# ISOLATION AND MOLECULAR IDENTIFICATION OF DENGUE AND CHIKUNGUNYA VIRUSES FROM FIELD-COLLECTED *AEDES* MOSQUITOES IN KOTA BHARU AREAS, KELANTAN

**TUAN NUR AKMALINA BINTI MAT JUSOH**

**UNIVERSITI SAINS MALAYSIA**

**2024**

# ISOLATION AND MOLECULAR IDENTIFICATION OF DENGUE AND CHIKUNGUNYA VIRUSES FROM FIELD-COLLECTED *AEDES* MOSQUITOES IN KOTA BHARU AREAS, KELANTAN

**by**

**TUAN NUR AKMALINA BINTI MAT JUSOH**

**Thesis submitted in fulfilment of the requirements**
**for the degree of**
**Master of Science**

**August 2024**

## ACKNOWLEDGEMENT

Alhamdulillah for His blessing for giving me the strength to finish the thesis. First and foremost, I would like to express my deepest gratitude, and respect to my supervisor, Associate Prof. Dr Rafidah Hanim Shueb for her continuous encouragement, assistance, guidance, patience, and support for me during the whole study and in completing my thesis.

Special thanks to my co-supervisor, Dr Zeti Norfidiyati Salmuna for the help and advice in the study. Besides, my appreciation is extended to all staff involved from the Entomology Unit, Makmal Kesihatan Awam (MKA) Kota Bharu especially Mr Izhan Shahrin Jaafar for their help in providing the mosquito samples.

I would like to express my special thanks to Dr Nik Zuraina, Yasmin, Syafiqah and Noreafifah for their advice in the labwork troubleshooting. To my beloved sisters and colleagues, Amalin, Hanifah, Najma, Amani, Hidayah, Alia, Alaa, Ahmad, Haziq, Bello, and others, thanks for the support through this journey.

My endless thanks and never-ending love go to my beloved family. All the struggles and challenges I faced during my research journey would have been impossible to overcome if not for the great support from all of you especially my parents, Mat Jusoh Deraman and Tuan Rosyati Raja Mat and my younger brothers, Hadi, Hakimie and Hazman.

Lastly, I would like to give my great appreciation to everyone who has been directly or indirectly involved in completing my thesis for their help and cooperation.

## TABLE OF CONTENTS

## LIST OF TABLES

**Page**

## LIST OF FIGURES

**Page**

## LIST OF SYMBOLS

| | |
|---|---|
| amps | Amperes |
| bp | Base pair |
| $cm^2$ | Centimetres squared |
| g | Gram |
| kb | Kilobase |
| L | Litre |
| m | Metre |
| mL | Millilitre |
| min | Minutes |
| n | Numbers |
| nm | Nanometre |
| s | Seconds |
| µg | Microgram |
| µL | Microlitre |
| µM | Micromolar |
| µm | Micron |
| **Δ** | Delta |
| $ | Dollar currency |
| °C | Degree Celcius |
| % | Percentage |
| ™ | Trademark |
| - | Minus |
| x *g* | Times gravity units |
| V | Voltage |

## LIST OF ABBREVIATIONS

| | |
|---|---|
| *Ae. albopictus* | *Aedes albopictus* |
| *Ae. aegypti* | *Aedes aegypti* |
| BLAST | Basic Local Alignment Search Tool |
| Bti | *Bacillus thuringiensis israelensis* |
| C | Capsid |
| CDC | Centers for Disease Control and Prevention |
| CHIKV | Chikungunya virus |
| CPE | Cytopathic effect |
| Dcon | Dengue conserved region |
| DENV | Dengue virus |
| DF | Dengue fever |
| DHF | Dengue haemorrhagic fever |
| DMSO | Dimethyl sulfoxide |
| DNA | Deoxyribonucleic acid |
| DSS | Dengue shock syndrome |
| E | Envelope |
| ECDC | The European Centre for Disease Prevention and Control |
| ECSA | East/Central/South/African |
| EGCG | Epigallocatechin gallate |
| ELISA | Enzyme-linked immunosorbent assay |
| FBS | Fetal bovine serum |
| FDA | Food and Drug Administration |
| GM | Genetically modified |
| HRP | Horseradish peroxide |
| IFA | Immunofluorescence assay |
| IgG | Immunoglobulin G |
| IgM | Immunoglobulin M |
| IOL | Indian Ocean Lineage |
| IMR | Institute for Medical Research |
| LAMP | Loop-mediated isothermal amplification |
| M | Membrane protein |

| | |
|---|---|
| MAb | Monoclonal antibody |
| MKA | Makmal Kesihatan Awam |
| MOH | Ministry of Health Malaysia |
| MTX | Methotrexate |
| NS | Non-structural protein |
| NSAIDs | Non-steroid anti-inflammatory drugs |
| nsP | Non-structural protein |
| ORF | Open reading frame |
| ORS | Outdoor residual spray |
| PBS | Phosphate buffer saline |
| PrM | Pre-membrane |
| RA | Rheumatic arthritis |
| RDT | Rapid diagnostic test |
| RNA | Ribonucleic acid |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| SIT | Sterile insect technique |
| TBE | Tris-borate-EDTA |
| TMB | Tramethylbenzidine |
| TPB | Tryptose Phosphate Broth |
| UiTM | Universiti Teknologi MARA |
| UniMAS | Universiti Malaysia Sarawak |
| USM | Universiti Sains Malaysia |
| UTR | Untranslated region |
| VLP | Virus-like particles |
| WA | West Africa |
| WHO | World Health Organisation |
| YFV | Yellow fever virus |
| ZIKV | Zika virus |

## LIST OF APPENDICES

**ISOLASI DAN PENGENALPASTIAN MOLEKUL VIRUS DENGGI DAN CHIKUNGUNYA DARIPADA NYAMUK *AEDES* YANG DIKUMPULKAN SECARA LAPANGAN DI KAWASAN KOTA BHARU, KELANTAN**

