carried out at Kampung Pulau Salak and Kampung Tanjong Bako, Kuching in which Kampung Tanjong Bako act as a negative control site in this study. Mosquito larvae were collected from ovitraps and discarded receptacles before being reared in the laboratory for emergence of adults while adult mosquitoes were sampled by using bare-leg catch method (BLC) with the help of mechanical aspirators. A total of 7834 *Aedes* mosquitoes were pooled according to species, sex and sampling location. The ovitrap index recorded on Kampung Pulau Salak was 84.3%, 96.7%, and 89.3% for the first, second and third sampling respectively. On the other hand, the ovitrap index recorded in Kampung Tanjong Bako was 67.6%, 50.0%, and 87.1% for the first, second and third sampling respectively. There was no significant difference ($p > 0.05$) of ovitrap indexes between Kampung Pulau Salak and Kampung Tanjong Bako. The highest number of species of mosquitoes collected in Kampung Pulau Salak was *Ae. albopictus* (90.07%) followed by *Ae. aegypti* (6.02%), *Culex* sp. (2.63%), and *Armigeres* sp. (1.28%) while the highest number of species of mosquitoes collected in Kampung Tanjong Bako was *Ae. albopictus* (99.93%) followed by *Ae. aegypti* (0.07%). RNA extraction and two-step Reverse Transcription Polymerase Chain Reaction (RT-PCR) were performed to detect CHIKV and DENV from the samples collected. There were 223 pools of *Aedes* mosquitoes

tested for the presence of the viruses. Chikungunya virus of East Central South African genotypes were detected in one pool of male and one pool of female (n=35) emerged *Aedes albopictus* mosquitoes collected from the ovitraps in Kampung Pulau Salak. DENV of serotype 2 was detected in one pool of male and two female pools (n=35) of emerged *Aedes albopictus* collected from Kampung Pulau Salak as well. There was no presence of CHIKV and DENV detected in the emerged *Aedes* mosquitoes collected from Kampung Tanjong Bako. None of the field-caught *Aedes* mosquitoes collected from Kampung Pulau Salak and Kampung Tanjong Bako were detected with CHIKV and DENV as well. The findings of this study showed evidence of transovarial transmission of CHIKV and DENV in *Aedes* mosquitoes in Kampung Pulau Salak. This finding may contribute to the effort in controlling the vector of CHIKV and DENV in the island as the status of the vector is currently unknown.

Keywords: Transovarial transmission, *Aedes* mosquitoes, RT-PCR, East Central South African genotypes, chikungunya virus, dengue virus

Status Vektor dan Transmisi Transovari Virus Chikungunya (CHIKV) dan Virus Denggi (DENV) dalam nyamuk Aedes di Kampung Pulau Salak dan Kampung Tanjong Bako, Sarawak

ABSTRAK

Virus chikungunya dan virus denggi merupakan virus bawaan vektor yang boleh disebarkan melalui vektor seperti nyamuk Ae. aegypti dan Ae. albopictus. Pulau Salak di Sarawak merupakan salah satu kawasan yang dijangkiti oleh wabak chikungunya pada tahun 2009. Oleh yang demikian, kajian ini dijalankan untuk mengesan status vektor dan penyebaran transovari virus chikungunya dalam nyamuk Aedes di Kampung Pulau Salak, Kuching, Sarawak. Proses pengesanan virus denggi juga dijalankan dalam kajian ini. Kajian dijalankan di Kampung Pulau Salak dan Kampung Tanjong Bako di mana Kampung Tanjong Bako berfungsi sebagai kawasan kawalan negatif. Larva nyamuk dikutip daripada pemasangan ovitrap dan bekas-bekas terbuang sebelum dipelihara di makmal sehingga peringkat dewasa manakala nyamuk dewasa ditangkap dengan menggunakan kaedah bare leg catching (BLC) dengan bantuan aspirator mekanikal. Sebanyak 7834 sampel nyamuk Aedes diasingkan dalam longgokan mengikut spesies, jantina, dan lokasi sampling. Indeks ovitrap dari Kampung Pulau Salak adalah sebanyak 84.3% dan 96.7% bagi pensampelan kali pertama dan kedua manakala indeks ovitrap untuk pensampelan kali ketiga adalah sebanyak 89.3%. Selain itu, indeks ovitrap dari Kampung Tanjong Bako adalah sebanyak 67.7% dan 50.0% bagi pensampelan kali pertama manakala indeks ovitrap untuk pensampelan kali ketiga adalah sebanyak 87.1%. Tiada sebarang perbezaan ketara ($p > 0.05$) di antara indeks ovitrap Kampung Pulau Salak dan Kampung Tanjong Bako. Spesies nyamuk yang paling banyak dikutip daripada Kampung Pulau Salak adalah Ae. albopictus (90.07%) diikuti oleh Ae. aegypti (6.02%), Culex sp. (2.63%) dan Armigeres sp. (1.28%) manakala spesies nyamuk yang paling banyak dikutip dari Kampung Tanjong Bako adalah

Ae. albopictus (99.93%) dan diikuti oleh *Ae. aegypti* (0.07%). Pengekstrakan RNA dan tindak balas rantaian polimerase dengan menggunakan transkripsi berbalik (RT-PCR) secara dua langkah telah dilaksanakan untuk mengesan virus chikungunya dan virus denggi dalam sampel yang dikutip. Sebanyak 223 longgokan nyamuk *Aedes* telah dikaji pengesanan virus chikungunya dan denggi. Virus chikungunya dari genotip East/ Central/ South/ African telah dikesan dalam satu longgokan nyamuk jantan dan satu longgokan nyamuk betina *Ae. albopictus* (n=35) yang dikutip dari Kampung Pulau Salak. Virus denggi serotip-2 juga telah dikesan dalam satu longgokan nyamuk jantan dan dua longgokan nyamuk betina (n=35) *Ae. albopictus* dari Kampung Pulau Salak. Tiada virus chikungunya dan denggi dikesan dari nyamuk *Aedes* yang dibesarkan dan dikutip dari Kampung Tanjong Bako. Tiada virus chikungunya dan denggi juga dikesan dari nyamuk dewasa yang ditangkap di lapangan dari kedua-dua buah kampung tersebut. Penemuan kajian ini menunjukkan bahawa berlakunya penyebaran transovari dalam nyamuk *Aedes* di Kampung Pulau Salak. Kajian ini boleh menyumbang kepada usaha untuk mengawal vektor virus chikungunya dan denggi di Kampung Pulau Salak memandangkan status vektor yang terkini di kawasan tersebut masih belum dikenalpasti.

Kata kunci: Penyebaran transovari, nyamuk *Aedes*, RT-PCR, genotip East Central South African, virus chikungunya dan denggi

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celcius
μL	Microliter
<i>Ae.</i>	<i>Aedes</i>
c-DNA	Complementary deoxyribonucleic acid
Bp	Base pair
CHIKV	Chikungunya virus
DENV	Dengue virus
DHF	Dengue haemorrhagic fever
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleic phosphate
MgCl ₂	Magnesium chloride
MIR	Minimum infection rate
MOH	Ministry of Health
ml	Milliliter
Mm	Millimeter
NCBI	National Center for Biotechnology Information
ORF	Open reading frame
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
Rnasin	Rnase inhibitor
Rpm	Revolutions per minute

CHAPTER 1

INTRODUCTION

1.1 Study Background

Vector-borne diseases are diseases that are caused by pathogens such as parasites, viruses, and bacteria, which are transmitted by living organisms classified as vector. Most of these vectors are blood-sucking insects such as mosquitoes, blackflies, and ticks (WHO, 2020). Vector-borne diseases are responsible for about 17% of the estimated global burden of infectious diseases and it had affected the poorer populations as well. The emergence of vector-borne diseases will hinder economic development through direct medical costs and indirect costs, which include loss of productivity and tourism (WHO, 2017). Vector-borne diseases such as malaria, leishmaniasis and dengue had caused great health risks and fatalities widely (Wilson et al., 2020).

Malaysia is one of those countries that are affected by vector-borne diseases. Some of the diseases that are commonly found in the country include dengue, Zika, malaria and Japanese encephalitis, which are transmitted by vector mosquitoes. Two known vector mosquitoes in Malaysia that are responsible for the transmission of dengue virus (DENV) and chikungunya virus (CHIKV) are *Ae. albopictus* and *Ae. aegypti* (Tham., 1993; Lee et al., 2015). *Ae. aegypti* is the primary vector of both diseases while *Ae. albopictus* acts as the secondary vector (Lee et al., 2015). Although *Ae. albopictus* is classified a secondary vector, it has been reported that this species of mosquito is an efficient vector for DENV and CHIKV (Scholte et al., 2007). In addition, the population of *Ae. albopictus* is more widespread in Sarawak compared to *Ae. albopictus* (Chang & Jute, 1982). Both species of mosquitoes have

different preferences of habitat as *Ae. aegypti* prefers indoor environment while *Ae. albopictus* prefers outdoor environment (Camara, 2010; Kweka et al., 2019).

Dengue fever and chikungunya are vector-borne diseases that spread widely and are imposing public health treats to both industrialized and developing nations worldwide. It is expected that mosquito-borne diseases such as DENV and CHIKV will invade new areas of the world including the United States since the *Aedes* vectors had spread worldwide (Fredericks & Fernandez-Sesma, 2014). Both diseases can cause morbidity and are endemic in several areas in the tropics (Azeredo et al., 2015; Dhenni et al., 2021).

Various control methods through chemical, physical, biological or an integrical approach have been adopted to handle the impact of *Aedes*-borne diseases. The control of vector-borne diseases is one of the hardest challenges on the agenda of global health. One of the first chemical approach that had been used to control adult dengue vectors is by using Dichlorodiphenyl trichloroethane (DDT) which led to the introduction of second and third generation insecticides (Sarimin et al., 2020).

On the other hand, an example of the biological approach is through the release of *Wolbachia*-infected *Ae. aegypti*. The infected mosquitoes will have a shorter life span and it cannot support the development of dengue virus. Another control innovation that were created to handle these mosquito-borne diseases includes outdoor residual spraying, insecticidal paint, ovitrap, autocidal adult and larva trap (Lee et al., 2015). Integrated vector management (IVM) approach, which involved both chemical and non-chemical method, had also been carried out in Malaysia in order to control *Aedes* vectors (Sarimin et al., 2020).

Both CHIKV and DENV could be transmitted by *Aedes* vectors through vertical transmission and venereal transmission (Sánchez-Vargaz et al., 2018; Monteiro et al., 2019).

Vertical transmission took place due to infection and dissemination of the virus within the female from ovarian cells to the eggs during the process of fertilization while venereal transmission occurs during mating (Pan American Health Organization, 2011; Sánchez-Vargas et al., 2018). Both viruses could be maintained through enzootic and epidemic cycle/urban cycle. Enzootic cycle involved the natural transmission of the virus between wild animals and *Aedes* vectors while epidemic cycle is maintained between human and *Aedes* mosquitoes (WHO, 2011; Go et al., 2014).

Pulau Salak, Kuching was one of the areas affected due to the outbreak of CHIKV in 2009 (Nor Aliza, 2012). There were no published data regarding the vector status of CHIKV and DENV that have been circulating in Pulau Salak. Thus, this study served as a reference in case of future CHIKV and DENV outbreaks. The *Aedes* mosquitoes collected in the villages were also tested for the presence of DENV. Kampung Tanjong Bako, Kuching was chosen as a control site for this research in which the *Aedes* mosquitoes collected from the area were also tested for the presence of CHIKV and DENV.

1.2 Problem Statement

It was observed that the number of dengue and chikungunya cases have been increasing in Malaysia based on the data reported by the Ministry of Health, Malaysia from year 2015 to 2018 (MOH, 2019; MOH 2020; MOH n.d.). However, there are not many studies done regarding the vector status and transovarial transmission of CHIKV and DENV in Sarawak. Nor Aliza et al. (2019) have reported the first natural evidence of transovarial transmission of DENV in the natural population of *Ae. albopictus* in selected areas in Kuching and Kota Samarahan divisions and the presence of dengue virus serotype 2 was detected in the study. On the other hand, the number of chikungunya cases recorded it peaked

in 2009 in Sarawak and there were no chikungunya cases reported by the end of November 2011 (Chua, 2010; MOH 2019). The presence of chikungunya cases, however resurfaced in Sarawak in the year 2017 and have been showing an increasing trend in Malaysia since 2015 (MOH, 2019; MOH, 2020).

There are no published data regarding the vector status and transovarial transmission of CHIKV and DENV on Kampung Pulau Salak, Kuching despite the increasing trend in the number of cases of chikungunya and dengue in Sarawak. This study could serve as a reference in case of CHIKV and DENV outbreaks in the area and could be beneficial to the effort of handling the outbreaks in the future.

1.3 Aims

This study aims to provide information regarding the vector status, to determine the occurrence of transovarial transmission of CHIKV and DENV among the mosquito, and to determine the parameters of immature mosquitoes breeding containers in Kampung Pulau Salak and Kampung Tanjong Bako.

1.4 Research Objectives

The objectives of this study are:

- i. To document the container breeding and field-caught mosquitoes on Kampung Pulau Salak and Kampung Tanjong Bako.
- ii. To investigate CHIKV and DENV status of the container breeding and field-caught mosquitoes at the two locations.

- iii. To determine the occurrence of transovarial transmission and the minimum infection rate of both CHIKV and DENV in mosquitoes.
- iv. To document the environmental parameters of immature mosquitoes breeding site

CHAPTER 2

LITERATURE REVIEW

2.1 Vector-borne Diseases

Vectors can be defined as living organisms that are responsible for the transmission of infectious diseases between humans or from animals to humans. Most of these vectors are insects, which blood feed from infected host and transfer disease-producing microorganisms into another host during their subsequent blood meal (World Health Organization, 2017).

Vector-borne diseases (VBDs) are illnesses that are caused by pathogens such as parasites, viruses, and bacteria that are transmitted by a vector such as mosquitoes, blackflies, sandflies, triatomine bugs, ticks, tsetse flies, mites, lice, and snails (Table 2.1). According to WHO (2017), VBDs such as malaria, dengue, Human African trypanosomiasis, Chagas disease, schistosomiasis, leishmaniasis, Japanese encephalitis, Yellow fever, and onchocerciasis have caused more than 700 000 deaths annually around the world and that the most common vector of VBDs are mosquitoes. It was first discovered in 1877 that mosquitoes could transmit diseases such as filariasis from human to human followed by the discovery of the fact that they could be the vector of malaria, Yellow fever and dengue from the year 1878 to 1903 (Gubler, 1998).

Generally, arthropods are accountable for hundreds of millions of cases of vector-borne diseases in humans and animals every year. The process of reversing the emergence of vector-borne diseases would be a great challenge and some available vaccines for VBD are not widely used as vaccine prospects for major vector-borne diseases are not progressing well.

Table 2.1: Vector-borne diseases and their vectors

Vector	Disease	References
<i>Aedes</i> mosquitoes	Chikungunya	Gratz (1999), WHO (2020)
	Dengue fever	Gratz (1999), WHO (2020)
	Yellow fever	Gratz (1999), WHO (2020)
	Lymphatic filiriasis	WHO (2020)
	Rift Valley fever	Gratz (1999), WHO (2020)
	Zika	WHO (2020)
<i>Anopheles</i> mosquitoes	Malaria	Gratz (1999), WHO (2020)
	Lymphatic filiriasis	WHO (2020)
	O'nyong-nyong fever	Gratz (1999)
<i>Culex</i> mosquitoes	Japanese encephalitis	Gratz (1999)
	West Nile fever	WHO (2020)
	Lymphatic filiaris	WHO (2020)
<i>Ixodes</i> sp.	Lyme disease	Gratz (1999)
	Relapsing fever	WHO (2020)
	Tularaem	WHO (2020)
	Crimean-Congo haemorrhagic fever	WHO (2020)
	Rickettsial diseases	WHO (2020)
	Tick-borne diseases	WHO (2020)
Phlebotominae	Phelebotomus fever	WHO (2020)
	Leishmaniasis	Gratz (1999)
<i>Glossina</i> sp.	African trypanosomiasis (sleeping sickness)	WHO (2020)
Triatominae	Chagas disease	WHO (2020)
<i>Xenopsylla cheopis</i> (flea)	Typhus	WHO (2020)
	Plague	WHO (2020)
<i>Pediculus humanus humanus</i> (lice)	Thypus	WHO (2020)
	Louse-borne relapsing fever	WHO (2020)
<i>Simulium</i> spp.	Onchocerciasis (river blindness)	WHO (2020)
Planorbidae	Schistosomiasis (snail fever)	WHO (2020)

Vector control such as the usage of environmentally safe insecticides, which includes MosquitoSafe (commercial biopesticides), and essential oils (Mossa, 2016; Kaur & Kaur, 2017) and research on alternative ways such as biological control would be needed to prevent transmission of most emergent vector-borne diseases (Gubler, 1998; Gubler, 2009).

2.2 Pathogens of Vector-borne Diseases

Pathogens are microorganisms that cause diseases to their host and they can be transmitted by arthropods. There are various types of pathogens and it is known that every living organism affected by pathogens. The pathogens can be divided into five main taxa of microorganisms which are nematodes, protozoa, bacteria, fungi, and viruses (Maggenti & Allen, 1959; Janeway et al., 2001; Balloux et al., 2017). Nematodes can be categorized into human and animal parasites, plant parasites, species living in marine and brackish water, or free-living species in soil and freshwater. They are usually elongate cylindrical and tapered at both ends of their body (Maggenti & Allen, 1959). Examples of parasitic nematodes are *Dirofilaria immitis*, which caused heartworm disease in humans and *Ascaris lumbricoides*, which are parasitic to both pigs and humans (Dold & Holland, 2011). On the other hand, protozoa are one-celled animals and can be found in all higher animals such as humans. Not all protozoa are life threatening as the effect of infections depends on the species and strain of the parasites and the resistance of the host itself. Examples of protozoa include *Cryptosporidium* which could cause serious aftereffect in patients with acquired immunodeficiency syndrome (AIDS) and *Acanthamoeba* which may cause corneal ulcers in individuals who wears contact lenses (Yaeger, 1996).

Bacteria that are pathogenic will normally have various virulence factors, which could lead to damage of host cells. The virulence factors are important in establishing

infection and causing disease (Love & Jones, 2008). Some known pathogenic bacterias are *Actinobaculum suis* which is related to swine diseases and *Salmonella* which leads to diarrhea (Post, 2019). On the other hand, regarding the role of fungi as a pathogen, most fungi do not show pathogenic traits but some are causing harm to humans while some are known as plant pathogens. Some common pathogenic fungal are *Cryptococcus* that causes meningitis in adults and *Magnaporthe oryzae* which leads to rice blast disease (Sun et al., 2020). As mentioned previously, viruses are one of the main taxa of pathogens as well. Examples of viruses include chikungunya virus (CHIKV), dengue virus (DENV) and coronavirus 2019 (COVID-19) (Parvez & Parveen, 2017; Yu et al., 2020).

Pathogens can cause illness to their hosts through various methods and the most obvious method is through direct damage of tissues or cells of the host during the replication process. The production of toxins allows the pathogen to find its way to the new tissues or exit the cells inside which it replicated (Gubler, 2009; Pirofski & Casadevall, 2012; Balloux & Dorp, 2017). Some of those pathogens are true parasites of humans while mostly are zoonotic with animals serving as their main vertebrate host. Humans and animals usually act as incidental hosts for vector-borne pathogens and are not usually required for the survival of pathogens in nature (Gubler, 2009). Pathogens can be divided into facultative and obligate pathogens depending on how closely their life cycle is tied to their host (Balloux & Dorp, 2017). Facultative pathogens exploit their host for reproduction purpose and comprise of mainly environmental bacteria and fungi. Meanwhile, obligate pathogens need a host to complete their life cycle. All viruses are considered obligate pathogens as they depend on the cellular machinery of their host to reproduce (Balloux & Dorp, 2017).

Examples of classes of viruses are Togaviridae, Flaviviridae and Bunyaviridae. Chikungunya virus (CHIKV) and dengue virus (DENV) are viruses under the class Togaviridae and Flaviviridae respectively. Both CHIKV and DENV are transmitted by mosquitoes. On the other hand, an example of a virus under the class Bunyaviridae is the one that caused sandfly fever among humans (Gubler, 2009; Pirofski & Casadevall, 2012). Some of these obligate pathogens require several different hosts to complete their life cycle in which the definite host is usually a vertebrate while the intermediate host (vector) is an arthropod or a mollusc in general (Balloux & Dorp, 2017).

