



Institute of Health and Community Medicine

**Molecular Typing of Dengue Viruses Circulating in Kuching, Sarawak
from September 2014 to June 2016**

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Molecular Typing of Dengue Viruses Circulating in Kuching, Sarawak from
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A thesis submitted

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DECLARATION

I declare that the work in this thesis was done in accordance with the regulations of Universiti Malaysia Sarawak. It is original and is the result of my personal work, unless otherwise indicated or acknowledged as referenced work from other sources. This thesis has not been accepted for any degree and is not concurrently submitted in candidature for any other degree.

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ABSTRACT

The aim of this study is to investigate the distribution and diversity of dengue viruses circulating in Kuching division of the Sarawak state. Dengue is an acute systemic viral infection which is associated with high mortality and morbidity. In Malaysia, dengue transmission has been a major concern with continuous upsurge of dengue incidences nationally with higher prevalence in urban and peri-urban areas. In this study, collected patient sera were separated into two groups; serologically dengue positive and serologically dengue negative, based on the results of preliminary serological test using commercial SD Bioline dengue kit (NS1 Ag + Ab combo). Pan-*flavivirus* RT-PCR screenings yielded 86 (26.6 %) positive results out of total 323 samples tested. Meanwhile, 467 serologically dengue negative samples were tested with Real-Time PCR (qPCR), which yielded another 4 (0.9 %) positive results. Overall, a higher rate of virus detection was observed from the group of patient samples with dengue NS1 antigen detected by commercial dengue kit. Age-wise analysis showed that adults of age 21-30 were most infected with dengue compared to other age groups. Besides, dengue isolates were successfully propagated for 61 (49.2 %) samples which were inoculated in C6/36 cell culture. Sequencing results of PCR generated amplicons showed circulation of DENV-1, DENV-2, DENV-3, with co-circulation of at least two serotypes at any one time. Phylogenetic analyses were performed on the basis of viral E gene sequence and C-prM gene junction sequence. Resulted phylogenetic trees revealed distinct clusters of individual dengue serotype with strong bootstrap support. DENV-1 isolate sequences of this study clustered within genotype I, showing high sequence homology to strains from neighboring countries in Southeast Asia. Such phylogenetic relationships indicate frequent exchanges of DENV-1 genotype I strains in the region. DENV-2 isolate sequences grouped within Cosmopolitan genotype. Among these, 11

DENV-2 Cosmopolitan isolates of one sub-lineage were related to Sarawak strains sampled previously. This clustering topology suggests local transmission of the Cosmopolitan strains. Another 10 DENV-2 Cosmopolitan isolates formed a sub-lineage with strains notably from Peninsular Malaysia, Singapore, China and Philippines, suggesting importation from these neighboring localities in Asia. Interestingly, DENV-3 isolate sequences identified in this study clustered within two distinct genotypes; genotype III and genotype I. Among the DENV-3 genotype III isolates, 3 of them were linked to a Singapore strain whereas another 4 formed a sub-lineage with reference strains from Southeast Asia. Similarly, these findings indicate frequent exchanges of dengue strains in the Southeast Asian region. The sole DENV-3 genotype I isolate closely related to a Singapore strains, strongly supports an importation event that is corroborated by the patient's recent travel history to Singapore. Both phylogenetic analysis approaches based on dengue viral C-prM and E gene sequences resulted in similar clustering topology suggesting that the C-prM sequence would be a useful tool for quick results of molecular typing. In conclusion, this study identified co-circulation of different DENV serotypes and genotypes in Kuching as a result of frequent exchanges of strains among neighboring localities in Southeast Asia.

Keywords: Dengue viruses, E gene sequence, C-prM gene junction sequence, phylogenetic analyses, serotype, genotype