## ABSTRAK

Jangkitan denggi dan chikungunya telah menjadi ancaman kesihatan awam yang utama di Malaysia dengan jumlah kematian yang ketara dilaporkan pada setiap tahun. Kedua-dua penyakit ini disebarkan terutamanya oleh *Ae. aegypti* dan *Ae. albopictus* dan menyebabkan pelbagai manifestasi klinikal bermula dari demam ringan hingga kes maut yang teruk. Kajian ini bertujuan untuk menyiasat peredaran CHIKV dan serotaip DENV daripada nyamuk *Aedes* dewasa yang dikumpulkan secara lapangan di kawasan Kota Bharu. Sebanyak 114 kumpulan nyamuk (610) terdiri daripada 30 kumpulan *Ae. aegypti* (69 ekor nyamuk jantan dan 40 ekor nyamuk betina) dan 84 kumpulan *Ae. albopictus* (208 ekor nyamuk jantan dan 293 ekor nyamuk betina) dikumpulkan daripada lokaliti wabak DENV di Kota Bharu dari Januari hingga Ogos 2019 menggunakan jaring sapu dan ovitrap melekit. Isolasi virus telah dilakukan di dalam sel C6/36 dan pengesanan DENV telah disahkan menggunakan antigen bukan struktur (NS1) denggi dan ujian DENV multiplek tindak balas rantai polimerase transkriptase berbalik (RT-PCR). Manakala, ujian satu langkah RT-PCR telah dilakukan untuk mengesan CHIKV. Lokaliti Badang menunjukkan populasi nyamuk *Ae. albopictus* yang paling tinggi, manakala tiada pengumpulan nyamuk daripada lokaliti Panchor. Nyamuk *Ae. aegypti* paling banyak direkodkan di Kampung Demit Sungai. Untuk ujian DENV multiplek RT-PCR, keadaan optimum suhu penyepuhlindapan adalah 60ºC, bagi kepekatan primer adalah 0.1 µM (DENV 1 dan DENV 2) dan 0.2 µM (DENV 3 dan DENV 4). Empat daripada 91 kumpulan nyamuk

telah positif antigen denggi NS1 bagi ujian ELISA yang terdiri daripada dua (2.2%) kumpulan nyamuk *Ae. albopictus* (lokaliti Pulau Melaka dan Kubang Siput) dan dua (2.2%) kumpulan nyamuk *Ae. aegypti* (Kampung Demit Sungai). DENV 1 telah dikesan dalam satu (0.9%) kumpulan nyamuk betina *Ae. albopictus* diantara 114 kumpulan nyamuk *Aedes* yang diuji. Dua (1.7%) kumpulan nyamuk *Ae. aegypti* dan nyamuk *Ae. albopictus* telah dikesan positif dengan serotaip berganda, DENV 1 dan DENV 2 (Pulau Melaka). Kebanyakan kumpulan nyamuk yang dikesan positif bagi ujian antigen denggi NS1 adalah negatif oleh ujian RT-PCR kecuali satu kumpulan nyamuk jantan *Ae. albopictus* dari Pulau Melaka. Walaubagaimanapun, tiada kumpulan nyamuk positif untuk CHIKV dalam kajian ini. Dapatan kajian ini mendedahkan bahawa DENV 1 dan DENV 2 adalah virus yang beredar semasa di kawasan kajian. Selain itu, nyamuk *Ae. albopictus* merupakan spesies yang paling banyak ditemui di Kota Bharu. Kehadiran DENV dan vektor utama DENV di Kota Bharu adalah petunjuk penting untuk menambah baik kawalan vektor bagi mengurangkan jangkitan DENV dalam kalangan penduduk di lokaliti-lokaliti.

**ISOLATION AND MOLECULAR IDENTIFICATION OF DENGUE AND CHIKUNGUNYA VIRUSES FROM FIELD-COLLECTED *AEDES* MOSQUITOES IN KOTA BHARU AREAS, KELANTAN**

## ABSTRACT

Dengue and chikungunya infections have been a major public health threat in Malaysia with a significant number of deaths reported every year. Both diseases are transmitted mainly by *Ae. aegypti* and *Ae. albopictus* and cause a wide range of clinical manifestations from mild fever to severe fatal cases. This study aimed to investigate the circulating CHIKV and DENV serotypes from field-collected adult *Aedes* mosquitoes in the Kota Bharu area. A total of 114 mosquito pools (610) comprising 30 pools (69 male and 40 female mosquitoes) of *Ae. aegypti* and 84 pools (208 male and 293 female) of *Ae. albopictus* were collected at DENV outbreak localities in Kota Bharu from January to August 2019 using a sweep net and sticky ovitrap. Virus isolation was carried out in C6/36 cells and the presence of DENV was confirmed using dengue non-structural (NS1) antigen and DENV multiplex reverse transcriptase polymerase chain reaction (RT-PCR) assay. On the other hand, a one-step RT-PCR assay was performed to detect CHIKV. Badang locality showed the highest population of *Ae. albopictus* while no collection from the Panchor locality. Most of *Ae. aegypti* was recorded at Kampung Demit Sungai. For DENV multiplex RT-PCR assay, the optimum condition of annealing temperature was 60ºC, 0.1 µM (DENV 1 and DENV 2) and 0.2 µM (DENV 3 and DENV 4) for primer concentration. Of 91, four mosquito pools were positive for dengue NS1 antigen by ELISA comprising two pools (2.2%) of male *Ae. albopictus* (Pulau Melaka and Kubang Siput locality) and two pools (2.2%) of *Ae. aegypti* (Kampung Demit Sungai). DENV 1 was detected in one pool (0.9%) of

female *Ae. albopictus* among 114 tested *Aedes* pools. Two pools (1.7%) from male *Ae. aegypti and Ae. albopictus* were positive with double serotypes, DENV 1 and DENV 2 (Pulau Melaka). Most of the positive mosquito pools by dengue NS1 antigen were negative by RT-PCR except one pool of male *Ae. albopictus* from Pulau Melaka. However, no mosquito pool was positive for CHIKV in the study. The findings revealed that DENV 1 and DENV 2 were the current circulating virus in the study area. In addition, *Ae. albopictus* was the predominant species in the Kota Bharu area. The presence of DENV and the main vectors of DENV in Kota Bharu are pertinent indicators of the need to improve vector controls to reduce arbovirus infections among people in the localities.