2.3 Modes of Transmission of Vector-borne Diseases

The modes of vector-borne diseases transmission can be divided into mechanical transmission and biological transmission via the vector (Singh et al., 2016). Mechanical transmission involves a simple transfer of pathogen on contaminated mouthparts or other parts of the vector. There are no multiplication or developmental change of the pathogen on or in the vector during the transmission process (Gubler, 2009; Singh et al., 2016).

Various enteroviruses, protozoa, and bacteria that have a direct faecal-oral transmission cycle are transmitted through mechanical transmission. For instance, arthropods such as houseflies may transport these pathogens to the food of humans while feeding on faeces (Gubler, 2009). There will be no replication or developmental changes in the arthropods when the pathogens are transmitted via this method (Singh et al., 2016). In contrast to mechanical transmission, the pathogen will undergo biological development in the body of its vector in order to complete its life cycle (Gubler, 2009).

There are four types of biological transmission that include propagative transmission, cyclopropagative transmission, cyclodevelopmental transmission, vertical and direct

transmission. Propagative transmission occurs when the pathogen ingested with blood meal undergoes simple multiplication in the body of the vector before being transmitted to a new host when the vector had their blood meal (Gubler et al, 2009). For instance, arboviruses go through propagative transmission by limiting themselves to only multiply in tissues of the vectors before being transferred to the host (Singh et al., 2016). Meanwhile, cyclopropagative transmission is a condition where the pathogen undergoes developmental changes and multiplies in the body of the vector (Gubler, 2009). An example of pathogens transmitted via this route is *Plasmodium* sp. (Singh et al., 2016).

Similar to cyclopropagative transmission, the pathogen also undergoes developmental changes through cyclodevelopmental transmission but it does not multiply in the body of the vector. An example of pathogens that undergo this type of transmission is nematode like *Filariodea* sp. (Gubler, 2009; Singh et al., 2016). On the other hand, vertical and direct transmission of a pathogen can be divided into transovarial transmission and venereal transmission. Transovarial transmission occurs when the pathogen infects the developing egg and the female vector passes the virus to the offspring while venereal transmission takes place during copulation. For example, infected male mosquitoes can transfer the virus to uninfected female mosquitoes in the seminal fluid (Gubler, 2009).

The pathogen would need time to develop inside the vector during biological transmission which allows it to progress to the stage at which it becomes infective and can be transmitted. This period needed is known as extrinsic incubation period which generally took place for 7-14 days depending on the pathogen, the vector, and environmental factor such as temperature (Gubler, 2009). For instance, during the process of transmission of arboviruses such as dengue virus via biological transmission, the virus will undergo

replication in the vector's body before being transmitted to the host (WHO, 2011; Laura et al., 2019).

The ability to transmit a pathogen biologically can be greatly different among species of vectors, and even among geographical strains within a species. For instance, within a single species of mosquitoes, it is not uncommon to discover geographical strains that would be good vectors while the other strains would be poor vectors. (Gubler, 2009). This was shown in the study conducted by Gubler et al. (1979) to test the difference in susceptibility to oral infection with dengue viruses among geographical strains of *Ae. aegypti*. The mosquito strains used were Nieu, Jakarta, Kuala Lumpur, Rangoon, Singapore, Bangkok, Manila, Tonga, Rabai, Shimba hills, Koro, Bobo, and Kari. It was discovered that the strains from Nieu Island and Jakarta have the highest susceptibility to the virus. Most of the other strains from Southeast Asia had only a slightly lower infection rate compared to strains from Nieu Island and Jakarta (Gubler et al., 1979).

2.4 Chikungunya

2.4.1 Epidemiology of Chikungunya

The first outbreak of CHIKV was in southern Tanzania in 1952. The disease had then been identified in over 60 countries, which include Asia, Europe, Africa, and America (WHO, 2017). There is a study that suggests CHIKV originated from Africa and has subsequently spread to Asia (Sam & Abu Bakar, 2006). A significant outbreak of chikungunya was recorded in Thailand in the late 1950's and early 1960's and in India between 1963 to 1973 (Petersen et al., 2016). There is no major outbreak of CHIKV observed since then until the year 2004 where a significant outbreak of the virus took place at the coast of Kenya. There were two cases of outbreaks of CHIKV in Kenya which involved Lamu

island and the city of Mombasa. The outbreak in Lamu Island was mistakenly thought to be malaria in the first place (Staples et al., 2009). The outbreak lasted for 4 years, which caused CHIKV to spread throughout various islands of the Indian Ocean, India, and some areas of Southeast Asia. Transportation of items and people among the islands of the Indian Ocean have introduced the virus into La Reunion Island. The outbreak was the first that caused a number of neurologic deficits, infections in pregnancy, and deaths related to CHIKV (Staples et al., 2009).

It was suspected that CHIKV was transmitted by *Ae. albopictus* primarily as there were limited numbers of *Ae. aegypti* present on the island. CHIKV was also recorded in at least 18 countries throughout Asia, Europe, and North America after the major outbreak of CHIKV at Lamu Island in 2004 (Staples et al., 2009). Up to September 2019, chikungunya cases have been reported in some countries and territories in Africa, Asia, Europe, the Americas, and Oceania/ Pacific Islands (Figure 2.1, Table 2.2).

The first outbreak of CHIKV in Malaysia was in Klang, Selangor around the year 1998 to 1999 (Lam et al., 2001). The second outbreak occurred in Bagan Panchor and Kinta, Perak in 2006 (Abu Bakar et al., 2007; Noridah et al., 2007). Subsequently, third outbreak of CHIKV in Malaysia was recorded in Tangkak, Johor in 2008 (Apandi et al., 2011). The number of chikungunya cases peaked in Kelantan, Kedah, Terengganu and Perlis in 2009 (Chua, 2010) and Sarawak experienced a major outbreak of CHIKV in the same year (Diop et al., 2015). Pulau Salak, Kuching was one of the areas affected due to the outbreak of CHIKV in 2009.

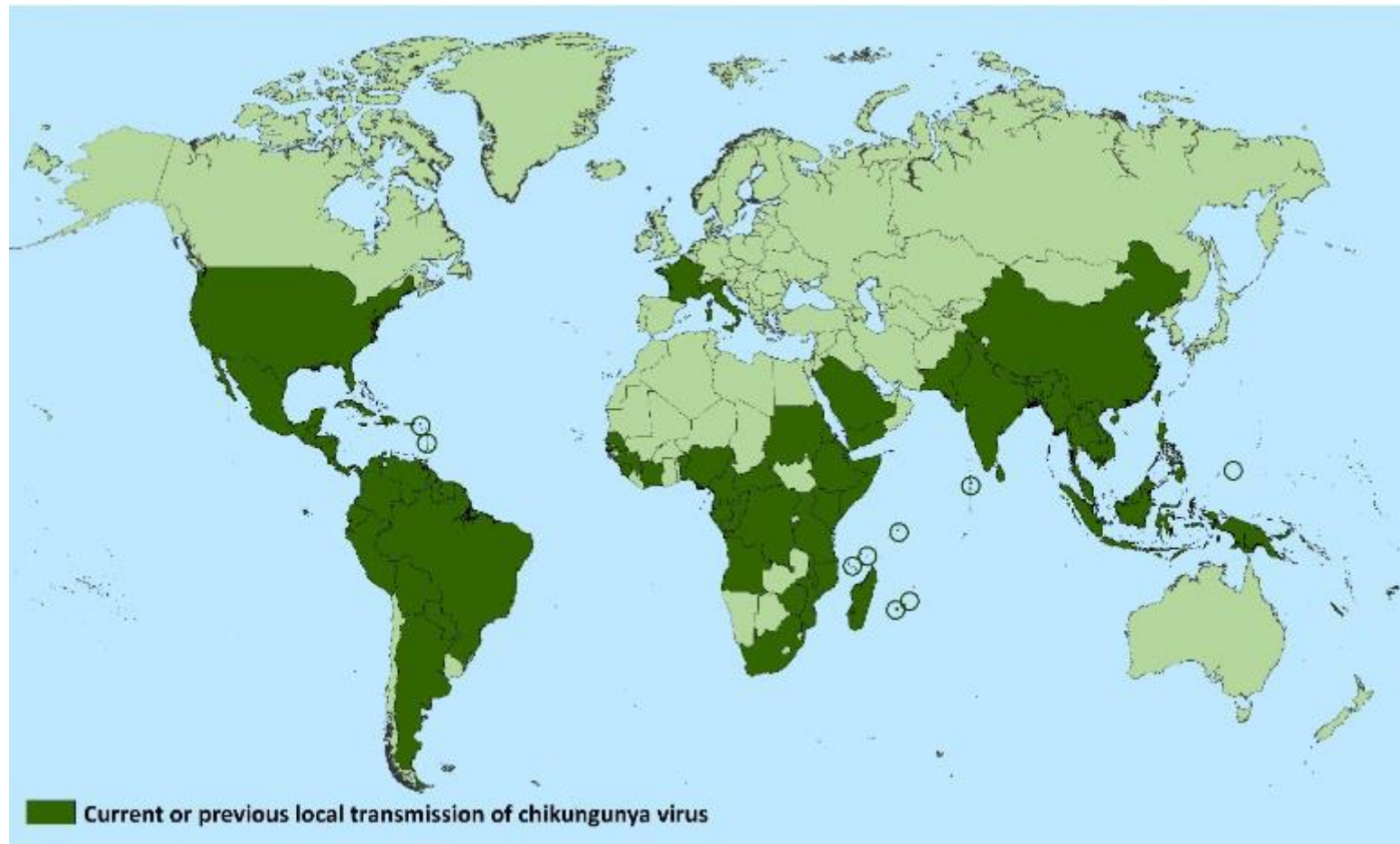


Figure 2.1: Map showing the countries and territories in which chikungunya cases have been reported (as of 17 September, 2019)
(Centers for Disease Control and Prevention, 2019)

Table 2.2: Countries and territories in which chikungunya cases have been reported (up to date until 17 September, 2019) (Center for Disease Control and Prevention, 2019)

Data table: Countries and territories where chikungunya cases have been reported			
AFRICA	ASIA	AMERICAS	
Angola	Bangladesh	Anguilla	Paraguay
Benin	Bhutan	Antigua and Barbuda	Peru
Burundi	Cambodia	Argentina	Puerto Rico
Cameroon	China	Aruba	Saint Barthelemy
Central Asian Republic	India	Bahamas	Saint Kitts and Nevis
Comoros	Indonesia	Barbados	Saint Lucia
Cote d'Ivoire	Laos	Belize	Saint Martin
Dem. Republic of the Congo	Malaysia	Bolivia	Saint Vincent & the Grenadines
Djibouti	Maldives	Brazil	Sint Maarten
Equatorial Guinea	Myanmar (Burma)	British Virgin Islands	Suriname
Eritrea	Nepal	Cayman Islands	Trinidad and Tobago
Ethiopia	Pakistan	Colombia	Turks and Caicos Islands
Gabon	Philippines	Costa Rica	United States
Guinea	Saudi Arabia	Cuba	US Virgin Islands
Kenya	Singapore	Curacao	Venezuela
Madagascar	Sri Lanka	Dominica	
Malawi	Taiwan	Dominican Republic	
Mauritius	Thailand	Ecuador	OCEANIA/PACIFIC ISLANDS
Mayotte	Timor-Leste	El Salvador	American Samoa
Mozambique	Vietnam	French Guiana	Cook Islands
Nigeria	Yemen	Grenada	Federal States of Micronesia
Republic of the Congo		Guadeloupe	Fiji
Reunion		Guatemala	French Polynesia
Senegal	EUROPE	Guyana	Kiribati
Seychelles	France	Haiti	Marshall Islands
Sierra Leone	Italy	Honduras	New Caledonia
Somalia		Jamaica	Papua New Guinea
South Africa		Martinique	Samoa
Sudan		Mexico	Tokelau
Tanzania		Montserrat	Tonga
Uganda		Nicaragua	
Zimbabwe		Panama	

The number of chikungunya cases recorded in Malaysia has been decreasing from 2010 until 2015 before showing an increasing trend from 2016 until 2020 (Sabah Control Unit of Vector-Borne Diseases (RKPBV), 2016; MOH, 2019). A number of 804 cases of chikungunya were reported in 2010 while 30 chikungunya cases were reported in 2011. The number of chikungunya cases recorded increased slightly in the year 2012 with a number of 93 cases before decreasing to only 7 cases in 2013 and 2014. The highest number of chikungunya cases recorded up to date was in 2020 (Table 2.3).

Table 2.3: The number of chikungunya cases reported in each state within Malaysia from 2015 until 18th July 2020 (MOH, 2019; MOH, 2020)

No	State	2015	2016	2017	2018	2019	2020 (up to 18 th July)
1	Perlis	0	1	0	1	5	0
2	Kedah	0	0	190	5	60	18
3	Pulau Pinang	0	1	0	0	5	947
4	Perak	3	0	2	0	635	899
5	Selangor	0	1	33	2	184	4
6	Wilayah Persekutuan, Kuala Lumpur & Putrajaya	0	0	0	0	1	3
7	N.Sembilan	0	9	0	0	8	0
8	Melaka	0	0	0	0	11	0
9	Johor	0	0	24	0	0	6
10	Pahang	0	0	0	0	1	2
11	Terengganu	0	0	0	0	0	0
12	Kelantan	0	0	19	77	74	82
13	Sarawak	0	0	2	1	4	0
14	Sabah	0	0	0	1	1	1
15	WP Labuan	0	0	0	0	1	0
Total		3	12	270	87	990	1973

2.4.2 Chikungunya Fever

Chikungunya fever might be mistaken for dengue fever since the symptoms are quite similar to classical dengue fever. It is hard to diagnose because the symptoms of infection are similar to diseases such as dengue, malaria, and other arboviral diseases (Lam et al., 2001; Horwood et al., 2015). The name “Chikungunya” was derived from a language used by the Makonde people which has the meaning of stooped posture that was experienced by those who get the fever due to frequent joint pain. Those who get infected with CHIKV have been reported to have joint pains for a longer period depending on their age. An older patient will take a longer time to recover (Bisen et al., 2013).

The symptoms of chikungunya include high fever, polyarthralgia, backache, fatigue headaches and rashes (Bisen et al., 2013; Thiberville et al., 2013). Rare symptoms of chikungunya such haemorrhaging and myocarditis were observed during early outbreak of chikungunya. However, not all individuals that are infected with CHIKV will have the symptoms as serosurveys showed that 25%- 30% of individuals who were infected and have antibodies to CHIKV have asymptomatic infections. Chikungunya generally does not cause deaths and the incubation period of the disease may take around 3 to 12 days (Staples et al., 2009; WHO, 2019).

2.4.3 Chikungunya Virus and its Genotypes

Chikungunya virus is an alphavirus that belongs to the *Togaviridae* family (Apandi et al., 2010). It is enclosed by a lipid envelope and is approximately 11.8 Kb in size. It consists of positive sense, single-stranded RNA genome with two open reading frames (ORF). The virus encodes for two polyproteins which are the non-structural polyprotein and the structural polyprotein. The non-structural polyproteins are nsP1, nsP2, nsP3, and nsP4

while the structural proteins are the capsid (C), E3, E2, 6K, and E1 (Singh & Unni, 2011; Slavov et al., 2015). The genome structure of CHIKV is shown in Figure 2.2.

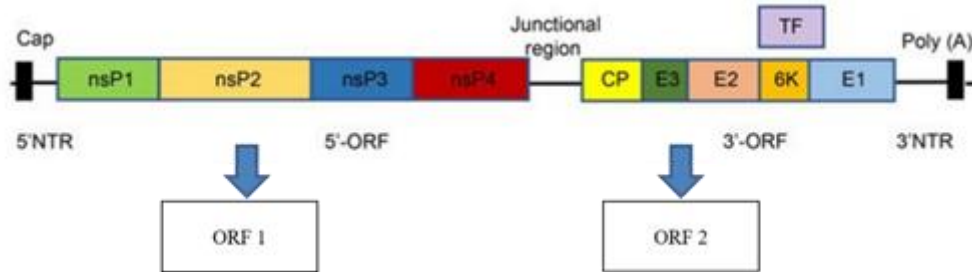


Figure 2.2: Genome structure of CHIKV (An et al., 2017)

CHIKV can be categorized into three genotypes, which include the West African, East/Central/South African (ECSA), and Asian genotypes. All the genotypes of CHIKV have been identified since its occurrence in 1952 (Costa-da-Silva et al., 2017). The West African and ECSA genotypes of CHIKV have been circulating in sub-Saharan Africa. CHIKV of ECSA genotype has spread widely across the world following the outbreak of the virus on Indian Ocean islands since 2005 (Ayu et al., 2010). The Asian genotype of CHIKV was introduced into the Americas in late 2013 and it has quickly spread across the Caribbean, South American, and Central American countries (Tan et al., 2018).

2.5 Dengue

2.5.1 Epidemiology of Dengue

A history of symptoms matched with dengue was recorded in Chin Dynasty of 265-460 A.D (Murray et al., 2013). The disease was known as ‘water poison’ in that period of time and was associated with flying insects (Gubler, 1998 cited in Anne et al., 2013). Dengue or epidemics which matches the symptoms of dengue were observed throughout the nineteenth or early twentieth centuries in the Americas, North Africa, Australia and Asia,

Southern Europe, the eastern Mediterranean, and on several islands in the Indian Ocean, the south and central Pacific and Caribbean (WHO, 2011).

The first epidemic of dengue was reported in 1653 in the French West Indies (WHO, 2011) while the first dengue haemorrhagic fever (DHF) was reported in the Philippines in 1953 (WHO, 1997). A severe form of haemorrhagic fever, which is similar to DHF, has emerged in some Asian countries due to World War II (WHO, 2011). Up to date until December 2019, dengue cases were reported in North America, South America, Africa, Europe, Asia, and Australia (Figure 2.3). Majority of the cases of dengue was reported in Brazil, the Philippines, Mexico, Nicaragua and Malaysia.

Dengue virus of serotype 1 (DENV-1) was discovered in 1977 while a new strain of dengue virus of serotype 2 was discovered in 1981. Meanwhile, dengue virus of serotype 4 and a new strain of dengue virus of serotype 3 (DENV-4 and DENV-3) were discovered in 1981 and 1994 respectively (WHO, 1986 cited in Gubler, 1998). DENV-2, DENV-3, and DENV-4 were detected in patients in the Philippines in 1956 (WHO, 1997). The four serotypes of dengue virus have been circulating in Asia, Africa, and the Americas as reported in WHO, 2011.

A new serotype of dengue which is known as DENV-5 was reported afterward in 2013 and this serotype was involved in the sylvatic cycle rather than the epidemic cycle. The new serotype of dengue was thought to be a variant of dengue 4 serotype. This was later proved wrong when rhesus macaque monkeys that have recovered from being pre-infected with DENV-1 to DENV-4 produced a different set of antibodies when they are being infected with DENV-5. This discovery had proved that the new virus of dengue was a new serotype instead of being a variant of DENV-4 (Mustafa et al., 2015).

Dengue fever (DF) was first described in Malaysia in 1902 after the outbreak of the disease in Penang in 1901 (Skae, 1902 cited in Abu Bakar & Shafee, 2002). The disease has become endemic in Malaysia by 1960's and the first laboratory-confirmed DHF case was announced in Penang in November 1962 (Rudnick et al., 1965 cited in Abu Bakar & Shafee, 2002). Bujang et al. 2017 have carried out a study involving the trend of dengue infection in Malaysia from 1995 until 2014. It had been stated in the study that the lowest incidence of dengue cases was in 1995 with 6543 cases while the highest incidence was in 2014 with 108698 reported cases.

Based on data retrieved from the Dengue Surveillance System by the Ministry of Health, Malaysia (Figure 2.4), it was observed that the number of cases recorded in 2015 was higher than the previous year with a total of 120836 cases. The total number of dengue cases reported has been decreasing from 2015 until 2018 before rising again in the year 2019 as seen in the data reported by Ministry of Health Malaysia. The total number of cases rises in 2019 with 130101 dengue cases reported. Up to date from 29th December 2019 until 15th August 2020, a total of 68097 dengue cases have been reported in Malaysia by the Ministry of Health, Malaysia. The highest number of dengue cases reported in 2020 (up until 15th August 2020) was from Selangor, which totals up to 35401 cases.