# CHAPTER 1

# INTRODUCTION

## 1.1 Background of the study

The emerging arbovirus infections become a public health concern for humans and animals as more than a million people are affected worldwide yearly (Kading et al., 2020; WHO, 2014). Arboviruses are viruses that are transmitted by arthropods such as mosquitoes and ticks. *Aedes* mosquitoes are the responsible vectors that transmit various arboviruses such as dengue virus (DENV), chikungunya virus (CHIKV), Zika virus (ZIKV), and yellow fever virus (YFV) to humans (Cholleti et al., 2018). Rapid urbanisation, economic, and population growth are associated with increased vector populations and arbovirus infections (Chew et al., 2012; Socha et al., 2022; Tajudeen et al., 2021).

Dengue, caused by the DENV, belongs to the *Flavivirus* genus and is classified into four serotypes: DENV 1, 2, 3, and 4 (Ferreira-de-Lima et al., 2020). Not all DENV serotypes exist in dengue-endemic and epidemic countries (Harvie et al., 2020). The disease can be found in tropical and subtropical regions (Nonyong et al., 2021). DENV is endemic in 128 countries with an estimated 390 million DENV infections yearly (Bhatt et al., 2013; Elia-Amira et al., 2023). In Southeast Asia, dengue has become the most important arboviral disease, especially in the Philippines, Indonesia, Malaysia, and Thailand due to its climate conditions that facilitate the proliferation of DENV (Najri et al., 2019). Additionally, Malaysia, Thailand, and most Southeast Asian countries are dengue hyperendemic countries where all four serotypes are present (Harvie et al., 2020; Nonyong et al., 2021). During 2015-2021, DENV 2 and DENV 1 were the most common serotypes circulating in Malaysia, followed by DENV

3 and DENV 4 (Chem et al., 2024). However, different serotypes were dominant within those years: DENV 1 in 2015 and 2016, DENV 3 in 2017 and 2020, DENV 2 in 2018 and 2019 and DENV 4 in 2021.

Between 2012 to 2019, Malaysia recorded the highest number of dengue cases in 2019 with 130,101 cases (AbuBakar et al., 2022). As for Kota Bharu, 3365 cases were reported in 2019 with Panji (21.2%) showing the highest number of recorded cases, followed by Kubang Kerian (15.7%) and Badang (12.5%) (Mohd Kamaruzaman et al., 2023). The clinical manifestation of dengue varies from asymptomatic to severe complications or death. According to Pang et al. (2017), the World Health Organisation (WHO) (1997) classified dengue into dengue fever (DF), dengue haemorrhagic fever (DHF) and dengue shock syndrome (DSS). However, this classification was then modified in 2009 into dengue, dengue with warning signs and severe dengue. The treatment depends on supportive care and fluid therapy to relieve dengue symptoms.

Chikungunya is a mosquito-borne disease caused by CHIKV, a member of the alphavirus in the *Togaviridae* family, first found in Tanzania in 1952 (Azami et al., 2013). CHIKV is further divided into four genotypes: Asian, West African (WA), East/Central/South/African (ECSA) and the newly emerged genotypes Indian Ocean Lineage (IOL), which evolved from the ECSA genotype (Anggraeni et al., 2021; Maeda et al., 2022). The classification is based on their genomic diversity. Genotype Asian, ECSA and IOL were found to circulate during the outbreaks in Malaysia (Wahid et al., 2017). Similar to DENV, CHIKV is transmitted by the *Aedes* mosquito. The presentation of fever, nausea, headache, joint pain, and vomiting manifest the

clinical symptoms of chikungunya. However, the disease is often misdiagnosed and underreported as these symptoms overlap with dengue. The most common symptoms of CHIKV infection are fever and joint pain (CDC, 2023). In addition, the clinical forms of symptomatic chikungunya reported in patients are classified into acute, atypical acute, severe acute and chronic (Suhrbier, 2019).

CHIKV is endemic in Africa, India, China, the Caribbean, and several Southeast Asian countries including Indonesia, Thailand, and the Philippines (Abubakar et al., 2007; Kendall et al., 2019). In Asia, the first case of chikungunya was reported in Thailand in 1958 (Anggraeni et al., 2021). Malaysia experienced the first CHIKV outbreak in Port Klang (1998), followed by the second outbreak in Bagan Panchor (2006), Ipoh (2006) and Johor (2008) (Azami et al., 2013). Additionally, a recent outbreak with a low number of cases was reported in Tanjung Sepat (2019) (Khor et al., 2023). The abundance of vectors and travellers from endemic regions might contribute to the outbreak. A new outbreak may occur when a CHIKV-infected person from the endemic region travels to the non-endemic region and introduces the virus to new populations.

In terms of diagnosis, chikungunya and dengue are diagnosed using several methods including viral isolation, reverse transcriptase polymerase chain reaction (RT-PCR) and serological assay (Álvarez-Argüelles et al., 2019; Muller et al., 2017). RT-PCR showed high sensitivity and specificity for detecting viral ribonucleic acid (RNA) in a short period making it a reliable assay (Raafat et al., 2019). A serological diagnosis such as enzyme-linked immunosorbent assay (ELISA) is commonly used to detect DENV and CHIKV antibodies or antigens (Álvarez-Argüelles et al., 2019;

Muller et al., 2017). Variety immunochromatography assays are available to detect DENV and CHIKV antigens which provide a fast result in less than 20 minutes. However, the sensitivity of these assays is affected by host and viral factors including days of fever and viral serotype (Caicedo-Borrero et al., 2020).

## 1.2 Problem statement

Malaysia experiences a higher number of chikungunya and dengue cases that lead to public health concerns, particularly when the number of death cases increases. According to the Ministry of Health Malaysia, the cumulative cases of dengue in Malaysia in 2019 were 127,407 cases compared to 78,066 cases in 2018. At the same time, 176 death cases were recorded in 2019. Due to the high number of dengue cases, Kelantan ranks fourth among the states in Malaysia following Selangor, Kuala Lumpur and Johor (MOH, 2020). For chikungunya, 935 cumulative cases were reported in Malaysia in 2019 (MOH, 2019). In Kelantan, Kota Bharu reported the highest number of CHIKV cases, followed by Tumpat, Bachok and Tanah Merah (MOH, 2019).

DENV may sometimes cause severe dengue, a potentially lethal complication. In primary infection, patients develop a lifelong immunity to the infecting serotype. However, secondary infection with other serotypes may put the patient at greater risk of severe dengue (Soo et al., 2016). Previous studies found that the severity of dengue is associated with different DENV serotypes (Tsai et al., 2023). For example, a higher proportion of severe cases was shown in DENV 2 than in DENV 1 and DENV 4 (Vicente et al., 2016). Hence, it is important to identify the serotypes of DENV to ensure accurate diagnosis and treatment. Besides, it aids in understanding the epidemiology and tailoring public health responses. According to the Pan American

Health Organization (2023), serious complications of chikungunya are uncommon however, severe cases may cause death. Lethal cases of chikungunya have been reported with 149 deaths in the Caribbean region in 2014 (Torres-Flores et al., 2022). Clinical manifestations of CHIKV and DENV are hard to distinguish, making accurate diagnosis challenging as the symptoms are almost similar.

Unfortunately, no antiviral drugs or effective vaccines are available for dengue and chikungunya disease in Malaysia. Current treatment focuses only on symptomatic relief with non-steroid anti-inflammatory drugs (NSAIDs) and fluid therapy. Thus, until effective treatment is established, mosquito surveillance remains among the primary options and is important in controlling and preventing diseases from spreading. Many surveillance studies of arboviruses in mosquitoes have been done previously involving two stages of the mosquito's life cycle, including larvae and adults (Ababa, 2021). The selection of both larvae and adult stages for the detection of arboviruses is crucial as the choice depends on the goals of the surveillance program, ease of handling, required expertise of staff, detection method used and the laboratory facilities (CDC, 2022). Detection of viruses using larvae is often used for control purposes as it targets breeding sites while detection of viruses in adult mosquitoes is crucial for assessing the risk of human infection (Liu et al., 2023). Therefore, combining both larva and adult surveillance can provide a better understanding of arbovirus risk in the areas.

However, studies on the arboviruses in adult mosquitoes are still lacking in Kelantan. Previously, surveillance of CHIKV using larvae and adult mosquitoes in Kelantan was conducted in 2009 that involved Jeli, Pasir Mas, Tumpat and Tanah Merah districts (Rozilawati et al., 2011). Azlinda et al. (2018) reported the presence of DENV in adult mosquitoes in Universiti Sains Malaysia (USM), Kubang Kerian with an unknown serotype. Since then, no latest report was documented on recent CHIKV and DENV infection from adult mosquitoes in Kelantan. Furthermore, the undetectable DENV in mosquito larvae during a DENV surveillance study conducted in dengue hotspots after the post-big flood in 2015 (Abu Yazid, 2015) has led to a sign of seeking a more sensitive detection assay for DENV and CHIKV. Therefore, this present study will provide information on the circulating DENV and CHIKV from adult mosquitoes captured in the outbreak localities, Kota Bharu using several detection methods.

## 1.3 Research questions

i) What is the optimum RT-PCR assay for DENV and CHIKV?

ii) What is the prevalence of DENV and CHIKV in field-collected *Aedes* mosquitoes in Kota Bharu?

iii) What DENV serotypes are more prevalent in *Aedes* mosquitoes in Kota Bharu?

iv) What are suitable detection assays for DENV and CHIKV in field-collected *Aedes* in Kota Bharu?

v) How do the detection rates of DENV in *Aedes* mosquitoes compare between the NS1 antigen assay and RT-PCR assay?

## 1.4 Research hypothesis

i) $H_0$: Dengue and chikungunya virus cannot be isolated from the *Aedes* mosquito.

$H_a$: Dengue and chikungunya virus can be isolated from the *Aedes* mosquito.

ii) $H_0$: Different DENV serotypes will not be detected in adult *Aedes* mosquitoes.

$H_a$: Different DENV serotypes will be detected in adult *Aedes* mosquitoes.

## 1.5 Objective of study

### General objective

To investigate the circulating DENV and CHIKV from field-collected *Aedes* sp. mosquitoes in Kota Bharu.

### Specific objectives

1. To isolate DENV and CHIKV from field-captured *Aedes* mosquitoes.
2. To optimise reverse transcription polymerase chain reaction (RT-PCR) assay for DENV and CHIKV in field-captured *Aedes* mosquitoes.
3. To detect and identify DENV serotypes from the isolated culture by NS1 detection and RT-PCR in field-captured *Aedes* mosquitoes.
4. To identify CHIKV from the isolated culture by RT-PCR.