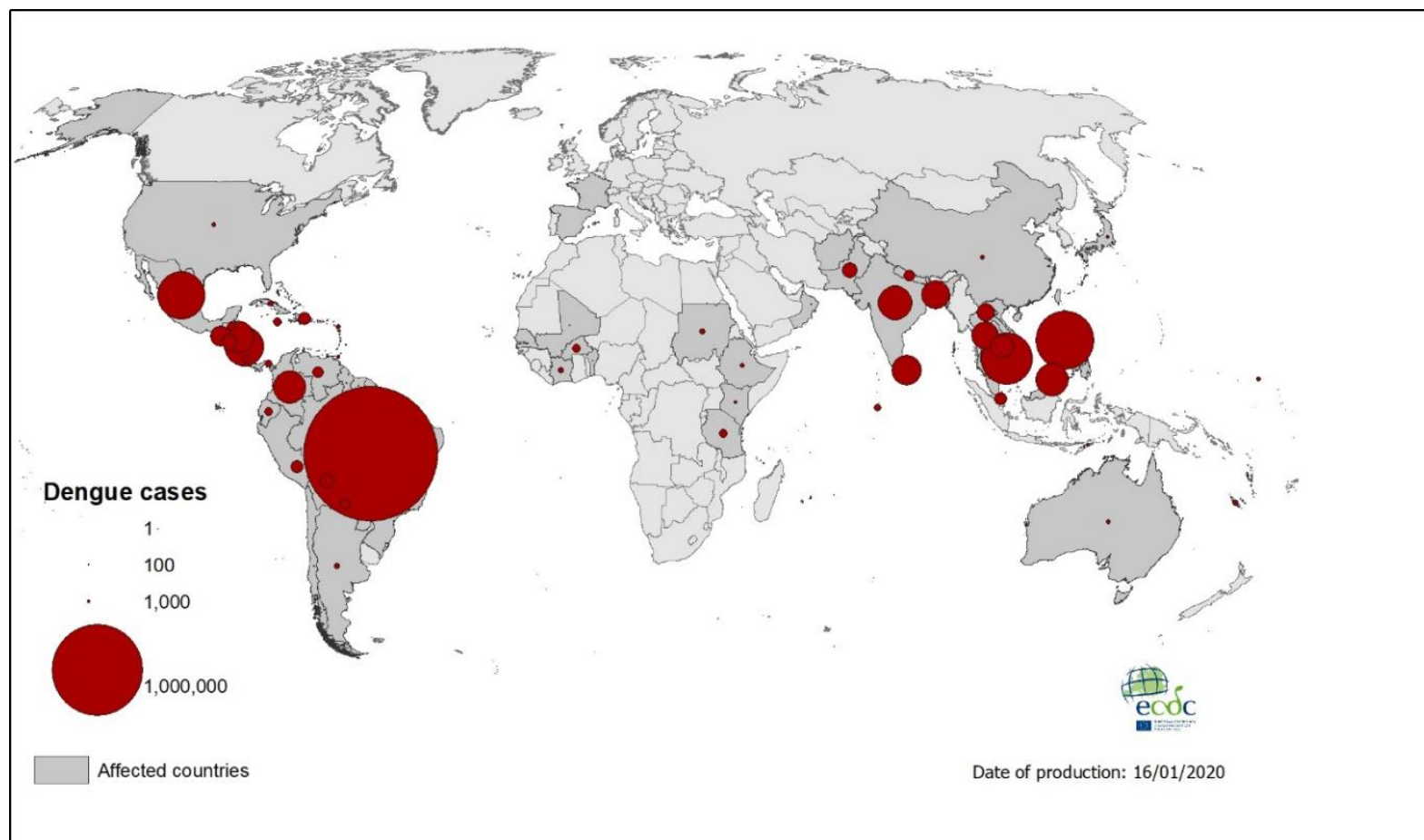


Figure 2.3: Dengue cases reported worldwide up to December 2019 (European Centre for Disease Prevention and Control, Jan 2020)

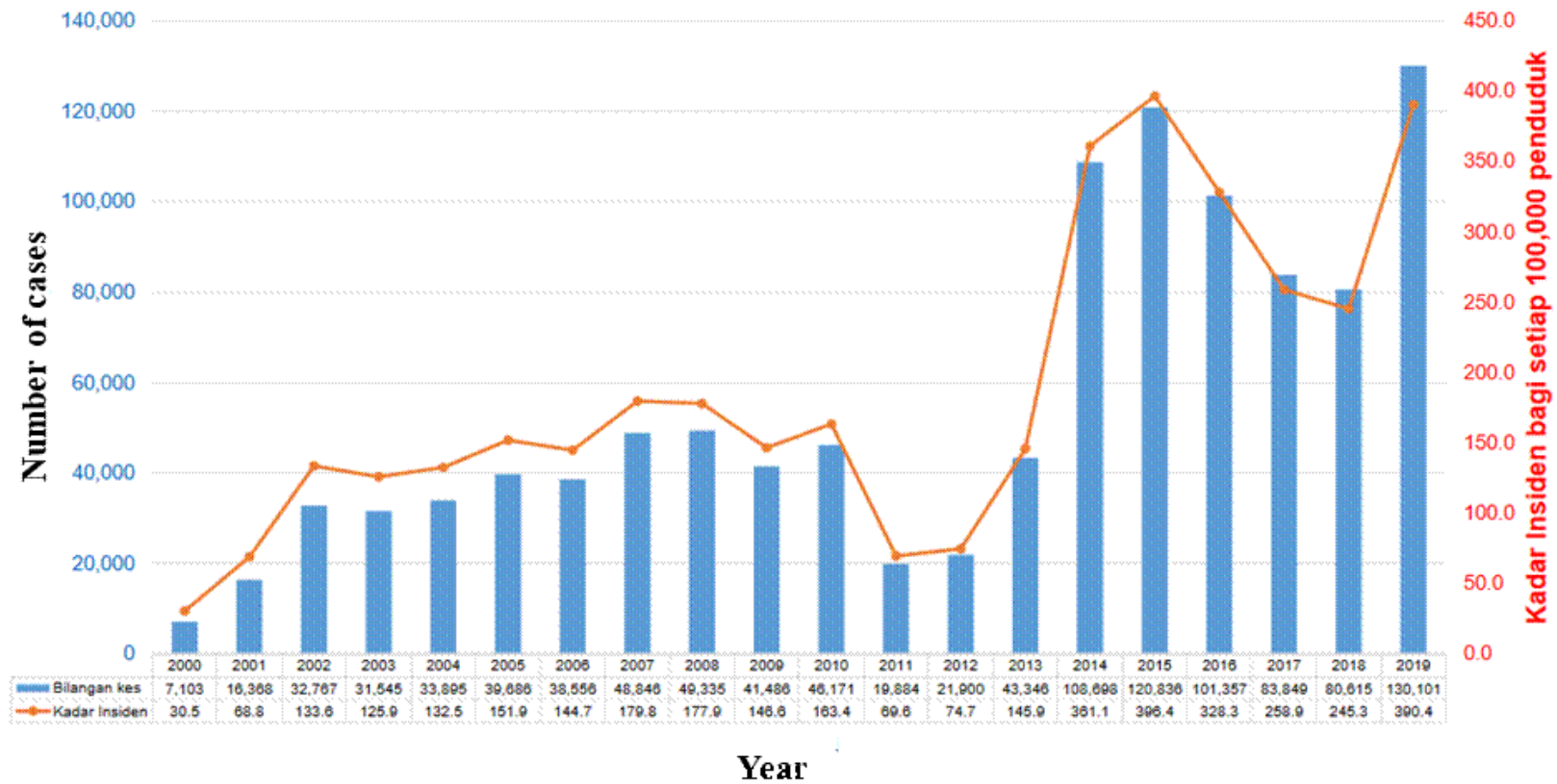


Figure 2.4: Number of dengue cases reported and the rate of incidence in Malaysia from the year 2000 until 2019 (Ministry of Health Malaysia, n.d.)

2.5.2 Dengue Fever

Dengue fever (DF) can be described by acute onset of high fever within 3 to 14 days after the bite of a mosquito being infected with dengue virus and the symptoms of DF includes frontal headache, pain in the area behind the orbit of the eyes, myalgias (pain in the muscles), arthralgias (joint pain), rash, haemorrhagic manifestations and low count of white blood cells (Gubler & Hayes, 1992). The symptoms of DF shown in the patient are depending on their age. For instance, groups of people like infants and young children might have an undifferentiated febrile disease, which is often accompanied by maculopapular rash.

On the other hand, older children and adults might have symptoms such as mild febrile symptoms or high fever of abrupt onset, severe headaches, nausea, vomiting, and rash. The appearance of skin haemorrhages among patients with DF is also uncommon (WHO, 1997). The fatality rate of cases involving DF is, however, less than 1% and it is vital to distinguish the case of DF with unusual bleeding from dengue haemorrhagic fever (DHF) which results in the increase of vascular permeability (WHO, 1997). DF is usually self-limiting and rarely causes death (Gubler, 1998).

Some patients infected with DF could develop dengue haemorrhagic fever (DHF) which is a severe form of the disease and it might cause death as well (Centers for Disease Control and Prevention, n.d.). It was reported that DHF is more common in children of age less than 15 years old in areas with high levels of the disease occurrence, which is associated with repeated dengue infections (WHO, 2011). Common symptoms of DHF include high fever, bleeding phenomena, abnormal enlargement of the liver (hepatomegaly), and failure in the circulation (WHO, 1997).

One of the typical clinical laboratory findings of the disease includes a moderate to pronounced low count of blood platelet (thrombocytopenia) with a simultaneous increase in the number of red blood cells in the blood (haemoconcentration) (WHO, 1997). The patients suffering from DHF might have early symptoms of shock which includes restlessness, cold sweaty skin, rapid weak pulse, and decrease of the pulse pressure (Centers for Disease Control and Prevention, n.d.) and the patients might die from the shock unless they are properly managed (Gubler, 1998). The duration of the shock is commonly short and may cause fatality among the patients within 8 to 24 hours, but the recovery process could be rapid if the patients were given antishock therapy (WHO, 1986 cited in Gubler, 1998).

2.5.3 Dengue Virus and its Serotypes

Dengue virus (DENV 1 to 4) is a type of virus, which belongs to the family Flaviviridae, and different serotypes of the virus can be sorted out through serological method (WHO, 1997; Rodenhuis-Zybert et al., 2010). Similar to other flavivirus, DENV consists of single-stranded RNA genome, which is surrounded by an icosahedral nucleocapsid and enclosed by a lipid envelope. The virion of DENV is approximately 50nm in diameter and it contains three structural proteins, which are the capsid protein (C), a membrane protein, and an envelope protein (E) (WHO, 1997; Rodenhuis-Zybert et al., 2010).

The virion is also composed of seven non-structural (NS) proteins besides the structural proteins mentioned. The viral genome (Figure 2.5) is around 11 kb in term of length and the RNA consists of type I cap structure (m7Gpp-pAmpN2) at its 5' end and lacks of poly (A) tail at its 3'-end (WHO, 1997; Alcaraz-Estrada et al., 2010). The order of the proteins starting from the 5'end is the capsid protein (C), prM, envelope protein (E),

followed by nonstructural proteins which are NS1, NS2, NS3, NS4, and NS5 towards the 3'end (WHO, 1997).

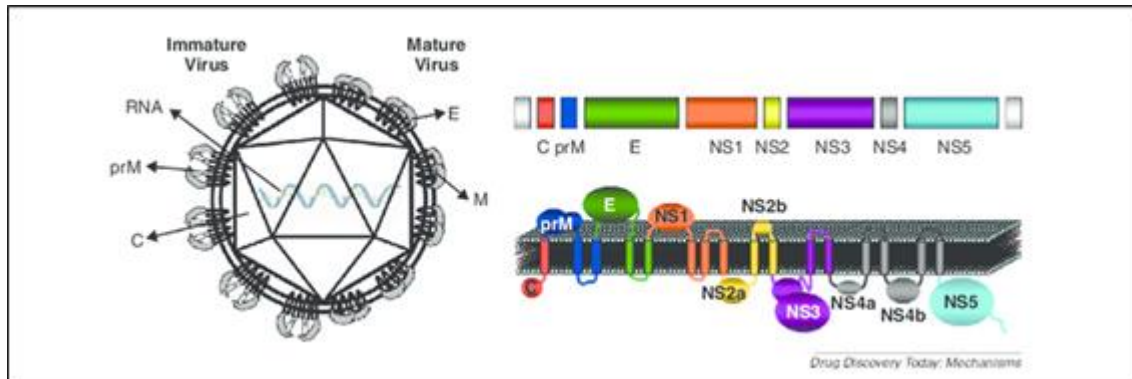


Figure 2.5: Structure and genome of dengue virus (Hottz et al., 2011)

2.6 Vector Mosquitoes

Chikungunya fever and dengue fever are mosquito-borne diseases that have caused increasing concern as the mosquito ranges are expanding which will, in turn, increase the risk of spread of the diseases (Manore et al., 2014). The primary vector of both CHIKV and DENV is *Ae. aegypti* while the secondary vector is *Ae. albopictus* (Lee et al., 2015). Both of the mosquito species are known to be diurnal in which they mostly bite in the morning and evening and less at night (Manore et al., 2014). Although the two species of *Aedes* mosquitoes are reported to be the common vectors that are responsible for the transmissions of both CHIKV and DENV in Malaysia, there are a number of other potential vectors, being reported in other countries as well (Gubler et al., 1985; Lee et al., 2015; Higgs & Vanlandingham, 2015).

During the interepidemic interval in Africa, CHIKV was maintained by a number of *Aedes* mosquitoes which are known as *Ae. aegypti*, *Ae. africanus*, *Ae. neoaffricanus*, *Ae. luteocephalus* and *Ae. furcifer-taylori* (Jupp & McIntosh, 1988 cited in Higgs &

Vanlandingham, 2015). Laboratory-based study regarding vector competence of CHIKV has demonstrated the ability of other species of mosquitoes in transmitting the virus. Meanwhile, the virus is maintained between *Ae. aegypti* and *Ae. albopictus* in Asia (Coffey et al., 2014; Higgs & Vanlandingham, 2015).

On the other hand, another possible vector of DENV had been reported in a study done by Gubler et al. (1958) in Puerto Rico. It was shown in the study that *Aedes mediovittatus* has the ability to support dengue virus replication similar to *Ae. aegypti*. *Ae. mediovittatus* was also reported to show higher susceptibility to oral infection with dengue serotype-2 compared to *Ae. aegypti*. The study suggests that *Ae. mediovittatus* could be a competent host for dengue virus and it might involve in the maintenance of dengue viruses in Puerto Rico during the interepidemic period. In addition, another potential vector of DENV would be *Aedes polynesiensis* (Gubler, 1998). Although there are many potential vectors of DENV, it was shown through field isolation and epidemiological evidence that both *Ae. aegypti* and *Ae. albopictus* are responsible for most of dengue transmission (Gubler, 1998 cited in Higa, 2011).

Although *Ae. aegypti* is the primary vector of both DENV and CHIKV, the population of *Ae. albopictus* is more widespread in Sarawak, Malaysia especially in rural areas compared to *Ae. aegypti* (Chang & Jute, 1982). There was no *Ae. aegypti* detected in the latest ovitrap surveillance done in Sarawak in contrast to the finding in 1982 (Lau et al., 2017). *Ae. albopictus* was reported to be the dominant vector of dengue (DENV) in Sarawak state (Lau et al., 2017). Since it is known that CHIKV shares the same mosquito vectors with DENV (Sam & Abu Bakar., 2006), this may suggest *Ae. albopictus* to be the main vector of CHIKV in Sarawak as well.

2.7 Life cycle of Mosquitoes

Mosquitoes are small-midge similar to flies that consist of the family Culicidae and the two subfamilies of Culicidae are the Anophelinae and the Culicinae. *Aedes* is the largest genus of mosquitoes in the subfamily Culicinae and its main habitat is aquatic (Kweka et al., 2019). There are four stages in the life cycle of *Aedes* mosquito which are known as the egg, larva, pupa and adult stage (Figure 2.6). Mosquitoes are able to live and breed inside and outside the home and the time taken for the mosquitoes to complete their life cycle (Figure 2.6) is between 8 to 10 days (Centers for Disease Control and Prevention, n.d.). Female *Aedes* mosquitoes will lay their eggs on the internal wet walls of containers filled with water, above the waterline. The eggs are able to survive desiccation for up to eight months. A very little amount of water is enough to attract a female *Aedes* mosquito to breed in a container storing water. *Aedes* larvae will emerge from the eggs after the water level rises to cover the eggs (Centers for Disease Control and Prevention, n.d.). It has been known that the larvae of *Aedes* mosquito are able to feed on microorganisms such as fungi, protozoa, bacteria, algae, crustaceans, plant debris, and insect exuviae in the water (Souza et al., 2019; Chersoni et al., 2021). The larva will emerge to be a pupa after undergoing the process of moulting three times (Centers for Disease Control and Prevention, n.d.). Adult *Aedes* mosquitoes will emerge from the pupa after 1 to 2 days (Kweka et al., 2019). After emergence from the pupae stage, adult mosquitoes will feed on nectar from flower (Centers for Disease Control and Prevention, n.d.; Kweka et al., 2019).

Although nectar from flower is known to be a common source of energy for mosquitoes, the female mosquitoes still prefer blood meal to nectar (Barredo & DeGennaro, 2020). Female mosquitoes will feed on human and animal blood for the production of eggs while the males will only feed on nectar and other plant juices. The female mosquitoes will

look for a breeding ground with water sources after the feeding process to lay their eggs (Centers for Disease Control and Prevention, n.d.; Kenney et al., 2017). *Aedes* mosquitoes also exhibit sexual dimorphism as the antennal flagellum of the males consists of a large number of long hairs compared to their female counterpart (Gopfert et al., 1999).

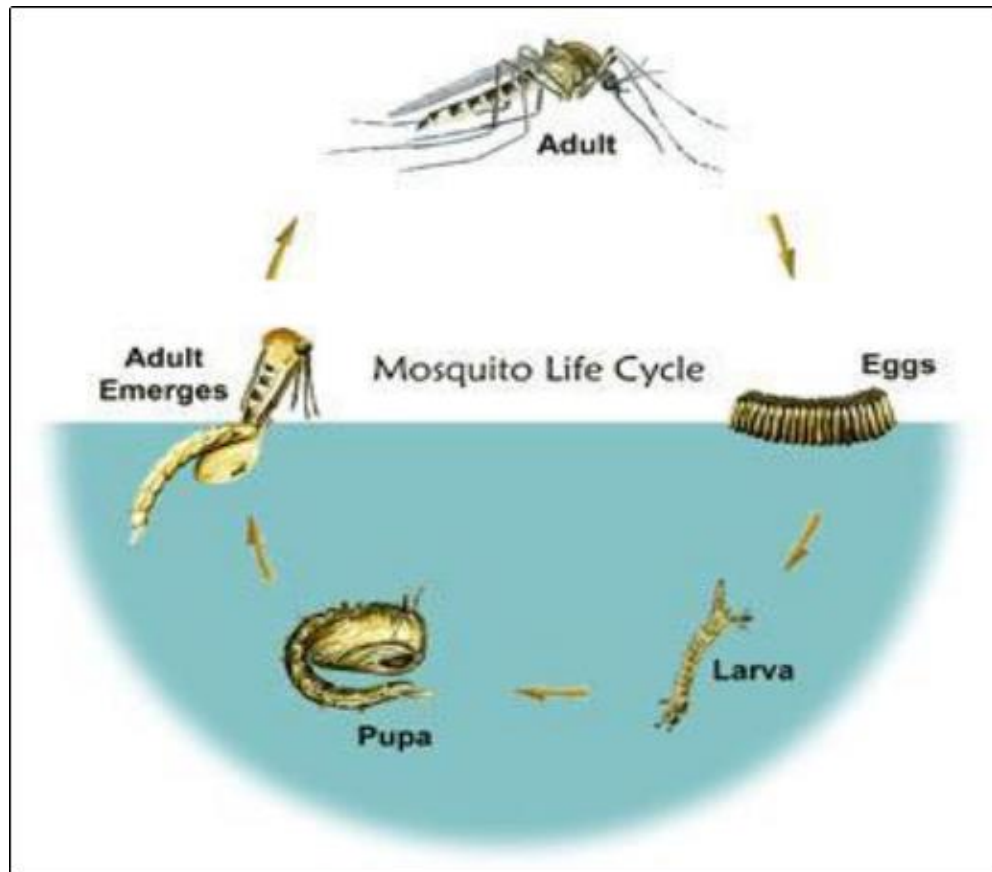


Figure 2.6: Life cycle of mosquitoes (The American Mosquito Control Association, n.d.)