# CHAPTER 2

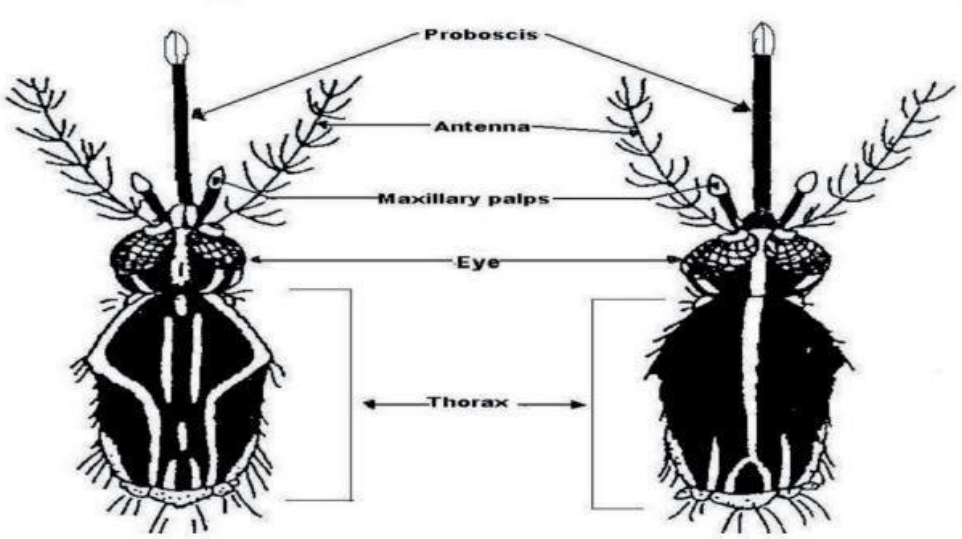
# LITERATURE REVIEW

## 2.1 *Aedes* mosquitoes

The genus *Aedes* belongs to the family *Culicidae*, comprising approximately more than 950 species and is one of the most widespread mosquito genera worldwide (Minwuyelet et al., 2023; Naim et al., 2020). *Aedes* mosquitoes generally have striped bodies that are more distinct in the legs and scutellum, with short palpi and a pale pointed abdomen band (Das et al., 2019). These remarkable criteria distinguish them from other mosquitoes. Among *Aedes* species, *Ae. aegypti* and *Ae. albopictus* are the most important and efficient vectors for spreading various arboviruses in humans (Leta et al., 2018; Näslund et al., 2021).

*Aedes aegypti* (Linnaeus) can be identified by the presence of lyred-shape silver scales on the thorax, while a single line of white scales pattern in the middle of the thorax is the feature of *Ae. albopictus* (Skuse) (Figure 2.1). Both species are commonly found in temperate and tropical regions on every continent except Antarctica (Laporta et al., 2023; Liang et al., 2015; Swan et al., 2022).*Aedes aegypti* is prevalent in urban areas, close to human dwellings with less vegetation (Athaillah et al., 2020) and tends to breed in artificial containers that collect and hold water, such as discarded tires, containers, flowerpots, buckets, and concrete tanks (Ferdousi et al., 2015). Unlike *Ae. aegypti*, *Ae. albopictus* has a high preference for areas with dense vegetation and is more adaptable to various habitats, including urban, suburban, and rural environments (Athaillah et al., 2020). Due to that, it can breed in both natural and artificial water-holding containers. Containers with organic debris, plant stalks, tree holes, animal shells, concrete tanks discarded tires and bamboo stumps served as breeding sites for

*Ae. albopictus* (Adnan et al., 2021; Higa, 2011). In addition, both species are considered highly anthropophilic due to their behaviour of feeding mammals (preferably humans) and avians when there are no humans around (Egid et al., 2022; Garcia-Rejon et al., 2021; Reinhold et al., 2018). *Aedes aegypti* is known as the primary vector for DENV and CHIKV (Novelo et al., 2023). However, *Ae. albopictus* was found as a responsible vector for the CHIKV outbreak in Reunion, Europe, and Gabon in the absence of *Ae. aegypti* (Ganesan et al., 2017). Moreover, a single mutation in the envelope viral gene E1 of an ECSA strain can increase the fitness of CHIKV in *Ae. albopictus* and the consequent acquisition of a more effective vector competence (Matusali et al., 2019).




(A) (B)

Figure 2.1 Thorax of adult female *Aedes* mosquitoes (A) *Ae. aegypti* (B) *Ae. albopictus* **(**adopted from Das et al. (2019)).

### 2.1.1 Life cycle of *Aedes* mosquitoes

*Aedes* mosquitoes undergo a complete metamorphosis process consisting of four stages: eggs, larvae, pupae, and adult mosquitoes (Rogers, 2023). According to Das et al. (2019), female mosquitoes bite and feed the blood from people or animals to produce 100-200 eggs per batch on average. The female lays their egg on a moist surface or above the waterline and soaks the eggs before hatching which may take several days and weeks depending on the environmental condition. The larvae then mature through four instars transition before developing into the pupae stages. At this stage, larvae hang upside down from the water surface and take oxygen through a siphon. Following that, the larvae development took less than 3 days for the first three instars and up to 3 days for the fourth instar. Later, a pupa emerges into an adult mosquito at the water surface in 2-3 days.

### 2.1.2 Disease-causing *Aedes* mosquitoes

Mosquitoes are vectors that transmit harmful pathogens and parasites from one host to another for both humans and animals (Socha et al., 2022). The genera *Aedes*, *Anopheles* and *Culex* are the important vectors that cause various mosquito-borne diseases (WHO, 2020). According to WHO, more than 17% of all infectious diseases are caused by vector-borne diseases with more than 700 000 deaths annually. Furthermore, mosquito-borne disease is reported to be the largest contributor to vector-borne diseases, and it is said that more than 80% of the global population is at risk of being infected (Aryaprema et al., 2023). Arboviral diseases such as dengue, chikungunya, Zika, and yellow fever are transmitted by *Aedes* mosquitoes (Bamou et al., 2021; Onen et al., 2023). The diseases have been spread worldwide and their occurrence is shown in Figure 2.2. These diseases cause major public health concerns

across the world with some arboviral infections causing severe conditions such as haemorrhage, neurological conditions, as well as death (Liang et al., 2015; Munoz et al., 2017). Most arboviral diseases share similar clinical manifestations and are often co-circulated in the same areas (Evans-Gilbert, 2020). However, overlapping symptoms such as fever, headache and muscle pain are present among the diseases, making it challenging to differentiate them based solely on clinical presentation. A proper diagnosis and management plan are needed to counter the overlapping symptoms, especially in areas where the viruses are prevalent.

Arboviruses are mainly transmitted horizontally among human hosts by the blood-feeding of an infected *Aedes* mosquito while vertical transmission of arboviruses from infected female mosquitoes to offspring is common (Lequime & Lambrechts, 2014; Paixão et al., 2018). However, vertical transmission of yellow fever is uncommon. Despite that, the transmission of CHIKV, DENV and ZIKV vertically in humans was reported (Brasil et al., 2020; Evans-Gilbert, 2020; Guzeloglu-Kayisli et al., 2022; Robillard et al., 2006).

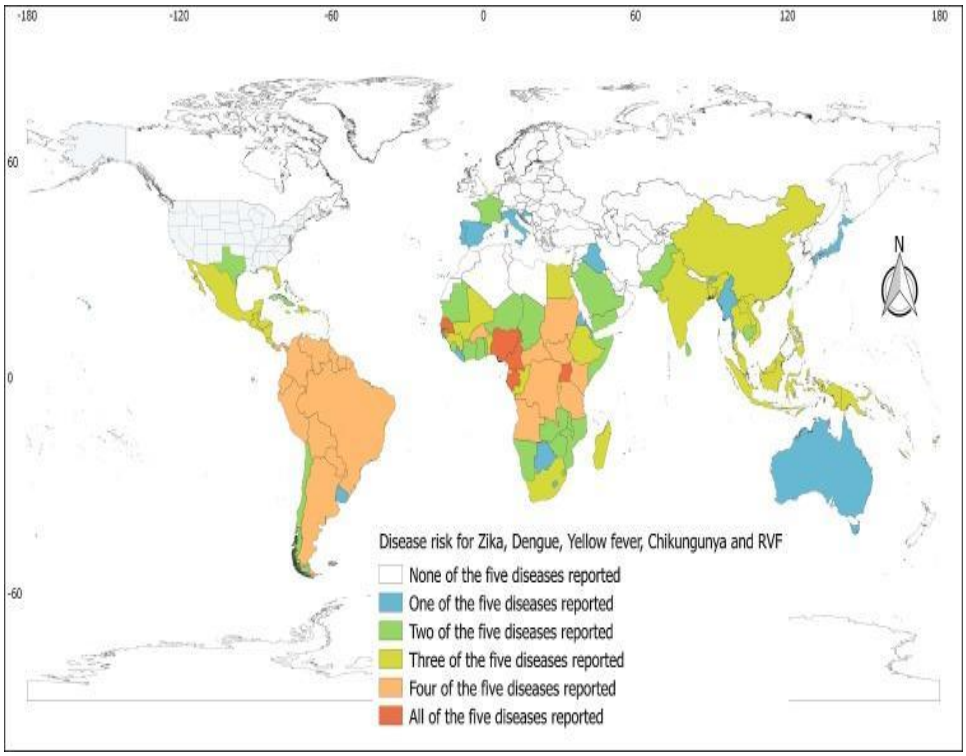



Figure 2.2 Map of the occurrences of arboviral diseases in the world (Adopted from Leta et al. (2018)).

### 2.1.3 Vector Control Program in Malaysia

As of now, the development of effective vaccines against mosquito-borne diseases is still in progress and management of the diseases relies on the control and prevention program. MOH has introduced a vector control approach to combat the disease (MOH, 2022). Vector control aims to reduce the vector population to the level that it may not allow any virus transmission (MOH, 2022). So, understanding the characteristics and habits of *Aedes* mosquitoes is essential for developing effective strategies to control its population and mitigate the risk of disease transmission.

There are six strategies under vector control approaches which are source reduction, larvaciding, space spraying, targeted outdoor residual spray (ORS), health promotion and law enforcement (MOH, 2022). Source reduction of *Aedes* larvae and space spraying of adult *Aedes* both outdoors and indoors is done within 24 hours after the case is notified in eDengue (an online system for management and monitoring of dengue surveillance across the country in real-time). For larvaciding, temephos or *Bacillus thuringiensis israelensis* (Bti) is used at the site that has potential breeding containers. ORS is done every 3 months by applying a low dose of pyrethroid onto the outer of the mosquito place to hide and rest. Many health promotions including mass media, posters, and health education at kiosks have been carried out to create and increase civilian awareness. Not only that, a compound is also given to the person who deliberately breeds under the Destruction of Disease-Bearing Insects Act 1975 (Caballero-Anthony et al., 2015).

Furthermore, new innovative approaches for dengue control have been introduced by the Institute for Medical Research (IMR) Malaysia (MOH, 2022). This includes the release of *Wolbachia*-infected mosquitoes to selected localities with a high dengue burden. *Wolbachia* is a bacterium that primarily infects arthropods, such as insects and other invertebrates and is known for their ability to manipulate the reproduction of their hosts (Stevens et al., 2001). *Wolbachia* is injected into *Aedes* eggs, and the bacteria will eventually grow inside the mosquito's abdomen and act to block the virus replication. *Wolbachia*-infected mosquitoes have been released at 22 dengue hotspots involving Selangor, Pulau Pinang, Wilayah Persekutuan Kuala Lumpur, Kelantan, Johor, Melaka, Negeri Sembilan and Pahang (MOH, 2022). Interestingly, the release of *Wolbachia* showed success after a decrease in dengue incidence was observed (Chrostek et al., 2020; Nazni et al., 2019).

Another biological control, the sterile insect technique (SIT) is used to control the vector population (MOH, 2022). This technique uses radiation to sterile the male mosquito which then makes it a genetically modified (GM) mosquito. Briefly, the sterile male mosquito will then be released to the targeted area and compete with wild males to mate with females. As a result, the eggs laid by females are infertile and this will indirectly reduce the vector population. A previous study by IMR demonstrated the ability of *Ae. aegypti* OX513A (My1) as a vector control to mate with female wild *Ae. aegypti* and produce offspring that will die as larvae (Subramaniam et al., 2012). Despite that, implementing and sustaining effective vector control programs can be expensive and laborious. This includes the costs associated with purchasing and applying insecticides, conducting surveillance, and monitoring, and educating communities about preventive measures.