2.7.1 *Aedes albopictus*

Aedes albopictus is also known as the Asian Tiger mosquito. It has a single longitudinal silver dorsal line on its thorax and banded legs (Figure 2.7 & Figure 2.8). This species was originated from the Asian continent and it was first reported in India before invading the Americas during the '80s. This presence of this species was reported for the first time in Africa in 1989. *Ae. albopictus* are the main dengue vectors in some areas but is mostly known as secondary vector (CDC, n.d; Hawley et al., 1987; Camara, 2010).



Figure 2.7: Male *Aedes albopictus* with magnification (Ellis, n.d.)



Figure 2.8: Female *Aedes albopictus* (40x magnification)

It has been proven in the field that this species of mosquito is an efficient vector for chikungunya and dengue virus (Scholte et al., 2007). *Ae. albopictus* usually bites aggressively during daytime and is spreading around the world as a public threat due to its role as a vector of both CHIKV and DENV (Scholte et al., 2007; Bonizonni et al., 2013). This species bites humans and a range of available domestic and wild vertebrates. *Ae. albopictus* can usually be found outdoor and are linked to thickets and arboreal plants. It will make use of water-filled container around or further away from households for breeding purposes (Kweka et al., 2019).

Although this species usually breeds in natural habitats such as tree holes and bamboo stumps, this species was well adapted to both suburban and urban areas as the larvae are able to survive in artificial containers such as tires and vases (Caputo et al., 2012; Bonizonni et al., 2013). This species had also become the main vector in South China and the sole vector in countries such as France (La Réunion Island) and India (Kerala) (Wu et al., 2010; Caputo et al., 2012). The expansion in the range of the population of *Ae. albopictus* are linked to climate change along with the global movements of goods and humans. It will be hard to eradicate the species once they are established in an area (Bonizonni et al., 2013).

2.7.2 *Aedes aegypti*

Aedes aegypti is a tiny, dark mosquito with white lyre-shaped markings and banded legs (Figure 2.9 & Figure 2.10). It is an African mosquito and was first reported in Egypt before invading the Americas rapidly. Currently, *Ae. aegypti* is widely distributed across the world and plays a significant role in dengue virus transmission in various countries. This species is a primary vector of contagion diseases such as dengue fever, chikungunya and yellow fever and they are usually found indoors (Kweka et al., 2019).

Ae. aegypti occupies urban areas and they can be generally found in areas lacking of piped water systems. They depend greatly on water storage containers to lay their eggs. Humans are creating a conducive environment for the reproduction of this species through the disposal of water-holding receptacles around the housing areas, as the mosquitoes will lay their eggs on the sides of receptacles. The eggs will then hatch into larvae after rain or through flooding (Kweka et al., 2019).



Figure 2.9: Male *Aedes aegypti* with magnification (Entomology Today, 2018)



Figure 2.10: Female *Aedes aegypti* (40x magnification)

This species of mosquito prefers dark-coloured receptacles located in the shadow for breeding purposes and those receptacles can usually be found within or in close proximity to houses (Centers for Disease Control and Prevention, n.d; Camara, 2010) as *Ae. aegypti* is anthropophilic and they prefer to get their blood meals from humans (Kweka et al., 2019). According to Kweka et al. (2019), *Ae. aegypti* is well adapted to the environment to the point that they are able to rapidly bounce back to their initial number after facing disasters such as drought and human intervention. It was mentioned that the species will be expected to continue to adapt to environmental changes.

2.8 Control of Aedes Mosquitoes

Chikungunya and dengue are mosquito-borne diseases that are classified as global public threats and are transmitted by the same *Aedes* vectors (Kajeguka et al., 2017; Sánchez-Vargaz et al., 2018). Various methods have been invented to control the spread of *Aedes* vectors such as focusing on individual protection against mosquito bites, treatment of patients, and control of mosquito population (Moulay et al., 2012; Lee et al., 2015). Mosquito control in a dengue programme usually consists of three major components, which are control of vectors and surveillance, participation of community, and enforcement (Lee et al., 2015).

Lee et al. (2015) had done a study about various innovations on control of *Aedes* mosquitoes which includes gene-based sterile technique using the (Release of insects carrying dominant lethal) RIDL technology, the release of *Wolbachia*-infected *Ae. aegypti* mosquitoes, auto-dissemination trap, autocidal trap, outdoor residual spraying, insecticidal paint, and biocontrol agent. Gene-based sterile technique is a condition where *Ae. aegypti* is genetically modified so that the mosquitoes will die at larval or pupal stage unless provided

with tetracycline in their rearing medium. Meanwhile, *Wolbachia*-infected *Ae. aegypti* mosquitoes will be having difficulty in supporting the development of dengue virus and has a shorter life span. On the other hand, auto-dissemination trap involved the usage of insect growth-regulator such as pyriproxyfen. Autocidal trap is first designed by Lee et al. (2015) and it is a modification of the usual ovitrap. It is able to trap both of the larvae and of gravid female mosquitoes as it had a sticky plastic strip attached on top of the floater. Another method that can be utilized to control the population of Aedes vectors is the usage of microbial control agent such as *Bacillus thuriangiensis* H-14 which can cause fatality at larval stage of mosquitoes. This method would not affect the environment and non-target organisms. *Bacillus thuriangiensis* H-14 is a larvicide agent that be can used on Aedes larvae. Delta endotoxin released from the larvicide agent will cause disruption to the osmotic balance in the mid gut epithelial cells of the larvae (Ahmad Zaki et al., 2020). Surveillance is usually done through house-to-house larval survey and the results collected will be used to design several indicators such as the House Index and Breteau Index. These indices are useful in denoting the risk areas. However, the larval survey is utilized as a form of enforcement rather than indicators of Aedes breeding in most countries (Lee et al., 2015).

2.9 Virus Maintenance and Transovarial Transmission

2.9.1 Virus Maintenance and Transovarial Transmission of CHIKV

Chikungunya virus is prevalent in countries of Sub-Saharan, Africa, India, and Southeast Asia (Coffey et al., 2014). The primary vectors of CHIKV are *Ae. aegypti* and *Ae. albopictus*. Different transmission cycle of CHIKV has been observed in Africa and Asia (Figure 2.11). CHIKV have been circulating in an enzootic cycle in Africa which involves forest-dwelling mosquitoes and non-human primates (Horwood et al., 2015; Honorio et al., 2018). Enzootic/sylvatic cycle is a process that involved the natural transmission of the virus

between wild animals and insect vectors, which can cause the amplification of the virus in the vector (Go et al., 2014). CHIKV have been detected in vectors of yellow fever in Senegal, which includes *Ae. furcifer*, *Ae. taylori*, *Ae. luteocephalus*, *Ae. africanus* and *Ae. neoaffricanus*. Besides, CHIKV has also been detected in African green monkeys (*Chlorocebus sabaeus*), patas monkeys (*Erythrocebus patas*), Guinea baboons (*Papio papio*), a bushbaby (*Galago senegalensis*), and guenons (*Cercopithecus aethiops*) in Senegal. The monkeys in the region play a role as amplification host of CHIKV in the sylvatic cycle (Coffey et al., 2014; Althouse et al., 2018). On the other hand, the virus was maintained by urban cycle (Figure 2.12) in Asia which took place between *Ae. aegypti*, *Ae. albopictus* and humans (Coffey et al., 2014). There are no sylvatic cycles observed in Asia (Higgs & Vanlandingham, 2015).

It is known that only the female *Aedes* mosquitoes are able to pass on CHIKV to the host since they are hematophagous. Two modes of transmission of CHIKV in the urban cycle are vertical and horizontal transmission. Vertical/transovarial transmission in vectors took place through infected eggs while horizontal transmission happened when the female mosquitoes ingest CHIKV particles during blood meal (Monteiro et al., 2019).

Infection and replication of CHIKV occur in the midgut cells of the mosquito as they feed on CHIKV infected blood (Figure 2.13). The virus was then disseminated to the hemocoel and the salivary glands of the vector before getting into the basal lamina of acinar cells. CHIKV replicates inside the cells before being deposited into the apical cavities which is a medium where mosquito saliva is stored until the next blood meal (Monteiro et al., 2019). After the virus has been injected into the host, it will travel directly into the subcutaneous capillaries where the process of replication will start instantly. Some of the viruses will then

infect susceptible cells in the skin such as fibroblasts and endothelial cells or macrophages (Figure 2.14). CHIKV is able to disseminate quite rapidly to the blood circulatory system. The virus formed in draining lymph nodes is released into the lymph circulation and then to the blood through the thoracic duct. The virus will gain access to different parts of the body including the liver, muscle, joints, and brain once it enters the blood. Penetration of mononuclear cells and viral replication in the muscles and joints are linked to very strong pain, with some patients presenting arthritis (Caglioti et al., 2013).

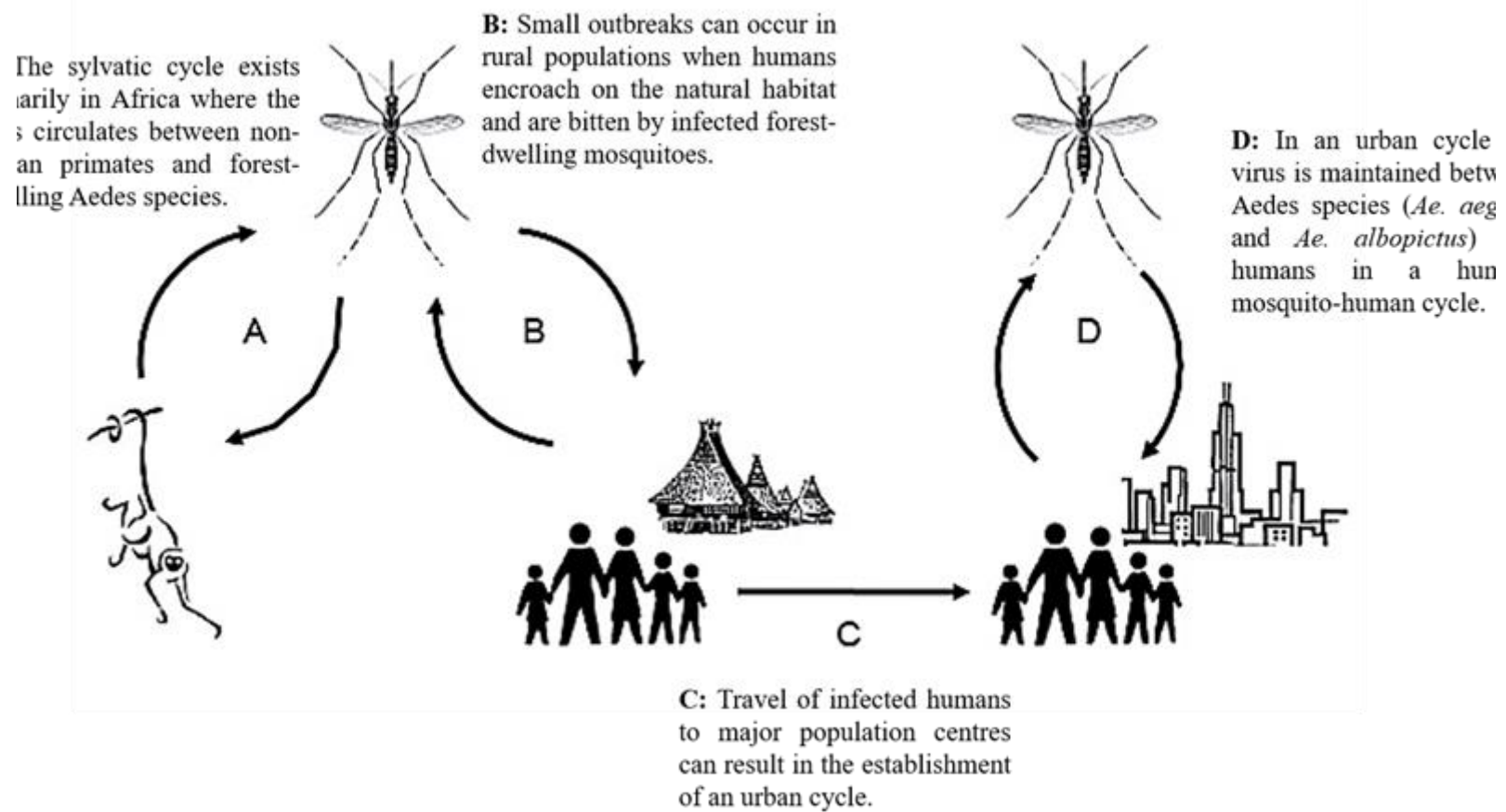


Figure 2.11: Transmission cycles of CHIKV (Adapted from Horwood et al., 2015)

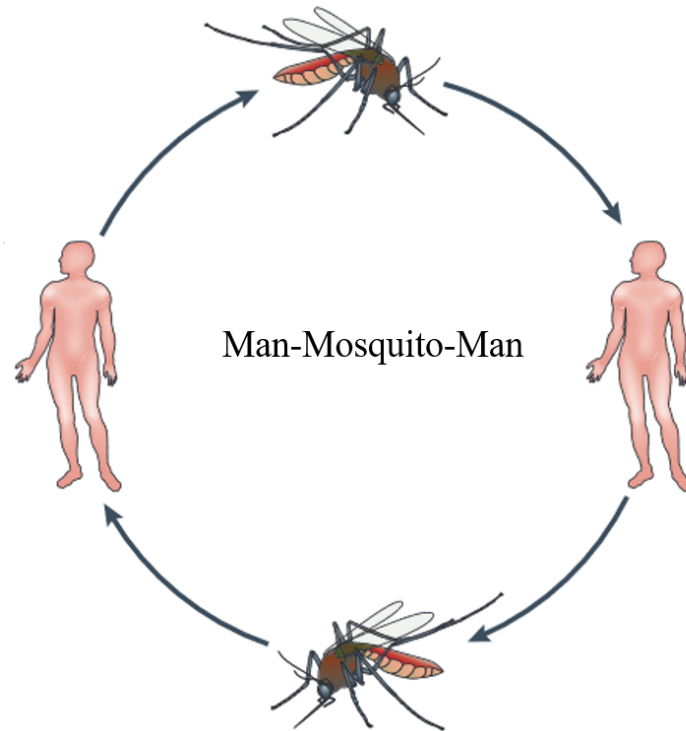


Figure 2.12: Urban cycle of CHIKV (Adapted from Whitehead, 2007)

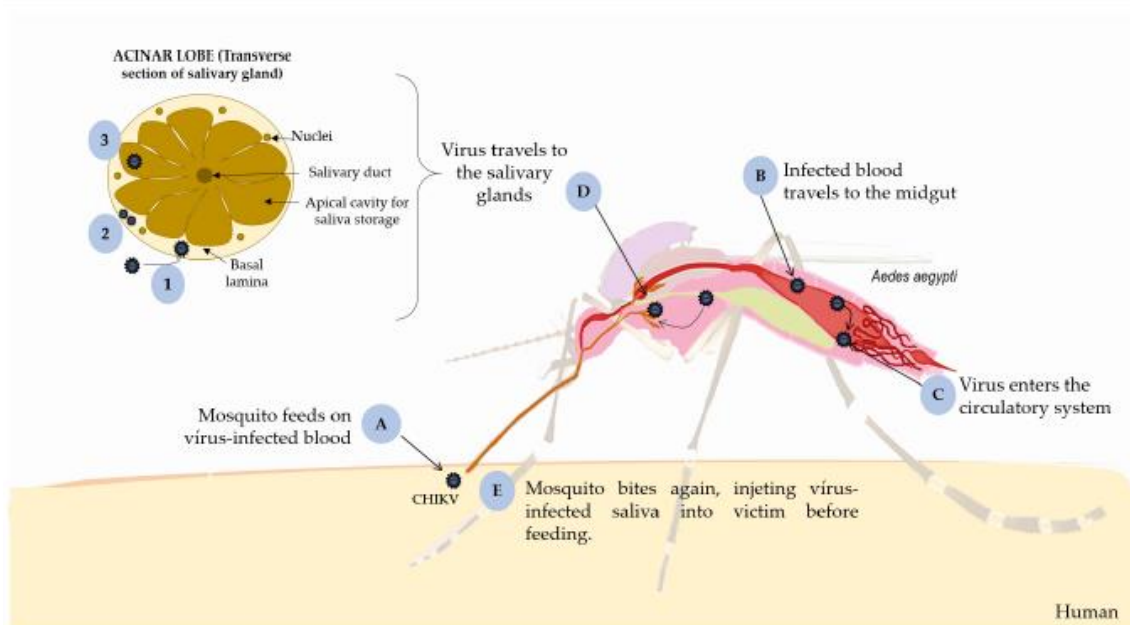


Figure 2.13: Process of replication of CHIKV (Monteiro et al., 2019)

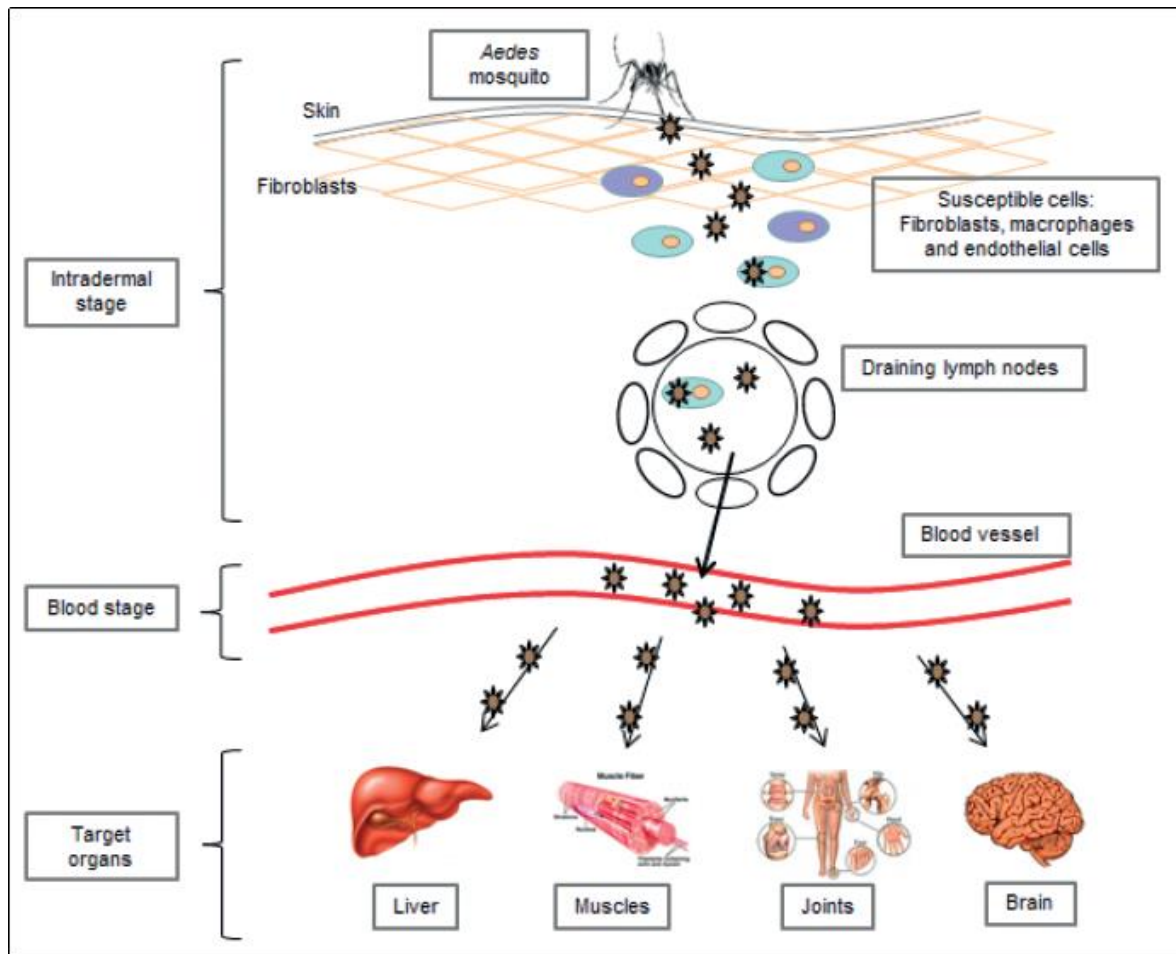


Figure 2.14: Dissemination of CHIKV to different tissues and organs (Caglioti et al., 2013)

2.9.2 Virus Maintenance and Transovarial Transmission of DENV

Transmission of DENV can occur in three different cycles, which are the enzootic cycle, epizootic cycle, and epidemic cycle (Costa-da-Silva et al., 2017). In the enzootic cycle, the sylvatic cycle is maintained between *Aedes* mosquitoes and monkey, and the relationship is reported in South Africa and Asia (Figure 2.10). It was reported that DENV is not pathogenic to monkeys and all the four dengue serotypes have been detected in monkeys (Gubler, 1998 cited in WHO, 2011).