## 2.2 Epidemiology of arboviral disease

### 2.2.1 Dengue

Dengue is the most common and widespread arboviral disease that causes significant public health concerns with approximately 400 million DENV cases and 22,000 deaths occurring worldwide (Roy & Bhattacharjee, 2021). Global dengue incidences have increased rapidly in the last several decades; in 2000, there were 505,430 cases reported to the WHO; by 2019, that number had risen to 5.2 million (WHO, 2023). Of these, 3.1 million cases were reported in America, followed by the Philippines (420,000), Vietnam (131,000), Malaysia (131,000) and Bangladesh (101,000). In the same period, Afghanistan reported DENV transmission for the first time (WHO, 2023). The spreading of DENV by the competent vector has caused several autochthonous transmissions in the Europe region with the first case occurring in Croatia and France in 2010. Since then, more local transmissions generated from viremia-travel cases were reported in France, Spain, and Italy from 2010-2023 (ECDC, 2023).

Currently, dengue is endemic in over 125 countries and the occurrence of epidemic annually in America, Asia, Africa, and Australia (Schaefer et al., 2022). The expansion of DENV to other parts of the world is driven by factors such as globalisation, urbanisation growth, climate and environmental change, and ineffective mosquito control (Zerfu et al., 2023). Furthermore, the interaction between vector populations, virus strains and the global environment increases the transmission efficacy of DENV (Cochet et al., 2022).

The first DENV outbreak was found in Malaysia in 1902, and major outbreaks occurred in 1974, 1978, 1982, 1990, and 2014–2016, which resulted in higher new cases and mortality (Abubakar & Shafee, 2002; Najri et al., 2019). DENV 1 was the first isolated dengue serotype in Malaysia, which was from a minor outbreak in Kuala Lumpur in 1954 (Suppiah et al., 2023), later, all four dengue serotypes were found to circulate in Malaysia, with several predominance shifts of serotype occurring in certain years (AbuBakar et al., 2022). For example, there was a switch from DENV 4 serotype to DENV2 observed in the early 1970s (Suppiah et al., 2023). In Malaysia, the incidence rate of dengue is the highest among communicable diseases with higher cases reported in Selangor, Wilayah Persekutuan Kuala Lumpur, and Johor (Mashudi et al., 2022). It has been shown that adolescents and children are the most affected by DENV infection (AbuBakar et al., 2022). In addition, higher dengue incidence resulted in economic burdens of $175.7 million in 2009/2010 for prevention and illness costs (AbuBakar et al., 2022).

### 2.2.2 Chikungunya

The name chikungunya is derived from the Makonde language word used by the local Tanzania people which means “that which bends up” to describe the posture of people suffering pain from the disease (de Lima Cavalcanti et al., 2022). CHIKV was first found in 1952-1953 during an outbreak in the Newala and Masisi districts in Southern Tanzania. Since then, several epidemics were reported in the Democratic Republic of the Congo (1958 and 1960), Zimbabwe (1957,1961-1962, and 1971), Zambia (1959), Senegal (1960), Uganda (1961-1962 and 1968), the Central African Republic (1978-1979), Guinea, Angola, Malawi, and others (Wahid et al., 2017; Zeller et al., 2016).

In Asia, the first outbreak was reported in 1958 and early 1960 in Thailand (Anggraeni et al., 2021), followed by an outbreak in Cambodia in 1961. Later, CHIKV was identified in the Philippines (1965), Vietnam (1966 and 1967), and Indonesia (1972) and minor outbreaks or sporadic cases were reported in South Asia and Southeast Asia including India, Myanmar, Malaysia, Sri Lanka, Pakistan, and others (de Lima Cavalcanti et al., 2022; Sharif et al., 2021). Malaysia experienced the first chikungunya outbreak in 1998 and then the re-emergence of CHIKV infection occurred in 2006 and 2008 (IMR, 2017). In 2004, a chikungunya outbreak was reported in Kenya with 70% of the population infected. CHIKV infection then spread to Mombasa and the Comoros which then caused a major outbreak in 2004-2005. Following that, several large epidemic waves occurred in La Reunion from 2005-2006 with 38.8 % of its 785,000 population were infected (Zavala-Colon & Gonzalez-Sanchez, 2019). Since 2004, CHIKV has spread to several islands in the Indian Ocean to India causing major outbreaks affecting over 1 million population during 2006-2009 (Zavala-Colon &

Gonzalez-Sanchez, 2019). The virus also has spread to other continents, such as the Pacific region, Europe, and the Americas (Sharif et al., 2021). Several autochthonous outbreaks/cases have been reported in Europe (2007), France (2010, 2013 and 2014), and America (2013) due to travelling activities from endemic regions (Wahid et al., 2017). These outbreaks were caused by the ECSA genotype that contains E1-226V mutation enhancing virus transmission by *Ae. albopictus* and is the responsible genotype for the re-emerged CHIKV outbreaks in Malaysia, Thailand, and Singapore (de Lima Cavalcanti et al., 2022).

The phylogenetic analysis of CHIKV revealed the virus diverged into four genotypes, the West African (WA), East/Central/South Africa/Asia (ECSA), and the newly emerged genotypes within the ECSA clade, the IOL (Rodriguez et al., 2019). WA genotype was reported to circulate in Guinea, Senegal, and India (Wahid et al., 2017). The ECSA is considered the ancestor strain, circulating in most countries in Africa and Asia and was reported to be the most prevalent genotype circulated in Asian countries such as Thailand, Malaysia, and Singapore from 2006-2010 (de Lima Cavalcanti et al., 2022; Sharif et al., 2021). The Asian strain was found in Asia and was most prevalent in the Philippines and Indonesia from 2007-2014 (de Lima Cavalcanti et al., 2022; Wahid et al., 2017). In addition, the outbreak in the Pacific region was caused by the Asian genotype involving New Caledonia, Yap State, Tonga, America Samoa, and Tokelau (Zeller et al., 2016). After 2004, the IOL strain rapidly spread in South Asia countries and caused an 80% outbreak in multiple countries including India, Cambodia, Thailand, China, Malaysia, Maldives, Sri Lanka, Bhutan, and Singapore. In Europe, the IOL circulates in France and Italy (Wahid et al., 2017).