On the other hand, DENV moves over to non-human primates from the epidemic cycle through bridge vectors. For instance, the occurrence of the cycle was observed among the population of toque macaques (*Macaca sinica*) during 1986 to 1987 in a study area in Sri Lanka (de Silva, 1999 mentioned in WHO, 2011). In the epidemic cycle, the virus is maintained between humans and *Aedes* mosquitoes (Figure 2.11) (WHO, 2011).

Dengue virus is an arbovirus that is transmitted to humans through the bite of DENV infected *Aedes* mosquitoes, primarily *Aedes aegypti*. It had been stated that infected mosquitoes will remain infected for life and they could transmit the virus to humans through probing and feeding (WHO, 1997). According to WHO (1997), the infected female mosquitoes can pass the virus to their next generation by transovarial transmission but the occurrence of this event is not that common. Transovarial transmission of DENV in *Aedes* mosquitoes is a significant mechanism to maintain the virus in nature (Costa-da-Silva et al., 2017) and WHO (1997) stated that human could be the main amplifying host of DENV.

Mosquitoes might get infected with the virus when they feed on infected individuals when they are viraemic. It is understood that after the ingestion of the infected blood meal, DENV will replicate in the epithelial cell lining of the midgut of the vector mosquitoes and passes into haemocoel to infect the salivary glands before entering the saliva causing infection during the process of probing. The genital tract of the mosquito will be infected as well which may allow the virus to enter the fully developed eggs during oviposition (WHO, 2011). When the mosquitoes get infected, the virus will develop in the mosquitoes for around 8 to 10 days before it could be transmitted to other individuals through probing and feeding. It is understood that the period required for the extrinsic incubation depends on the environmental conditions, especially on the ambient temperature (WHO, 1997).

2.10 Minimum Infection Rate

Minimum infection rate (MIR) is widely used in the estimation of proportion of infected individuals from pooled samples. MIR is the ratio of the number of positive pools to the total number of mosquitoes tested. It is assumed that there is only one infected individual in each positive mosquito pool. It is one of the methods that are usually used in arbovirus surveillance and one of the importances of arbovirus surveillance is to monitor the presence of a pathogen. Mosquito surveillance has been greatly carried out by local public health agencies and mosquito control districts as a means for monitoring arboviral transmission (Gu et al., 2003; Gu et al., 2008).

Rohani et al. (2005) have carried out rapid detection of chikungunya virus in laboratory infected *Aedes aegypti* by RT-PCR and recorded a minimum infection rate of 71% among the laboratory colonies of mosquitoes. The value of minimum infection rate is higher than those recorded from field-caught *Aedes* species by Diallo et al. (1999) which was 4.0% – 8.8%. On the other hand, Lee et al. (2005) had recorded the MIR value of DENV in their study with a range of 2.35% to 14.30% and 5.77% to 40.00% for *Ae. albopictus* and *Ae. aegypti* respectively. The value of the minimum rate recorded by Rohani et al. (2005) and Lee et al. (2005) was quite high and it was assumed that higher mosquito infection rate will increase the rate of arbovirus transmission (Bustamante & Lord, 2010).

CHAPTER 3

MATERIALS AND METHODS

3.1 Study Area

The sampling area, Kampung Pulau Salak is located in Kuching and about 32 km away from UNIMAS (Figure 3.1). This area was selected because it was affected by chikungunya outbreak in 2009. The island is located at a geographical location of 1°40'0.01" N and 110°16'60" E. There are approximately 100 houses located in Kampung Pulau Salak (Figure 3.2). The houses were located near the water bank and fishing has been the main source of income in this island. There used to be no supply of treated water on this island and the source of drinking water for the villagers was the stored rainwater. Boat services were provided by the villagers from Sungai Daun jetty to the village.

Kampung Tanjong Bako was chosen as a control site for this study. There are no records of chikungunya and dengue outbreaks in the area. The village is located approximately 16 km away from UNIMAS (Figure 3.3 & Figure 3.4). It is located at a geographical location of 1°34'26.9" N and 110°25'45.8" E. There are approximately 213 houses in the village. Fishing is one of the sources of income of the villagers. However, there were fewer water storage containers found in the village compared to those in Kampung Pulau Salak because this village has a tap water supply. The village is accessible by asphalt road.

3.2 Mosquito Sample Collection

3.2.1 Field Collection of Immatures

The collections of the immature mosquitoes were done at Kampung Pulau Salak and Kampung Tanjong Bako by using ovitraps. There were 130 ovitraps placed outside the houses of the villagers and around the vicinity of the village of Kampung Pulau Salak and Kampung Tanjong Bako in each sampling. Collections of immature mosquitoes were conducted three times each in both Kampung Pulau Salak and Kampung Tanjong Bako from September 2018 to November 2019. The ovitraps were prepared as described by Lee (1992) in Lau et al. (2017) with modifications. The outer surface of the ovitraps was sprayed with black colour sprays. Each of the ovitrap was lined with clear plastic bag and filled with approximately 150 ml cow-grass infusion solution prepared by using methods from Tang et al. (2007). A tongue depressor (1.7 cm × 14.9 cm) was placed slanting in each of the ovitrap to facilitate the oviposition of gravid female mosquitoes. The tongue depressor was wrapped with paper to allow the mosquito to lay eggs on the moist paper (Appendix EIV). All the prepared ovitraps were placed around the houses of the villagers and the vicinity of the village. Mosquito immatures were also collected randomly from discarded receptacles that can be found in the village. The pH level and temperature of the medium in the ovitraps and discarded receptacles were measured by using EcoTester (OAKTON, USA).

The ovitraps were left in the village for a week before they were collected and brought back to the laboratory at the Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak. The immature mosquitoes collected from both ovitraps and discarded receptacles were reared in the laboratory at room temperature in labelled plastic containers (according to the receptacles and village they are collected from) until the emergence of adult mosquitoes. Any remaining viable eggs on the tongue depressors in the ovitraps were

let hatched and the emerged larvae were reared until adult. The immature mosquitoes were fed with beef liver following the protocol in Lau et al. (2017). The emerged adult mosquitoes were kept in a -80°C freezer and pooled according to sex, species, and sampling location.

3.2.2 Field-caught *Aedes albopictus*

Adult *Aedes albopictus* were collected by bare leg-catch method with the help of two mechanical aspirators around 9.00 am to 10.30 am and around 4.30 pm to 5.30 pm. Sampling of adult *Aedes* mosquitoes were carried out as these hours as they are day-biting mosquitoes and they are active early in the morning and late afternoon (Reinhold et al., 2018). The field-caught *Aedes* mosquitoes were placed into the cryogen tubes and put inside the liquid nitrogen tank to prevent the samples from degrading before being brought back to the laboratory. They are pooled according to sex, species, and sampling location as well.

3.3 RNA Extraction/ Virus /Isolation

RNA extraction was carried out by using QIAGEN RNeasy Mini Kit (Qiagen, Germany) (Appendix EI). The kit contains RNeasy spin columns, Buffer RLT, Buffer RW1, Buffer RPE, and RNase free water. The extraction process was carried out according to the protocols provided by the manufacturers.

The mosquito samples were kept in 1500 µl microcentrifuge tubes and 350 µl of Buffer RLT were added to the tubes. Tubes that contain of more than 20 mosquitoes were filled with 600 µl of Buffer RLT. The mosquito samples were then homogenized uniformly by using a disposable homogenizer/Bio masher (Optima, Japan). The samples were then centrifuged for 3 minutes at 12600 rpm. The supernatants were then transferred into a new 1.5 ml microcentrifuge tubes and 700 µl of 70% ethanol were added into the tubes. The samples were mixed by pipetting. The mixtures were subsequently transferred to RNeasy

spin column which was attached to a 2ml collection tube. The mixtures were centrifuged at 12600 rpm for 15 seconds. 35 μ l of Buffer RW1 were added to the spin column and centrifuged at 12600 rpm for 15 seconds. 500 μ l Buffer RPE was then added into the spin column and centrifuged at 12600 rpm for 15 seconds.

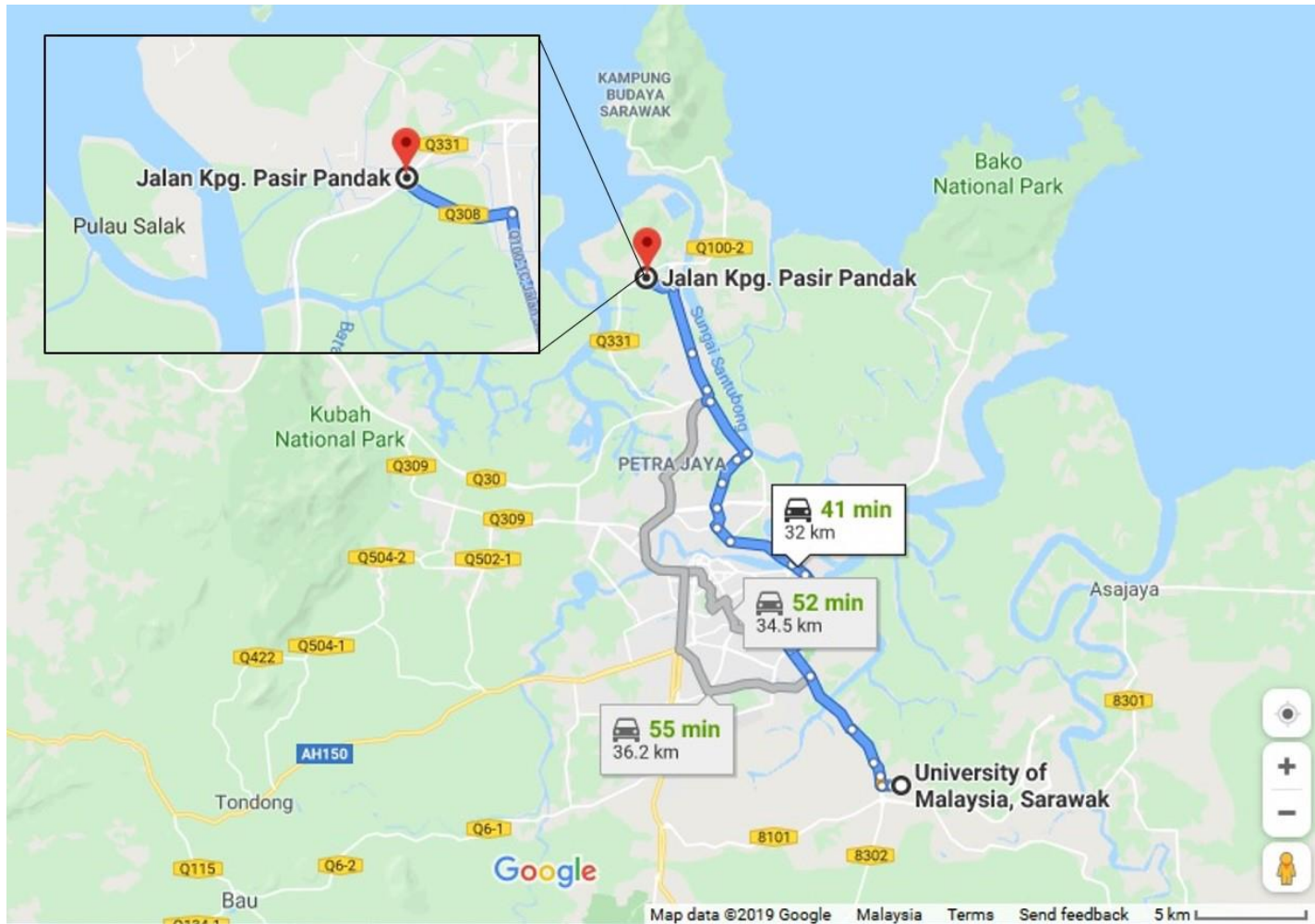


Figure 3.1: Map of Pulau Salak from UNIMAS (Photo courtesy from Google Map)



Figure 3.2: Map of Kampung Pulau Salak (Photo courtesy from Google Map)

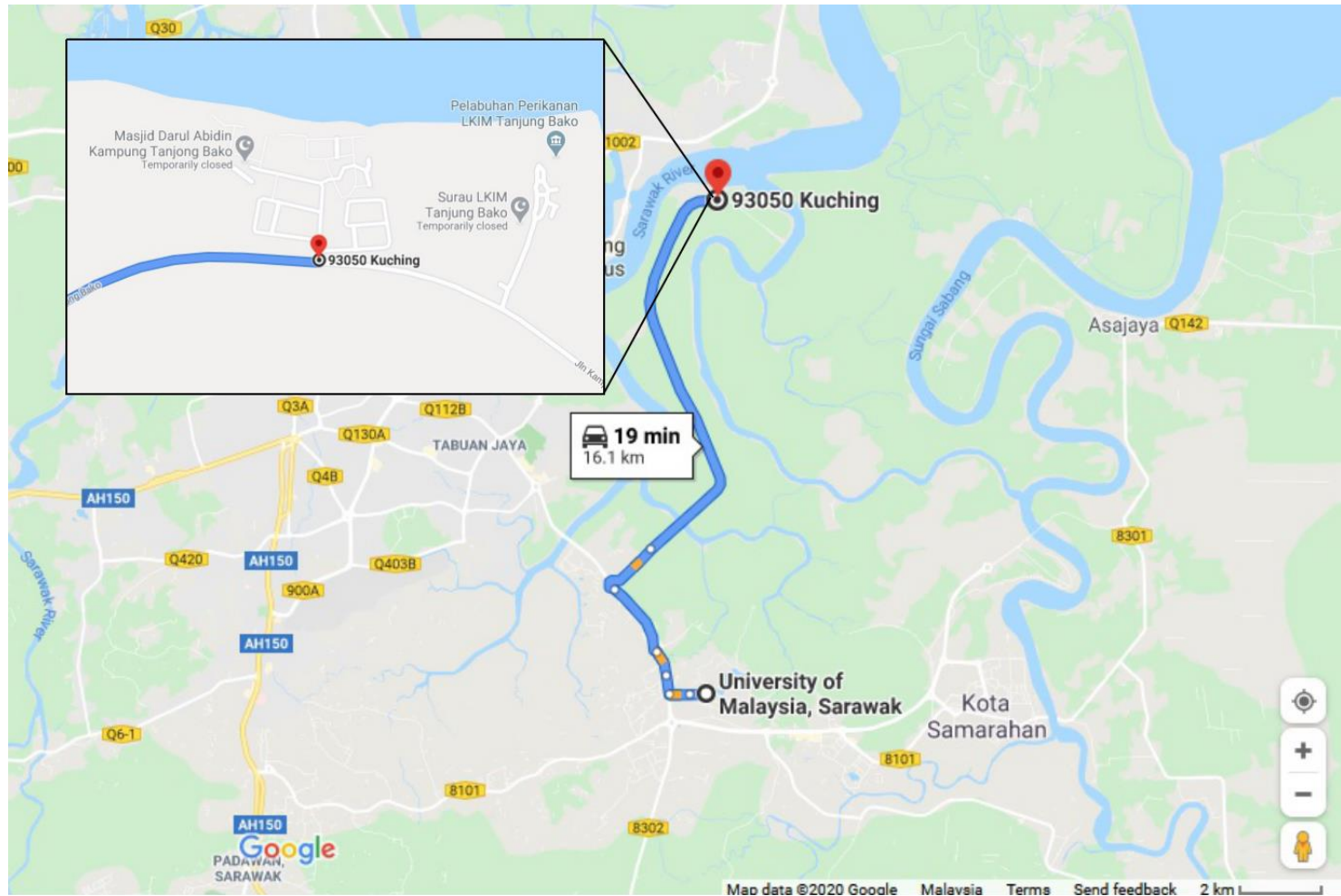


Figure 3.3: Map of Kampung Tanjong Bako from UNIMAS (Photo courtesy from Google Map)



Figure 3.4: Map of Kampung Tanjong Bako (Photo courtesy from Google Map)

Another 500 µl of Buffer RPE was added into the spin column after the process of centrifugation. The column was then centrifuged for 2 minutes at 12600 rpm to dry the column membrane. Then the column was attached to a new 1500 µl microcentrifuge tubes and 40 µl of RNase-free water was added into the spin column. The spin column was centrifuged for 1 minute at 12600 rpm. Another 40 µl of RNase free water was added into the spin column and centrifuged again for 1 minute at 12600 rpm to gain a higher concentration of RNA. The tube was labelled and stored in -80°C freezer pending further process.

3.4 Reverse Transcriptionpolymerase Chain Reaction of CHIKV

Two-step reverse transcription polymerase chain reaction (RT-PCR) was performed for the detection of chikungunya virus by using the protocol as stated in Thavara et al. (2009) and Nor Aliza et al. (2019) with modifications and the suggested primers followed Dayakar et al. (2015). The sequences of the primers used are shown in Table 3.1.

Table 3.1: Sequences of the primers (CHIKF and CHIKR) used for detection of chikungunya virus (Dayakar et al., 2015)

Primer	Sequence	Genome Position	Expected size (bp) of DNA product
CHIKF (Forward)	5'-ACCGGCGTCTACCCATTCATGT-3'	10237-10258	325
CHIKR (Reverse)	5'-GGGCGGGTAGTCCATGTTGTAGA-3'	10544-10566	325

3.4.1 Reverse transcription (RT) of CHIKV

Preparation of master mix was carried out in a biosafety cabinet (BSC) and all the reagents needed were kept on ice throughout the experiment. The master mix was placed in a 1500 µl microcentrifuge tubes and labelled. Besides, 200 µl PCR tubes were also prepared

and labelled for the experiment. The volumes of reagents in the master mix required for one sample are shown in Table 3.2. The total volume of the master mix prepared was calculated based on the number of samples. The master mix was mixed thoroughly by pipetting. The master mix was vortexed for 5 seconds and spun for 5 seconds to get rid of the bubbles.

Table 3.2: Reagents used for the preparation of master mix for the reverse transcription of CHIKV

Reagents	Volume per reaction	Final concentration
RNase-free water	4.4 μ l	-
5 $\times$ RT Buffer	3 μ l	5 $\times$
dNTP mix (10Mm each dNTP)	0.1875 μ l=0.2 μ l	500 μ M (125 μ M each)
CHIKR Primer (10 μ M)	0.375 μ l=0.4 μ l	0.25 μ M
Rnasin (20 unit/ μ l)	1 μ l	20 unit
Total	9 μl	

Nine μ l of the prepared master mix was added into the 200 μ l PCR tube. 5 μ l of the RNA template which was the extracted RNA was also added to the PCR tube. One μ l of Go-script reverse transcriptase was then added into the mixture for the process of transcription of chikungunya RNA into complementary-DNA. The PCR tube was spun briefly before being placed into a thermal cycler. The PCR machine or the thermal cycler (Applied Biosystems®Veriti® Thermal Cycler) was programmed as shown in Table 3.3. The RT products were kept on ice for further processing.

Table 3.3: Thermal cycler (Applied Biosystems® Veriti® Thermal Cycler) programmed for the RT process of CHIKV

Reaction step	Time (minute)	Temperature (°C)
Heating	0.5	48
RT	30	48

3.4.2 Polymerase Chain Reaction (PCR) of CHIKV

The preparation of the master mix for PCR is shown in Table 3.4. A 1500 μ l microcentrifuge tubes and 200 μ l PCR tubes were prepared and labelled. The master mix was placed in the 1500 μ l microcentrifuge tube. The total volume of the master mix prepared was calculated based on the number of samples. The master mix was mixed thoroughly by pipetting, vortexed for 5 seconds, and spun for another 5 seconds to get rid of the bubbles.

Table 3.4: Reagents used for the preparation of the master mix for polymerase chain reaction for detection of CHIKV

Reagents	Volume per reaction	Final concentration
RNase-free water	26.6 μ l	-
MgCl ₂ (25mM)	5 μ l	2.5 mM each
dNTP mix	1 μ l	0.2 mM each
CHIKF Primer (10 μ M)	1 μ l	0.2 Mm
CHIKR Primer (10 μ M)	1 μ l	0.2 mM
5 $\times$ Taq Green/ Colorless Buffer	10 μ l	1X
Final Total	44.6 μl	

A master mix of 44.6 μ l was added into the 200 μ l PCR tube. About 5 μ l of the RT product was added to the PCR tube as well. A volume of 0.4 μ l of Go Taq DNA Polymerase was then added into the mixture as well. The PCR tubes were spun briefly before being placed into a thermal cycler. A positive control and negative control were also prepared for this experiment. The PCR machine or the thermal cycler (Applied Biosystems®Veriti® Thermal Cycler) was programmed as shown in Table 3.5. The PCR products were then kept in a -20°C for further processing.

3.4.3 Visualization of PCR Products in Agarose Gel Electrophoresis of CHIKV

Agarose gel (1.6%) was prepared by mixing 1.6 grams of agarose powder (Vivantis, USA) with 100 ml of 0.5X Tris-Borate-EDTA (TBE) buffer. The mixture was heated in microwave for 2 minutes. The mixture was left for a while to cool down afterwards.

Table 3.5: Thermal Cycler (Applied Biosystems®Veriti® Thermal Cycler) programmed for PCR of CHIKV

Reaction step	Time (minute)	Temperature (°C)	Cycles
Heating	∞	50	-
Initial Denaturation	2	94	-
Denaturation	1	94	35
Annealing	1	54	35
Extension	1	72	35
Final extension	10	72	-
Hold	∞	4	-

SYBR DNA gel stain of 5 µl was added to the mixture and the mixture was poured into an electrophoresis casting tray with a comb. The mixture was left to solidify at room temperature for 30 minutes. The comb was removed after the gel solidified and the casting tray was placed in an electrophoresis tank filled with 0.5X TBE Buffer. Ten µl of the PCR products and 5 µl of 100 base pair (bp) DNA ladder (Promega Bench Top DNA Ladder) were loaded into the wells of the gel. The voltage used for the gel electrophoresis process was 100 volts for 75 minutes. The gel was visualized under the Syngene UV transilluminator in a dark room. The bands appearing in the gel image under the visualization were recorded and the image printed were kept and analysed.

3.5 Reverse-transcription Polymerase Chain Reaction (RT-PCR) of DENV

Two-step reverse transcription polymerase chain reaction (RT-PCR) was performed for the detection of dengue virus based on the protocol in Reynes et al. (2003), Ayers et al. (2006), and Nor Aliza et al. (2019) while the suggested primers followed Lanciotti et al. (1992). The same primers were used in the study by Nor Aliza et al. (2019) as well. The sequences of the primers used are shown in Table 3.6.

Table 3.6: Sequences of the primers (D1 and D2) used for detection of dengue virus (Lanciotti et al.1992)

Primer	Sequence	Genome Position	Expected size (bp) of DNA product
D1 (Forward)	5'-TCAATATGCCTGAAACGCCCGAGAAACCG-3'	131-161	511
D2 (Reverse)	5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3'	616-644	511

3.5.1 Reverse Transcription (RT) of DENV

Preparation of the master mix for reverse transcription of DENV is similar to the preparation of master mix used in the process of reverse transcription of CHIKV with the exception that the primers used were changed. The process was carried out in a Biosafety Cabinet (BSC) and all the reagents needed were kept on ice throughout the experiment. The master mix was placed in a 1500 µl microcentrifuge tubes and labelled. PCR tubes (200 µl) were also prepared and labelled for the experiment. The volumes of reagents in the master mix required for one sample are shown in Table 3.7. The total volume of the master mix prepared was calculated based on the number of samples. The master mix was mixed

thoroughly by pipetting. The master mix was vortexed for 5 seconds and spun for 5 seconds to get rid of the bubbles.

Nine μl of the prepared master mix was added into the 200 μl PCR tube. A volume of 5 μl of the RNA template which was the extracted RNA was also added to the PCR tube. One μl of Go-script reverse transcriptase was then added into the mixture for the process of transcription of dengue RNA into complementary-DNA. The PCR tube was spun briefly before being placed into a thermal cycler. The PCR machine or the thermal cycler (Applied Biosystems®Veriti® Thermal Cycler) was programmed as shown in Table 3.8 for reverse transcription process of DENV. The RT products were kept on ice for further processing.

Table 3.7: Reagents used for the preparation of the master mix for reverse transcription of DENV

Reagents	Volume per reaction	Final concentration
RNase-free water	4.4 μl	-
5 $\times$ RT Buffer	3 μl	5 $\times$
dNTP mix (10Mm each dNTP)	0.1875 μl =0.2 μl	500 μM (125 μM each)
D2 Primer (10 μM)	0.375 μl =0.4 μl	0.25 μM
Rnasin (20 unit/ μl)	1 μl	20 unit
Total	9 μl	

Table 3.8: Thermal cycler (Applied Biosystems® Veriti® Thermal Cycler) programmed for the RT step of DENV

Reaction step	Time (minute)	Temperature (°C)
Heating	1	42
RT	60	42

3.5.2 Polymerase chain reaction (PCR) of DENV

The preparation of the master mix for the PCR process of DENV is shown in Table 3.9. A 1500 µl microcentrifuge tubes and 200 ml PCR tubes were prepared and labelled. The master mix was placed in the 1500 µl microcentrifuge tube and the total volume of the master mix prepared was calculated based on the number of samples. The master mix was mixed thoroughly by pipetting. The master mix was vortexed for 5 seconds and spun for 5 seconds to get rid of the bubbles.

Table 3.9: Reagents used for the preparation of the master mix for polymerase chain reaction for detection of DENV

Reagents	Volume per reaction	Final concentration
RNase-free water	26.6 µl	-
MgCl ₂ (25mM)	5 µl	2.5 mM each
dNTP mix	1 µl	0.2 mM each
D1 Primer (10µM)	1 µl	0.2 mM
D2 Primer (10µM)	1 µl	0.2 mM
5×Taq Green/ Colorless Buffer	10 µl	1X
Final Total	44.6 µl	

A master mix of 44.6 µl was prepared for polymerase chain reaction of DENV and added into the 200 µl PCR tube. 5 µl of the RT product was also added to the PCR tube. Go Taq DNA Polymerase of 0.4 µl was added into the mixture as well. The PCR tubes were spun briefly before being placed into thermal cycler. A positive control and negative control were also prepared for this experiment. The PCR Machine or the Thermal Cycler (Applied Biosystems®Veriti® Thermal Cycler) was programmed as shown in Table 3.10. The PCR products were then kept in a -20°C freezer for further processing.

3.5.3 Visualization of PCR Products in Agarose Gel Electrophoresis of DENV

Agarose gel (1.6%) was prepared by mixing 1.6 grams of agarose powder (Vivantis, USA) with 100 ml of 0.5X Tris-Borate-EDTA (TBE) buffer. The mixture was heated in a microwave for 2 minutes and was left for a while to cool down. SYBR DNA (5 µl) gel stain was added to the mixture and the mixture was poured into an electrophoresis casting tray with a comb.

Table 3.10: Thermal Cycler (Applied Biosystems®Veriti® Thermal Cycler) programmed for PCR of DENV

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

The mixture was left to solidify at room temperature for 30 minutes. The comb was removed after the gel solidified and the casting tray was placed in an electrophoresis tank filled with 0.5X TBE Buffer and ten µl of the PCR products and 5 µl of 100 base pair (bp) DNA ladder (Promega Bench Top DNA Ladder) were loaded into the wells of the gel. The voltage used for the gel electrophoresis process is 100 vol for 60 minutes. The gel was visualized under the Syngene UV transilluminator in a dark room after the electrophoresis process. The bands appearing in the gel image under the visualization were recorded and the image printed were kept and analysed.

3.6 Data Analysis

3.6.1 Calculation of Ovitrap Index and Identification of Mosquitoes

The total number of ovitrap collected and the total number of ovitraps that were positive with larvae were recorded in each sampling in order to calculate the ovitrap index (OI). The OI were calculated with the formula used in Rozilawati et al. (2015).

$$OI = \frac{\text{Number of positive ovitrap}}{\text{Number of ovitrap collected}} \times 100$$

On the other hand, emerged adult mosquitoes collected from ovitraps, discarded receptacles, and field-caught adult mosquitoes were identified by using the keys from Division of Medical Entomology (IMR), Kuala Lumpur (Abdullah A.G., 2000). The species of the mosquitoes identified were recorded and tabulated in order to determine the variation of species of mosquitoes that were presence in the study area. Aedes mosquitoes were pooled according to sex and species before being subjected to RT-PCR.

3.6.2 Calculation of Minimum Infection Rate

Pools of female and male *Aedes albopictus* emerged from immatures that were detected positive with CHIKV and DENV were analyzed for its minimum infection rate (MIR). The MIR was calculated from the number of female/male mosquitoes in a positive pools divided by the total number of adult female/male mosquitoes multiplied by 1000.

$$\text{MIR} = \frac{\text{Number of pool with CHIKV/DENV positive mosquitoes}}{\text{Total number of mosquitoes tested}} \times 1000$$

3.6.3 Phylogenetic and Statistical Analysis

Samples that were detected positive for CHIKV and DENV during the visualization of agarose gel were sent for gene sequencing. The PCR product with positive chikungunya virus band and dengue virus band were sent to a commercial laboratory (First Base Malaysia) for gene sequencing together with the forward and reverse primers. The results of the gene sequencing were checked for its authenticity using National Centre for Biotechnology Information's (NCBI) nucleotide BLAST programme function and the gene sequences were compared and aligned with the sequences available in the GenBank. The sequences were aligned by using Multiple Sequence by Log-Expectation (MUSCLE) in Molecular Evolutionary Genetics Analysis (MEGA 7) software (Appendix A) before constructing a phylogenetic tree using the maximum likelihood method in MEGA 7 using 100 bootstrap replications.

On the other hand, statistical analysis using ANOVA test was performed to check whether there is a significant difference between the ovitrap indices in both of the villages. Statistical analysis was also performed by using one-way ANOVA to see the association

between captured mosquito species and the villages. All of the statistical analyses were performed by using IBM Statistical Package for the Social Sciences (SPSS) version 28.

CHAPTER 4

RESULTS

4.1 Species of Mosquitoes Collected

4.1.1 Immatures and Adult Mosquitoes Collected from Kampung Pulau Salak

Immatures of mosquitoes (larva and pupa) were collected in Kampung Pulau Salak, which totals up to 5222 in number of immatures that are reared for screening of CHIKV and DENV (Table 4.1). A total of 5000 immatures of mosquitoes were collected and identified from the ovitraps during the sampling at Kampung Pulau Salak. Four species of mosquitoes were identified from the ovitrap which are *Ae. albopictus*, *Ae. aegypti*, *Culex* sp. and *Armigeres* sp. in which *Ae. albopictus* is the dominant species of mosquitoes collected. A total of 4497 *Ae.albopictus*, 295 *Ae. aegypti*, 140 *Culex* sp. and 68 *Armigeres* sp. were collected from the ovitraps respectively.

Table 4.1: Mosquitoes species collected from Kampung Pulau Salak, Kuching

Species	Source of collection			Total	Percentage (%)
	Ovitrap	Discarded receptacles	Aspirator		
<i>Ae.albopictus</i>	4497	197	93	4787	90.07
<i>Ae.aegypti</i>	295	25	-	320	6.02
<i>Culex</i> sp.	140	-	-	140	2.63
<i>Armigeres</i> sp.	68	-	-	68	1.28
Total	5000	222	93	5315	100
Percentage (%)	94.07	4.18	1.75	100	

On the other hand, a total of 222 immatures were collected from discarded receptacles that can be found around the village. A total of 197 *Ae. albopictus* and 25 *Ae.*

aegypti have been collected from basin, plastic bowl, plastic container, and water storage containers around the village. Meanwhile, 93 adult *Ae. albopictus* have been collected from the field by using mechanical aspirators. The ovitrap index recorded from Kampung Pulau Salak were 84.3%, 96.7% and 89.3% for the first, second, and third sampling respectively.

4.1.2 Immatures and Adult Mosquitoes Collected from Kampung Tanjong Bako

A total of 2560 immatures of *Aedes* mosquitoes were collected from the ovitraps placed in Kampung Tanjong Bako (Table 4.2). Two species of mosquitoes were identified from the ovitraps which are *Ae. albopictus* and *Ae. aegypti*. Similar to Kampung Pulau Salak, *Ae. albopictus* is found to be the dominant species collected in Kampung Tanjong Bako. A total of 2558 immatures of *Ae. albopictus* and 2 immatures of *Ae. aegypti* have been collected from the ovitraps (Table 4.2).

Table 4.2: Mosquitoes species collected from Kampung Tanjong Bako, Kuching

Species	Source of collection			Total	Percentage (%)
	Ovitrap	Discarded receptacles	Aspirator		
<i>Ae.albopictus</i>	2558	136	31	2725	99.93
<i>Ae.aegypti</i>	2	-	-	2	0.07
Total	2560	136	31	2727	100
Percentage (%)	93.88	4.99	1.14	100	

Meanwhile, a total of 136 immatures of *Ae. albopictus* were collected from discarded receptacles that can be found around the village which includes containers and discarded kettle. On the other hand, 31 adult mosquitoes were collected from the field by using mechanical aspirators and the ovitrap index recorded from Kampung Tanjong Bako were 67.6%, 50.0% and 87.1% for the first, second and third sampling respectively. Statistical analysis by ANOVA (SPSS ver 28) revealed that there is no significant difference between

captured mosquito species between Kampung Pulau Salak and Kampung Tanjong Bako (($p > 0.05$).

4.2 Detection of CHIKV

4.2.1 Detection of CHIKV in Mosquitoes Collected from Kampung Pulau Salak

Chikungunya virus of East/Central/South/African (ECSA) genotype was detected in 2 pools out of 128 pools of *Ae. albopictus* collected from the ovitraps. The virus was detected in one male and one female pool ($n=35$) of the emerged adults of *Ae. albopictus*. Detection of CHIKV was indicated by the presence of 325 bp CHIKV DNA band in the gel electrophoresis image (Appendix A1).

The gene sequences of the positive CHIKV samples were retrieved from the laboratory and compared with Genbank by using BLAST function in NCBI. The sequences of the positive samples were aligned with 22 sequences from Genbank from the same genotype. The maximum likelihood tree constructed by using MEGA 7 software had shown that the isolate of positive female pool of *Aedes albopictus* (Pulau Salak/2018/FAe.alb) is closely related to the strain from Sarawak (KU196264/Sarawak/2009) while the isolate of positive male pool (Pulau Salak/2018/Mae.alb) is closely related to the strain from Thailand (KX009170/Thailand/2013) (Figure 4.1).

The minimum infection rate (MIR) which is the ratio of positive mosquito pool to the number of mosquitoes tested for the positive male pool and female pool in this study is 0.46% and 0.43% respectively. Presence of CHIKV was not detected in emerged adults of *Ae. aegypti*. Similarly, CHIKV was not detected in emerged adults of *Ae. albopictus* collected from discarded receptacles around the village. A total of 93 field-caught *Ae.*

albopictus were screened for the presence of CHIKV. There was no presence of CHIKV detected in the field-caught *Ae. Albopictus*.

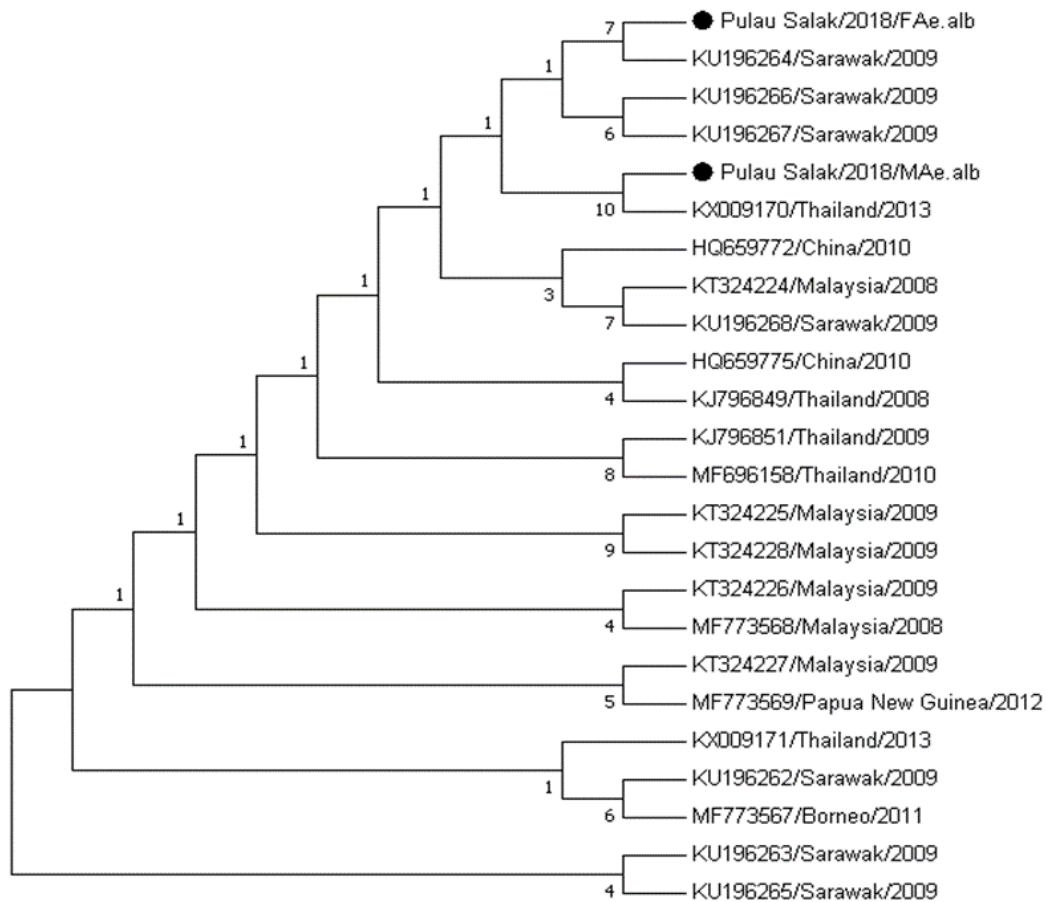


Figure 4.1: Maximum likelihood tree of 24 gene sequences of ECSA genotype of CHIKV created by using MEGA 7 (2 from this study, 22 from GenBank). The black dots represent the isolates from this study. The sequences were labelled according to the accession number/ country/ year of detection

4.2.2 Detection of CHIKV in mosquitoes collected from Kampung Tanjong Bako

There was no CHIKV detected in the emerged adult of *Ae. albopictus* and *Ae. aegypti* collected from the ovitraps as the 325 bp band did not appear in the gel electrophoresis image. Similarly, there is no presence of CHIKV detected in emerged adults of *Aedes* mosquitoes collected from discarded receptacles around the village. A total of 31 field-

caught *Ae. albopictus* were screened for the presence of CHIKV as well. There was no presence of CHIKV detected in the field-caught *Ae. albopictus*.

4.3 Detection of DENV

4.3.1 Detection of DENV in mosquitoes collected from Kampung Pulau Salak

Dengue virus (DENV) of serotype 2 was detected in 3 pools out of 128 pools of *Ae. albopictus* collected from the ovitraps. The virus was detected in one male and two female pools of the emerged adults of *Ae. albopictus*. Detection of DENV was indicated by the presence of 511 bp DENV DNA band in the gel electrophoresis image (Appendix AII)

The gene sequences of the positive DENV samples were retrieved from the laboratory and compared with Genbank by using BLAST function in NCBI. The sequences of the positive samples were aligned with 30 sequences from Genbank from the same genotype. The maximum likelihood tree constructed by using MEGA 7 software had shown that the isolate of positive male pool of *Ae. albopictus* (Pulau Salak/2018/20MAe.albo) and one of the isolates of positive female pool of *Ae. albopictus* (Pulau Salak/2018/20FAe.albo) are closely related to the strain from Malaysia (KU666948.1/Malaysia/TM296). On the other hand, one of the isolates of positive female pool (Pulau Salak/2018/19FAe.albo) is found to be closely related to the strain from India (MG885749.1/India/2017) (Figure 4.2).

The minimum infection rate (MIR) which is the ratio of positive mosquito pool to the number of mosquitoes tested for the positive male pool and female pool in this study is 0.46% and 0.86% respectively. The presence of DENV was not detected in emerged adult of *Ae. aegypti*. Similarly, DENV was not detected in emerged adults of *Ae. albopictus* collected from discarded receptacles around the village.