## 2.3 Virological characteristics

### 2.3.1 DENV structure

DENV is a single-stranded positive RNA, a member of the *Flavivirus* under the family *Flaviviridae* and causes dengue fever (Salles et al., 2018). The virus consists of four antigenically distinct serotypes with various genotypes (DENV 1-4) (Roy & Bhattacharjee, 2021). DENV is a 50 nm enveloped virus in a spherical shape with icosahedral nucleocapsid covered by the lipid bilayer (Figure 2.3) (Roy & Bhattacharjee, 2021). With a length of 11 kb, the DENV viral genome consists of untranslated regions (UTRs) at both the 5' and 3' end flanking the open reading frame (ORF). ORF encodes a polyprotein comprised of three structural proteins capsid (C), pre-membrane (PrM), and envelope (E) and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) (Figure 2.4) (Guzman et al., 2010).

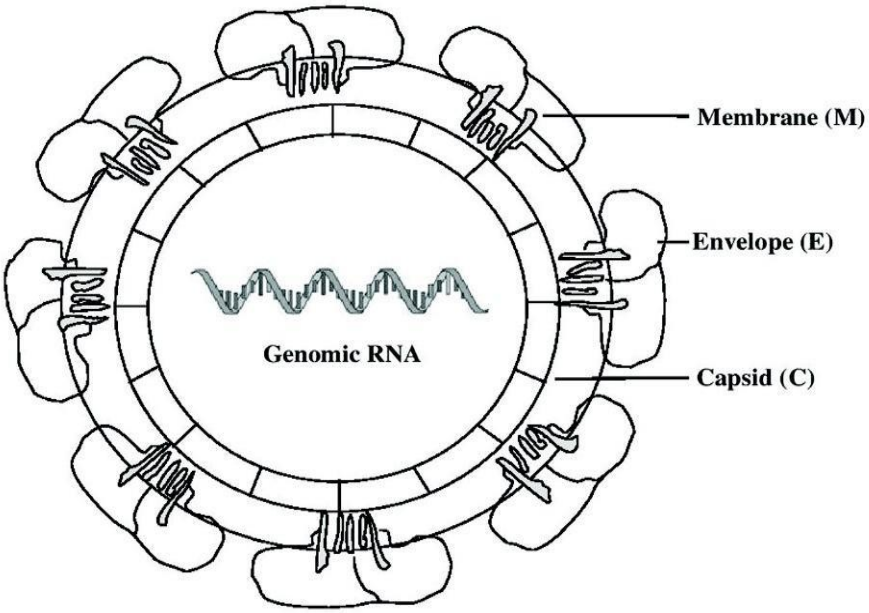



Figure 2.3 The structure of dengue virion (Adopted from Roy & Bhattacharjee, (2021)).

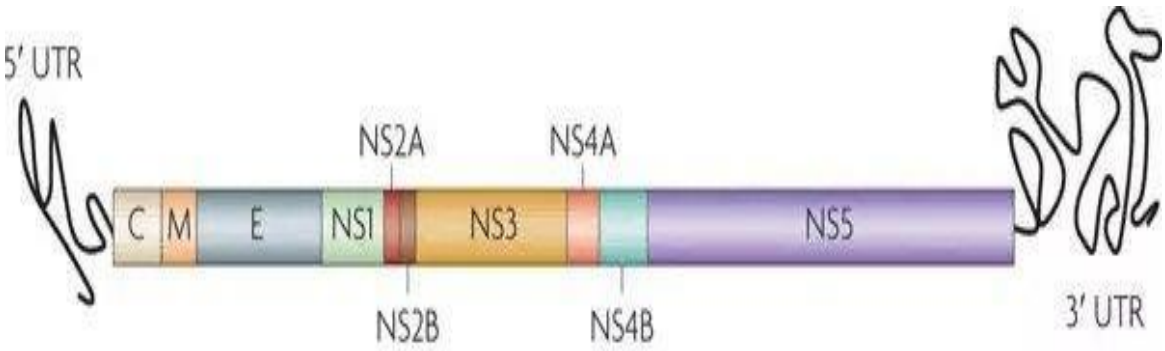



Figure 2.4 Genomic structure of DENV whole genome (Adopted from Guzman et al. (2010)).

Each protein has specific functions in producing new viral particles. The structural proteins play an important role during viral RNA replication. The C protein of DENV is involved in the encapsulation of viral RNA, the M protein for the arrangement and maturation of DENV particles and the E protein is essential for the attachment of the virus to the host cells (Dwivedi et al., 2017). Apart from that, the E gene of DENV is considered the molecular marker for viral pathogenicity due to the sequence differentiation between the strain DENV pathogenic and non-pathogenic (Dwivedi et al., 2017).

The seven non-structural proteins are characterised as water-soluble (NS1, NS3 and NS5) and membrane-bound (NS2A, NS2B, NS4A and NS4B) (Li & Kang, 2022). NS1 is a highly conserved protein, that plays an important role in RNA synthesis during viral replication and immune evasion (Li & Kang, 2022). Additionally, the interaction of NS1 with structural protein (E and M protein) suggest a novel role of NS1 in the production of infectious DENV particles (Dwivedi et al., 2017). A hydrophobic NS2A, 22kDa in weight, comprises two sets of different proteins involved in DENV RNA synthesis and virion assembly during the RNA replication process (Dwivedi et al., 2017). NS2B is slightly lighter than NS2A and has a function to regulate the activity of NS3 protease by acting as a cofactor of the NS3 protease domain (Li & Kang, 2022). In addition, the interactions of NS2B-NS3 in viral replication possess the most powerful therapeutic target for DENV-effective drug development (Dwivedi et al., 2017).