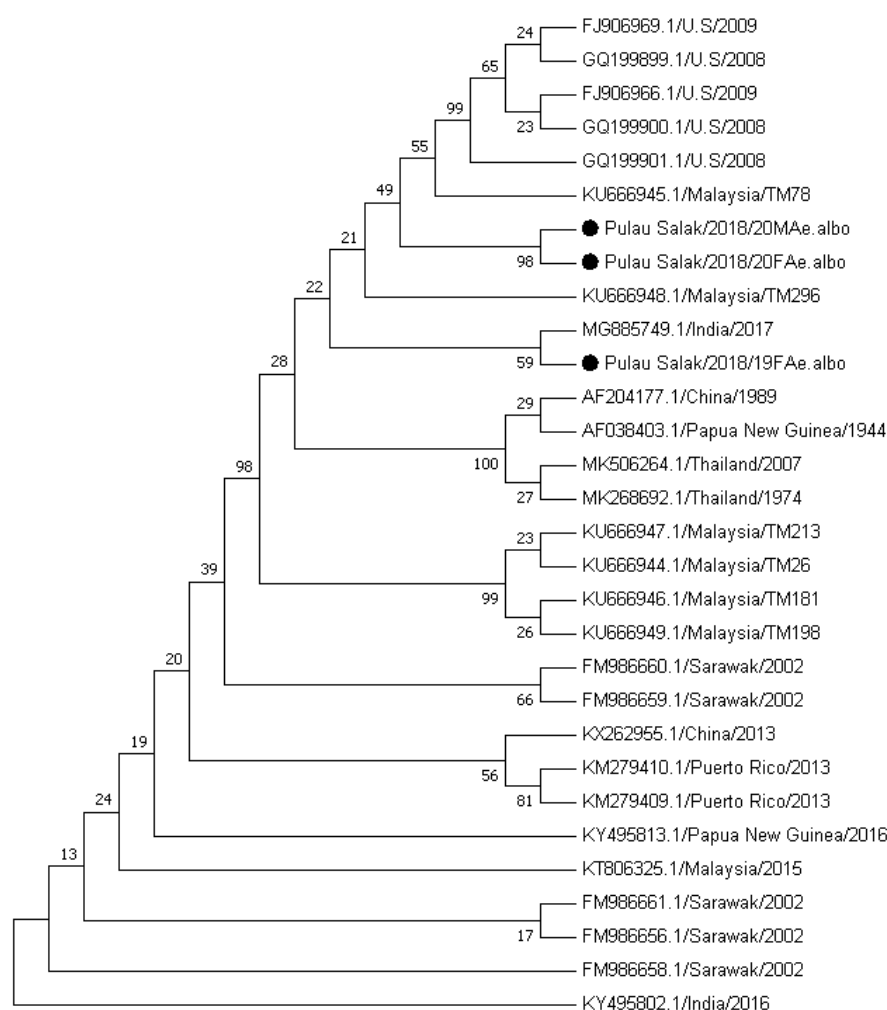


Figure 4.2: Maximum likelihood tree of 30 gene sequences of DENV-2 created by using MEGA 7 (3 from this study, 27 from GenBank). The black dots represent the isolates from this study. The sequences were labelled according to the accession number/ country/ year of detection or isolate number

4.3.2 Detection of DENV in mosquitoes collected from Kampung Tanjong Bako

There was no DENV detected in the emerged adult of *Ae. albopictus* and *Ae. aegypti* collected from the ovitraps as the 511bp band did not appeared in the gel electrophoresis image. Similarly, there are no presence of DENV detected in emerged adults of *Aedes* mosquitoes collected from discarded receptacles around the village. A total of 31 field-

caught *Ae. albopictus* were screened for the presence of DENV as well. There was no presence of DENV detected in the field-caught *Ae. albopictus*.

4.4 Temperature and pH Recorded in Ovitrap and Discarded Receptacles

4.4.1 Temperature and pH Recorded in Ovitrap and Discarded Receptacles in Kampung Pulau Salak

The temperature and pH of the water in the ovitrap were recorded after they are retrieved from the village. The range of temperature of ovitrap that are positive with the presence of mosquito larvae is around 26.4 °C to 28.4 °C with pH ranging from 6.6 to 7.9. The temperature and pH of the water in the discarded receptacles that are positive with the presence of immatures of mosquitoes were also recorded (Table 4.3). The range of temperature is around 27 °C to 28.2 °C while the pH ranges from 6.4 to 7.8.

Table 4.3: Range of temperature and pH recorded from discarded receptacles found around Kampung Pulau Salak

Type of container	Range of temperature (°C)	Mean $\pm$ SD	Range of pH	Mean $\pm$ SD
Basin	27.8-28.1	27.9667 $\pm$ 0.15275	6.7-7.8	7.2500 $\pm$ 0.77782
Plastic container	27-27.1	27.4333 $\pm$ 0.57735	6.8-6.9	6.8500 $\pm$ 0.7071
Plastic bowl	27.3	27.3 (* only one plastic bowl)	6.6	M: 6.6
Water storage container	27.3-28.2	27.5667 $\pm$ 0.55076	6.4-7.0	6.7333 $\pm$ 0.30551

* Notation: SD denotes standard deviation

4.4.2 Temperature and pH Recorded in Ovitrap and Discarded Receptacles in Kampung Tanjong Bako

The temperature and pH of the water in the ovitraps were recorded after they are retrieved from the village. The range of temperature of ovitraps that are positive with the presence of mosquito larvae is around 24.2 °C to 31.1 °C with pH ranging from 7.0 to 8.2. The temperature and pH of the water in the discarded receptacles that are positive with the presence of immatures of mosquitoes were also recorded (Table 4.4). The range of temperature is around 25.6 °C to 31.1 °C while the pH ranges from 7.1 to 8.0.

Table 4.4: Range of temperature and pH recorded from discarded receptacles found around Kampung Tanjong Bako

Type of container	Range of temperature (°C)	Mean ± SD	Range of pH	Mean ± SD
White container	30.1-31.	30.6000 ± 0.70711	7.1-8.0	7.5500 ± 0.63640
Discarded kettle	25.6	25.6 (*only one kettle)	7.6	7.6000

* Notation: SD denotes standard deviation

CHAPTER 5

DISCUSSION

5.1 The Diversity of Mosquitoes Found at Kampung Pulau Salak and Kampung Tanjong Bako

Species of mosquitoes that were collected from Kampung Pulau Salak were *Aedes albopictus*, *Ae. aegypti*, *Culex* sp. and *Armigeres* sp. Meanwhile, only *Ae. albopictus* and *Ae. aegypti* were collected from the sampling in Kampung Tanjong Bako. *Ae. albopictus* was found to be the dominant mosquito species collected in this study. This is not uncommon as Chang and Jute (1982) have mentioned that the population of *Ae. albopictus* is more widespread in Sarawak compared to *Ae. aegypti*. They carried out the study at 73 localities in seven divisions in Sarawak and only 2 out of the 73 localities were found to be without *Ae. albopictus*. On the other hand, *Ae. aegypti* was only detected in 36 localities in the study by Chang and Jute (1982). The population of *Ae. aegypti* seems to decrease compared to 37 years ago as studied by Lau et al. (2017) showed no presence of *Ae. aegypti* at all in 8 divisions of Sarawak. Similar to Lau et al. (2017), Afizah et al. (2015) also reported that there was no presence of *Ae. aegypti* during their ovitrap surveillance in 2012 and 2006 in Malay and Aboriginal Village on Carey Island in Kuala Langat District of Selangor. In contrast, our findings showed the presence of *Ae. aegypti* although it is less in number as compared to *Ae. albopictus*. Before 2007, there is no proper supply of water in the village of Salak Island. The absence of proper water supply system might have contributed to the presence of *Ae. aegypti* as the villagers stored rainwater into containers that were placed in the surrounding of their houses. These water containers could serve as a good breeding ground for *Ae. aegypti*.

Meanwhile, the low number of *Ae. aegypti* in this study may be due to certain communication barriers such as the absence of proper means of transportation between the village and the urban areas that could have affected migration of *Ae. aegypti* leading to uneven distribution of the species in the village. Besides, *Ae. aegypti* have not been able to establish their population well compared to *Ae. albopictus* in rural areas as they have limited flight range and they are usually found hitchhiking in human transportation networks such as cars and buses (Chang & Jute, 1982; Overgaard et al., 2017). Although *Ae. albopictus* can adapt well to the urban environment, this species still prefer areas of dense vegetation (Lwande et al., 2020). Another possible factor would be that the population of *Ae. albopictus* is so dominant that it does not allow the establishment of *Ae. aegypti* on the island as the establishment of *Ae. albopictus* was linked to the reduction in the abundance and range of *Ae. aegypti* (Afizah et al., 2015). It was known that both *Ae. albopictus* and *Ae. aegypti* are the vectors of CHIKV and DENV (Gratz, 1999) and since the population of *Ae. albopictus* is greater than those of *Ae. aegypti* in this study, the former could be the potential vector of the viruses in Kampung Pulau Salak.

Co-breeding was found in some of the ovitraps retrieved from Kampung Pulau Salak and Kampung Tanjong Bako. *Aedes* mosquitoes were found to co-breed with *Culex* sp. and *Armigeres* sp. in the ovitraps retrieved from Salak Island. This is not uncommon as it was reported in certain studies that *Aedes* mosquitoes are able to co-breed with *Culex* sp. and *Armigeres* sp. (Dieng et al., 2012; Getachew et al., 2015; Lau et al., 2017; Wan-Norafikah et al., 2018). The co-existence of more than a single species a habitat shows that they are sharing the same environmental conditions (Low et al., 2013). For instance, some *Armigeres* sp. such as *Armigeres kerselli* and *Armigeres subalbatus* may adapt to breeding sites similar to *Aedes* and *Culex* mosquitoes (Nurin-Zulkifli et al., 2015; Lau et al., 2017).

It was also found that *Ae. albopictus* was co-breeding with *Ae. aegypti* in the ovitraps recovered from Kampung Pulau Salak and Kampung Tanjong Bako. This is similar to findings by Seng and Jute (1994), Chen et al. (2006), and Hashim et al. (2018) in which they reported the presence of mixed breeding of *Ae. albopictus* and *Ae. aegypti* in their study. Seng and Jute (1994) have reported in their study that the total of shared containers that are positive with mixed breeding of both species of *Aedes* in a house survey are accounted to 9% which mainly placed outdoor. All of the ovitraps are placed outdoor at the village in Salak Island and this might have contributed to the co-breeding of both *Aedes* mosquitoes as well.

5.2 CHIKV in Aedes Mosquitoes

The presence of CHIKV was detected in emerged adult of *Ae. albopictus* which indicates the presence of transovarial transmission among the mosquito population in Kampung Pulau Salak. Transovarial transmission which is also known as vertical transmission is a condition where the infected female mosquitoes pass the virus to their offspring through their eggs. This type of transmission is believed to be a predominant way in which some arbovirus is maintained during harsh environmental conditions. It was found that eggs of *Aedes* mosquitoes could withstand dryness and remain viable for a longer period in harsh environmental conditions, which makes it possible for CHIKV to be retained in the eggs (Chompoosri et al., 2016). Besides, the ribonucleic acid (RNA) of CHIKV can be retained for prolonged period in mosquito eggs, which can be detected in emerged adults (Wong et al., 2016).

A study conducted by Niyas et al. (2010) was one of those findings that have demonstrated the presence of transovarial transmission in which they have detected the

presence of CHIKV in the emerged adults from the larvae that were collected in the households of CHIK patients in Kerala, India. Similar to this study, the larvae were reared in the laboratory before being subjected to RNA extraction and screened for detection of CHIKV. On the other hand, Thavara et al. (2009) have detected the presence of CHIKV in field-caught adult male *Aedes* mosquitoes that indicates the presence of transovarial transmission of the arbovirus in the field population of the mosquito vectors. It was also reported that infected females of both *Ae. albopictus* and *Ae. aegypti* are able to pass the virus to their offspring up to few generations under laboratory conditions (Chompoosri et al., 2016).

CHIKV was detected among *Ae. albopictus* collected from this study. It is not surprising as the mutation in the envelope protein gene of CHIKV, which is known as E1-A226V would contribute to the significant increase in CHIKV infectivity for *Ae. albopictus* (Tsetsarkin et al., 2007). A severe epidemic of CHIKV have taken place on Reunion Island which is located in the Southwestern part of the Indian Ocean in 2005-2006 and the epidemic was linked to a mutation of E1-A226V (Borgherini et al., 2007; Tsetsarkin et al., 2007). The outbreak in Reunion Island is a special case as it is the first reported case of CHIKV outbreak in which the main vector is *Ae. albopictus* (Tsetsarkin et al., 2007).

On the other hand, there is a difference found in the capability of transmitting the Indian Ocean lineage of CHIKV between the two species of mosquitoes (Chompoosri et al., 2016). *Ae. albopictus* is reported to be more competitive at infection and transmission of CHIKV compared to *Ae. aegypti*. It was also mentioned that higher cases of CHIKV have been reported in the southern provinces of Thailand where the population of *Ae. albopictus* was higher than those of *Ae. aegypti* (Chompoosri et al., 2016). Similarly, there were more

Ae. albopictus detected in Kampung Pulau Salak as compared to *Ae. aegypti*. All these factors could explain why CHIKV was only detected in the mosquito pool of *Ae. albopictus* in this study.

The genotype of CHIKV detected in this study is the East/ Central/ South/ African (ECSA) genotype. There are two genotypes of CHIKV detected in Malaysia namely, Asian and ECSA genotype. Asian genotype was detected during the first and second outbreak of CHIKV in Klang and Bagan Panchor respectively (Lam et al., 2001; Abu Bakar et al., 2007). The ECSA genotype in Malaysia was first detected in Kinta district, Perak in 2006 (Noridah et al., 2007; Sam et al., 2009). Similar to Malaysia, the shift of Asian genotype to ECSA genotype of CHIKV that caused the outbreak was observed in India (Yergolkar et al., 2006). The genotype caused outbreak in Malaysia in the year 2008 to 2010 which affected over 10000 people (Sam et al., 2012). The genotype might have been introduced in Malaysia due to the movement of people from Malaysia to India to visit their relatives in those outbreak areas of CHIKV (Noridah et al., 2007). This factor might contribute to the detection of CHIKV of ESCA genotype in Kampung Pulau Salak.

Faint PCR bands were detected in this study, which is similar to a study done by Rohani et al. 2005. Rohani et al. (2005) have carried out reverse transcription polymerase chain reaction (RT-PCR) on laboratory infected *Ae. aegypti* with CHIKV and one of the positive pools demonstrated weak PCR band. It is assumed that the presence of the faint band is due to a low number of infected mosquitoes in the pool. A stronger PCR band is expected to appear under certain conditions such as a higher number of infected mosquitoes in the pools, great dosage of viral infection, and the presence of a suitable vector (Rohani et al., 2005).

5.3 DENV in Aedes Mosquitoes

It is known that DENV can be transmitted by infected female adult mosquitoes to some part of their offspring (Ferreira-de-Lima & Lima-Camara, 2018). The presence of DENV was detected in emerged adults of *Ae. albopictus* in this study and this reveals the presence of transovarial transmission of DENV among the mosquito population in Kampung Pulau Salak. The first detection of DENV in emerged adults of *Ae. albopictus* through transovarial transmission in selected localities in Kuching and Samarahan divisions, Sarawak was reported by Nor Aliza et al. (2019). Dengue virus serotype 1 (DENV-1) and 2 (DENV-2) were detected in emerged adults of female *Ae. albopictus* in their study. On the other hand, only DENV-2 was detected in the emerged adults of *Ae. albopictus* in Kampung Pulau Salak which comprises of two pools of female and one pool of male *Ae. albopictus*.

Transovarial transmission of DENV is now a well-known phenomenon recorded in many parts of the endemic parts of the world in which the transmission played a significant role in initiating and maintaining the outbreak of the virus among human populations (Rohani et al., 2014). Several studies have been done regarding transovarial transmission of DENV, one of them is by Rohani et al. (1997) in which larvae, and mosquito adults were collected from 12 states on Peninsular Malaysia and screened for the presence of DENV. The majority of the positive samples for DENV came from *Ae. albopictus* in their study and one pool each from Kuala Lumpur and Perak in which the virus was isolated from *Ae. aegypti*. Their findings show the possibility of the presence of transovarial transmission by *Ae. albopictus* and *Ae. aegypti*.

Thenmozhi et al. (2007) have collected the larvae and adults of *Ae. albopictus* to study the natural transovarial transmission of dengue virus in Kerala which is state at

Southern India. DENV-2 was detected in the field-caught male adult mosquitoes in Kerala and it is suggested that the virus is maintained in *Ae. albopictus* during the dry season through transovarial transmission in nature. Lee et al. (2005) and Rohani et al. (2014) have also detected the occurrence of transovarial transmission in *Aedes* mosquitoes in certain areas in Malaysia in which the majority of the positive samples are from *Ae. albopictus*. On the other hand, Joanne et al. (2017) have stated that although *Ae. albopictus* is a less competent vector of DENV compared to *Ae. aegypti* as it has a lower virus load in its organ but due to the possible high-vertical transmission rate that they have discovered in their study, they are suggesting *Ae. albopictus* might play a vital role in maintaining the dengue infection throughout and between the epidemics depending on the circulation of the serotype. *Ae. albopictus* might be a potential vector of DENV in the island as it is the dominant species of *Aedes* mosquitoes collected and the virus was only detected in emerged adults of the species.

5.4 Minimum Infection Rate of CHIKV and DENV in Aedes Mosquitoes

5.4.1 Minimum Infection Rate of CHIKV in Aedes Mosquitoes

The minimum infection rate (MIR) of the positive male pool and female pool in this study is 0.28% and 0.26% respectively. MIR is one of the methods that are usually used in arbovirus surveillance and one of the importances of arbovirus surveillance is to monitor the presence of pathogens (Gu & Novak., 2003). The MIR value in this study is very low compared to the MIR value recorded in a study done by Rohani et al. 2005. In Rohani et al. 2005, colonies of mosquitoes were artificially infected with CHIKV in the laboratory and the MIR value recorded was 71% in the study. The low value of MIR indicates that an outbreak of CHIKV will be unlikely to occur in the village as it is assumed that a higher mosquito infection rate will increase the rate of arbovirus transmission (Bustamante & Lord,

2010). Thus, the maintenance of CHIKV among the vector mosquito population will be low due to the low infection rate.

5.4.2 Minimum Infection Rate of DENV in Aedes Mosquitoes

Similar to the MIR of CHIKV, the value of MIR of DENV in *Ae. albopictus* is low as well in this study. The minimum infection rate is the ratio of positive for the positive male pool and female pools in this study are 0.28% and 0.52% respectively. The MIR value is low compared to the MIR value of DENV for *Ae. albopictus* in Lee et al. (2005) which have the range of 2.35 to 14.30% and 5.77 to 40.00% for *Ae. albopictus* and *Ae. aegypti* respectively. The value of MIR in this study is very low as well compared to the MIR values recorded in Harvie et al. (2020) which ranges from 11.8 to 47.6%. It had been suggested that the higher the value of MIR, the higher the risk of dengue transmission will be (Vikram et al., 2015). Therefore, the maintenance of DENV among the mosquito population in the village might be low as well due to the low MIR value.

5.5 Temperature and pH of the Medium of Growth for Immatures of Aedes Mosquitoes

Temperature can affect the reproduction and development of the immature stages of mosquitoes (Couret & Benedict, 2014 cited in Reinhold et al., 2018). The larvae of mosquitoes will be stationary and they will die within a couple of days when they are in a medium with lower temperature such as 8 °C (Reinhold et al., 2018). On the other hand, it was discovered that the time taken by the immature of mosquitoes to complete their developmental stages was optimal at the temperature of 32 °C (Bar-Zeev, 1958 cited in Reinhold et al., 2018). In a study about the effect of temperature on the growth rate and survival of the immature stages of *Ae. aegypti*, it was discovered that the lowest and highest temperature which allows development from newly hatched larvae to adult mosquitoes were

14 °C and 36 °C respectively (Bar-Zeev, 1958). Meanwhile, it was reported that *Ae. albopictus* are able to fully develop within a temperature of 15 °C to 35 °C (Dellate et al., 2009 cited in Reinhold et al., 2018). The range of temperature recorded in the ovitraps for this study is 24.2 °C and 31.1 °C that allows the full development of the *Aedes* mosquitoes to take place.

The lowest and highest pH level recorded in this ovitrap in this study are 6.6 and 8.2 respectively. It was reported in Clark et al. (2004) that *Ae. aegypti* managed to survive and develop in water with pH level ranging from 4 to 11. It was also reported that another species of *Aedes* mosquitoes which is *Aedes vittatus* was able to survive in pH level ranging from 5.97-9.85 (Adebote et al., 2008 cited in Sequihod, 2016). It can be concluded that *Aedes* mosquitoes have wider level of pH tolerance compared to other mosquito species (Sequihod, 2016). Therefore, it can be deduced that the pH and temperature recorded in this study are still within the normal range for the development of *Aedes* mosquitoes.

CHAPTER 6

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The findings of this study indicated that there was presence of transovarial transmission of chikungunya virus (CHIKV) and dengue virus (DENV) among the population of *Aedes albopictus* in Kampung Pulau Salak, Kuching as the virus was detected in the emerged adults *Ae. albopictus* mosquitoes. CHIKV and DENV transovarial transmission occurred in both emerged adults of male and female of *Ae. albopictus* but not in *Ae. aegypti*. CHIKV of Eastern Central South African (ECSA) genotype was detected in one pool of females and one male pool of *Ae. albopictus*. On the other hand, DENV of serotype 2 was detected in two pools of female *Ae. albopictus* and one pool of male *Ae. albopictus*. This study also indicates that *Ae. albopictus* have the potential to be the main vector of CHIKV and DENV on Kampung Pulau Salak due to its dominance as the predominant mosquitoes found in these two villages are *Ae. albopictus*. *Ae. albopictus* have the potential to be the main vector responsible for the outbreak reported previously in Kampung Pulau Salak as well.

However, the minimum infection rate of both CHIKV and DENV recorded in this study are low compared with the previous reports and thus, the maintenance of CHIKV and DENV among the vector mosquito population might be low due to the low infection rate. In addition, it was also discovered that mosquito immatures can survive at a wide range of temperatures and pH levels in both villages. All the data obtained might help in the process of estimating the risk of infection of the viruses in both study locations. This study is the

first to demonstrate the presence of transovarial transmission of CHIKV and DENV in Kampung Pulau Salak, Kuching, Sarawak.

6.2 Limitations of Study

One of the limitations in this study is environmental factor. There were less field-caught adults and immature of mosquitoes collected in the village at Kampung Tanjong Bako as compared to Kampung Pulau Salak as the village had much less discarded receptacles for mosquito breeding receptacles and relatively cleaner. There was also less water storage containers in the village and the waste management is better because the village has asphalt road connected to the rest of Kuching. Another limitation would be the trapping method to collect the field-caught mosquitoes as BioGents (BG) Sentinel Mosquito Trap might be a better alternative compared to mechanical aspirator as interference of human is needed to catch the mosquitoes individually by using mechanical aspirator. More samples collected will yield results that are more accurate and better to represent the level of transmission of CHIKV and DENV in the mosquito population on the island.

6.3 Recommendations

More field-caught mosquitoes and immatures should be collected in the future study to extensively study the presence of transovarial transmission of CHIKV and DENV as it can serve as a reference in case of future outbreaks. Seroprevalence survey should be carried out in the future to determine the percentage of human population in Kampung Pulau Salak who have history of CHIKV infection since the outbreak in 2009. It is recommended since CHIKV was detected in Aedes mosquitoes on the island and a seroprevalence survey will add to the accuracy of the data as well. A seroprevalence study will extend the current findings of the research and serve as a reference of CHIKV and DENV status among the

island population. Besides, the number of villages sampled for Aedes mosquitoes should be increased too especially in those areas affected by CHIKV or DENV outbreaks in Sarawak. Finally, the vector surveillance and the risk of infections assessment study can also be extended to other areas for estimating the risk of infections.

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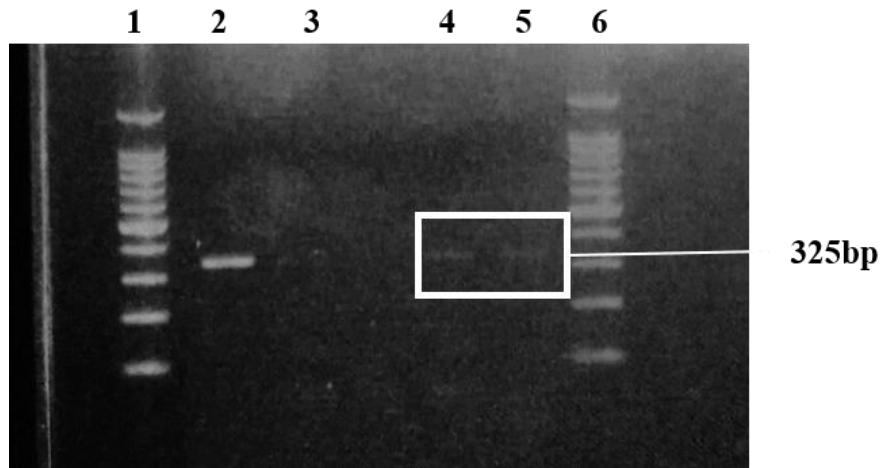
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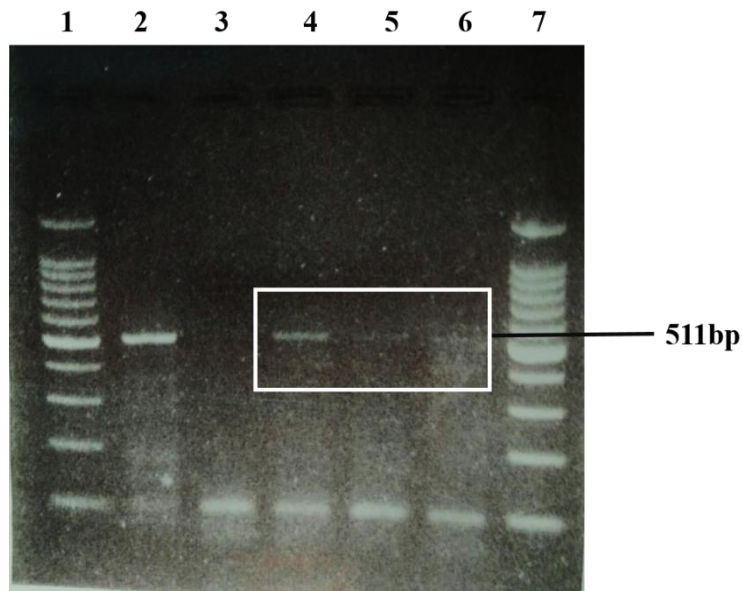
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APPENDICES

Appendix A: Gel Electrophoresis Images



Appendix AI: Gel electrophoresis image showing CHIKV positive mosquito pools from Kampung Pulau Salak , as indicated by a 325bp DNA band. (Lane 1,6: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4,5: Positive samples showing 325bp CHIKV c-DNA band).



Appendix AII: Gel electrophoresis image showing DENV positive mosquito pools from Kampung Pulau Salak, as indicated by a 511bp DNA band. (Lane 1,7: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4,5,6: Positive samples showing 511bp DENV c-DNA band).

Appendix B: Alignment of Nucleotide Sequences

Appendix BI: Nucleotide sequence alignments by MUSCLE of CHIKV-positive samples with reference sequences from GenBank

Samples	Nucleotide sequence alignments
Pulau Salak/2018/FAe.alb (Sample)	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KU196264/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KU196266/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KU196267/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
Pulau Salak/2018/MAe.alb (Sample)	TTTACGGGCGTCTACCCATTTCATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGA AGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGA AATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCMGTCACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAG CCTGGACACCTTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTACAACATGGACTACCCGCCCAA
KX009170/Thailand /2013	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
HQ659772/China/2010	- TTACCGGCGTCTACCCATTTCATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGAA GTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAA ATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAGCC TGGACACCTTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTACAACATGGACTACCCGCCCAA

Appendix BI continued

KT324224/Malaysia /2008	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KU196268/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
HQ659775/China/2 010	TTTACCGGCGTCTACCCATTATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGAA GTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAA ATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAGCC TGGACACCTTTGACAACAAAATTGTGGTGTACAAAGGTGACGTCTACAACATGGACTACCCGCCCA---
KJ796849/Thailand/ 2008	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KJ796851/Thailand/ 2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
MF696158/Thailand /2010	---- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KT324225/Malaysia /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KT324228/Malaysia /2009	TTCACCGGCGTCTACCCATTTATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGAA GTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGAA ATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAGCC TGGACACCTTTGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAACATGGACTACCCGCC--- ---
KT324226/Malaysia /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAAGTGCCTATGCAAACGGCGACCATGCCGTCACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---

Appendix BI

continued

MF773568/Malaysi a/2008	TTCACCGGCGTCTACCCATTTATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGA AGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGA AATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAGC CTGGACACCTTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAACATGGACTACCCGCCC---
KT324227/Malaysia /2009	TTCACCGGCGTCTACCCATTTATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGA AGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGA AATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAGC CTGGACACCTTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAACATGGACTACCCGCCC---
MF773569/Papua New Guinea/2012	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KX009171/Thailand /2013	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KU196262/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
MF773567/Borneo/ 2011	TTCACCGGCGTCTACCCATTTATGTGGGGCGGCGCCTACTGCTTCTGCGACGCTGAAAATACGCAGTTGAGCGAAGCACATGTGGAGA AGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGCATCAGCTAAGCTCCGCGTCCTTTACCAAGGA AATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGACGCCAAATTCATTGTGGGGCCAATGTCTTCAGC CTGGACACCTTTTCGACAACAAAATTGTGGTGTACAAAGGTGACGTCTATAACATGGACTACCCGCCC---
KU196263/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---
KU196265/Sarawak /2009	--- ATACGCAGTTGAGCGAAGCACATGTGGAGAAGTCCGAATCATGCAAAACAGAATTTGCATCAGCGTACAGGGCTCATACCGCATCTGC ATCAGCTAAGCTCCGCGTCCTTTACCAAGGAAATAACATCACTGTAACTGCCTATGCAAACGGCGACCATGCCGTACAGTTAAGGAC GCCAAATTCATTGTGGGGCCAATGTCTTCAGCCTGGACACCTT---

Appendix BII: Nucleotide sequence alignments by MUSCLE of DENV-positive samples with reference sequences from GenBank

Samples	Nucleotide sequence alignments
FJ906969.1/U.S/2009	ACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCTGATGAAT---
GQ199899.1/U.S/2008	ACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCTGATGAAT---
FJ906966.1/U.S/2009	ACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCTGATGAAT---
GQ199901.1/U. S/2008	ACAAAGACAGATTCTCTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATTAGAGAGCAGATCTCTGATGAAT---
KU666945.1/Malaysia/TM78	ATGGACCTCGGTGAATTGTGTGAAGACACAATCATTATAATTGTCCTCTTCTCAGGCAGAATGAACCAG---
Pulau Salak/2018/20MAe.albo	NNNNNNNNNGNANNNTATCTGCNCTNNCGGTTCTCGTCCACCTCGAACCTGAAGACATTGACTGACTGGTGCAAATGTTGT GAACCNNGGTGGCGTTCCTTCGTTTCCTAACAAACCCACCATGAGCAGGGATACTGAAGATGTGGGGAACAATTAATAAATC AAAAGCCTTTAATGTTTTGAGAGGGTTCAGGAAAGAGATTGGAAGGATGCTGAACATCTTGAACAGGAGACGCAGAACTG CAGGCATGATCATTATGCTGATTCCAACAGTGATGGCGTTCATTTAACACACGTAACGGAGAACCACACATGATCGTCA GTAGACAAGAGAAAGGGAAAAGTCTTCTGTTTAAACAGAGGATGGTGTGAACATGTGTACCCTCATGGCCATGGACCTTG GTGAATTGTGTGAAGATACAATCACGTACAAGTGTCTTTTCTCAAGCAGAATGAACCTGAAGACATTGACTGTTGGTGCA AA---
Pulau Salak/2018/20FAe.albo	NNNNNNNNNTNNNNNATCTGCACTANNCGGTTCTCGTCCACCTCGAACCTGAAGACATTGACTGTTGGTGCAAATGTTTCAT GGCCCTGGTGGCGTTCCTTCGTTTCCTAACAAATCCCAACAGCAGGGATACTGAAGAGNTGGGGAACAATTAATAAATC AAAAGCCATTAATGTTTTGAGAGGGTTCAGGAAAGAGATTGGAAGGATGCTGAACATCTTGAACAGGAGACGCAGAACTG CAGGCATGATCATTATGCTGATTCCAACAGTGATGGCGTTCATTTAACACACGTAACGGAGAACCACACATGATCGTCA GTAGACAAGAGAAAGGGAAAAGTCTTCTGTTTAAACAGAGGATGGTGTGAACATGTGTACCCTCATGGCCATGGACCTTG GTGAATTGTGTGAAGATACAATCACGTACAAGTGTCTTTTCTCAAGCAGAATGAACCTGAAGACATTGACTGTTGGTGCA AA---
KU666948.1/Malaysia/TM296	GCGTTCATTTAACCACACGCAATGGAGAACCACACATGATCGTCAGTAGACAAGAGAAAGGGAAAAGTC---
MG885749.1/ India/2017	TTTGTAGAAGGGGTTTCAGGAGGAAGTTGGGTTGACATAGTCTTAGAACATGGAAGCTGTGTGACGACGA---
Pulau Salak/2018/19FAe.albo	NNNNNNNNNTNNGNTCNNACTAGACGNTTCTCTGCTCCACTTGGAATNGNGANGACGTTGACTGACTGGTGCAAATGT TCATGGCCCTGGTGGCGTTCCTTCGTTTCCTAACAAATCCCAACAGCAGGGATACTGAAGAGATGGGGAACAATTAATAA AATCAAAAGCCATTAATGTTTTGAGAGGGTTCAGGAAAGAGATTGGAAGGATGCTGAACATCTTGAACAGGAGACGCAGA ACTGCAGGCATGATCATTATGCTGATTCCAACAGTGATGGCGTTCATTTAACACACGTAACGGAGAACCACACATGATC GTCAGTAGACAAGAGAAAGGGAAAAGTCTTCTGTTTAAACAGAGGATGGTGTGAACATGTGTACCCTCATGGCCATGGAC CTTGGTGAATTGTGTGAAGATACAATCACGTACAAGTGTCTTTTCTCAAGCAGAATGAACCTGAAGACATTGACTGTTGGT GCAAA

Appendix BII continued

AF204177.1/China/1989	AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATT
AF038403.1/Papua New Guinea/1944	AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATT---
MK506264.1/Thailand/2007	AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATT---
MK268692.1/Thailand/1974	AGTTGTTAGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACGTAGTTCTAACAGTTTTTTAATT---
KU666947.1/Malaysia/TM213	ATGCTGAAACGCGAGAGAGAAACCGCGTGTCAACTGTGCAGCAGCTGACAAAGAGATTCTCACTTGGAATGC---
KU666944.1/Malaysia/TM26	ATGCTGAAACGCGAGAGAGAAACCGCGTGTCAACTGTGCAGCAGCTGACAAAGAGATTCTCACTTGGAATGC---
KU666946.1/Malaysia/TM181	ATGCTGAAACGCGAGAGAGAAACCGCGTGTCAACTGTGCAGCAGCTGACAAAGAGATTCTCACTTGGAATGC---
KU666949.1/Malaysia/TM198	ATGCTGAAACGCGAGAGAGAAACCGCGTGTCAACTGTGCAGCAGCTGACAAAGAGATTCTCACTTGGAATGC---
FM986660.1/Sarawak/2002	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGATATAG---
FM986659.1/Sarawak/2002	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGATATAG---
KX262955.1/China/2013	ATGCGTTGCATAGGAATATCAAATAGAGACTTTGTAGAAGGGGTTTCAGGAGGAAGTTGGGTTGACATAG---
KM279410.1/Puerto Rico/2013	ATGCGCTGCATAGGAATATCAAATAGAGACTTCGTAGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAG---
KM279409.1/Puerto Rico/2013	ATGCGCTGCATAGGAATATCAAATAGAGACTTCGTAGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAG---
KY495813.1/Papua New Guinea/2016	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGGAGCTGGGTTGACATAG---
KT806325.1/Malaysia/2015	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTCTCAGGAGGAAGCTGGGTTGACATAG---
KT806325.1/Malaysia/2015	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTCTCAGGAGGAAGCTGGGTTGACATAG---
FM986661.1/Sarawak/2002	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAG---
FM986656.1/Sarawak/2002	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAG---
FM986658.1/Sarawak/2002	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAG---
KY495802.1/India/2016	ATGCGTTGTATAGGAATATCAAATAGAGACTTTGTGGAAGGGGTTTCAGGAGGAAGCTGGGTTGACATAG---

Appendix C: Data of Mosquitoes Collected

Appendix CI: Number of mosquitoes collected from ovitraps in Kampung Pulau Salak based on species and sex

Sampling	Species	Sex	Total
First sampling	<i>Aedes albopictus</i>	Male	1222
		Female	1286
	<i>Aedes aegypti</i>	Male	81
		Female	128
	<i>Culex</i> sp.	Male	32
		Female	56
	<i>Armigeres</i> sp.	Male	23
		Female	25
Second sampling	<i>Aedes albopictus</i>	Male	650
		Female	722
	<i>Aedes aegypti</i>	Male	38
		Female	35
	<i>Culex</i> sp.	Male	36
		Female	16
	<i>Armigeres</i> sp.	Male	6
		Female	14
Third sampling	<i>Aedes albopictus</i>	Male	303
		Female	314
	<i>Aedes aegypti</i>	Male	3
		Female	10
Total			5000

Appendix CII: Number of mosquitoes collected from ovitraps in Kampung Tanjong Bako based on species and sex

Sampling	Species	Sex	Total
First sampling	<i>Aedes albopictus</i>	Male	196
		Female	244
Second sampling	<i>Aedes albopictus</i>	Male	372
		Female	370
Third sampling	<i>Aedes albopictus</i>	Male	651
		Female	725
	<i>Aedes aegypti</i>	Male	0
		Female	2
Total			2560

Appendix CIII: Total mosquitoes collected from discarded receptacles and field from Kampung Pulau Salak and Kampung Tanjong Bako

Source	Species	Sex	Total
Basin	<i>Aedes albopictus</i>	Male	41
		Female	49
	<i>Aedes aegypti</i>	Male	17
		Female	8
Plastic bowl	<i>Aedes albopictus</i>	Male	13
		Female	11
Plastic container	<i>Aedes albopictus</i>	Male	11
		Female	13
Water storage container	<i>Aedes albopictus</i>	Male	79
		Female	88
Discarded kettle	<i>Aedes albopictus</i>	Male	13
		Female	15
Field-caught	<i>Aedes albopictus</i>	Male	70
		Female	54
Total			482

Appendix D: Species of Mosquitoes Collected



Appendix DI: Emerged adult of *Aedes albopictus* (Magnification : 40x)



Appendix DII: Emerged adult of *Aedes aegypti* ((Magnification : 40x)



Appendix DIII: Emerged adult of *Culex* sp. ((Magnification : 40x)



Appendix DIV: Emerged adult of *Armigeres* sp. ((Magnification : 40x)

Appendix E: Study Sites and Breeding Containers



Appendix EI: Sampling site at Kampung Pulau Salak



Appendix EII: Sampling site at Kampung Pulau Salak



Appendix EIII: Sampling site at Kampung Pulau Salak



Appendix EIV: Sampling site at Kampung Pulau Salak



Appendix EV: Sampling site at Kampung Tanjong Bako



Appendix EVI: Sampling site at Kampung Tanjong Bako



Appendix EVII: Sampling site at Kampung Tanjong Bako



Appendix EVIII: Sampling site at Kampung Tanjong Bako



Appendix EIX: Water storage containers around Kampung Tanjong Bako



Appendix EX: Water storage containers around Kampung Pulau Salak

Appendix F: Reagent Kits and Equipment



Appendix FI: QIAGEN RNeasy Mini Kit used in extraction of RNA from the mosquito samples



Appendix FII: QIAamp Viral Mini Kit used in extraction of RNA from supernatant of CHIKV-positive control and DENV-positive control



Appendix FIII: Mechanical aspirator used to collect adult mosquitoes from the field



Appendix FIV: Set up of the ovitrap with the wooden paddle and plastic