***Pengesanan Molekul bagi Virus Denggi yang Tersebar di Kuching, Sarawak dari
September 2014 hingga Jun 2016***

ABSTRAK

Kajian ini bertujuan untuk menyiasat penyebaran dan diversiti virus denggi di sekitar kawasan bahagian Kuching, Sarawak. Denggi adalah sejenis penyakit sistemik jangkitan virus denggi yang berhubungkait dengan kadar kematian dan morbiditi yang tinggi. Keadaan denggi di Malaysia semakin membimbangkan selaras dengan peningkatan berterusan kes denggi terutamanya di kawasan bandar dan peri-bandar. Dalam kajian ini, semua sampel serum yang dikumpulkan telah dibahagi kepada dua kategori iaitu sampel yang positif denggi dan negatif denggi berdasarkan keputusan daripada ujian serologi denggi dengan menggunakan kit komersial denggi kombo. Pan-flavi RT-PCR mengenalpasti 86 (26.6 %) keputusan positif daripada jumlah 323 sampel yang diuji. Sementara itu, sejumlah 467 sampel yang negatif serologi denggi telah diuji dengan qRT-PCR dan menghasilkan 4 keputusan positif. Secara keseluruhannya, kadar pengesanan virus denggi yang lebih tinggi dikenalpasti daripada kategori sampel yang dikesan dengan kehadiran antigen denggi NS1. Analisis berdasarkan umur menunjukkan bahawa dewasa dalam lingkungan umur 21-30 paling kerap terdedah kepada jangkitan denggi. Isolat virus denggi berjaya dikesan dan diasingkan daripada 61 (49.2 %) kultur. Keputusan penjujukan amplicon DNA membuktikan peredaran tiga serotip virus denggi (serotip 1, serotip 2, dan serotip 3) dan peredaran serentak yang melibatkan sekurang-kurangnya dua serotip virus denggi. Analisis filogenetik dilakukan melalui dua kaedah iaitu penjajaran sekuen gen E dan C-prM isolat-isolat denggi. Hasil analisis filogenetik menunjukkan klasifikasi virus denggi yang berlainan serotip dalam kelompok individu. Semua sekuen isolat denggi serotip 1 dikelompokkan dalam genotip 1 dan menunjukkan homologi sekuen dengan strain denggi dari negara sekeliling dalam kawasan Asia Tenggara. Hubungan filogenetik berkenaan

menunjukkan kekerapan pertukaran strain denggi genotip 1 dalam lingkungan Asia Tenggara. Semua sekuen isolat denggi serotip 2 dikelompokkan dalam genotip Kosmopolitan. Antaranya 11 isolat genotip Kosmopolitan membentuk satu sub-keturunan bersama strain denggi Sarawak yang dikesan dahulu. Hubungan topologi sebegini mencadangkan kemungkinan transmisi strain secara tempatan. Sementara itu, 10 isolat genotip Kosmopolitan membentuk sub-keturunan bersama strain-strain denggi yang dikesan dari Semenanjung Malaysia, Singapura, China, dan Filipina, menggambarkan pengimportan strain denggi dari negara-negara jiran di dalam kawasan Asia. Berbeza daripada denggi serotip 1 dan 2, isolat denggi serotip 3 yang dikesan dalam kajian ini berkelompok dalam dua genotip yang berlainan iaitu genotip III dan genotip I. Di antara genotip III, 3 isolat berkait rapat dengan satu strain Singapura manakala 4 isolat yang lain berkelompok dengan strain-strain dari Asia Tenggara. Secara samanya, hubungan filogenetik sebegini mengilustrasikan kerap berlakunya pertukaran strain-strain denggi dalam kawasan Asia Tenggara. Isolat denggi 3 genotip I yang tunggal berkait rapat dengan strain Singapura dan mencadangkan kemungkinan pengimportan disokongi dengan rekod pelawatan pesakit ke Singapura. Kedua-dua kaedah analisis filogenetik berdasarkan sekuen gen C-prM dan E menghasilkan percabangan pohon filogenetik yang semirip dan ini mencadangkan bahawa C-prM sekuen sesuai untuk pengesanan molekul virus denggi. Kesimpulannya, kajian ini mengesan pengedaran serentak pelbagai denggi serotip dan genotip dalam kawasan Kuching disebabkan kerap berlakunya pertukaran strain denggi di antara tempatan sekeliling dalam kawasan Asia Tenggara.

Kata kunci: Virus denggi, sekuen gen E, sekuen gen C-prM, analisis filogenetik, serotip, genotip

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LIST OF ABBREVIATIONS

Ab	Antibody
ADE	Antibody-dependent enhancement
AG	Antigen
AGE	Agarose Gel Electrophoresis
BCB	BigDye Cleaning Bead
BLAST	Basic Local Alignment Search Tool
cDNA	Complementary DNA
CHIKV	Chikungunya virus
cHP	Capsid region hairpin
CPE	Cytopathic effect
C-prM	Capsid-premembrane
CS	Conserved sequence
Ct	Cycle threshold
DENV	Dengue virus
DF	Dengue Fever
DHF	Dengue Hemorrhagic Fever
DNA	Deoxyribonucleic acid

dNTP	Deoxyribonucleotide Trisphosphate
DSS	Dengue Shock Syndrome
E gene	Envelope gene
EtBr	Ethidium Bromide
FBS	Fetal Bovine Serum
HCl	Hydrochloride
IDT	Integrated DNA Technologies
IgG	Immunoglobulin G
IgM	Immunoglobulin M
L-15	Leibovitz 15
MAFFT	Multiple Alignment using Fast Fourier Transform
MEGA	Molecular Evolutionary Genetic Analysis
MgCl ₂	Magnesium Chloride
NaCl	Sodium Chloride
NS	Non-structural
ORF	Open reading frame
prM-E	Premembrane-envelope
qRT-PCR	Real-Time Reverse Transcription-Polymerase Chain Reaction

RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic Acid
RT-PCR	Reverse Transcription-Polymerase Chain Reaction
SLA	Stem-loop A
SLB	Stem-loop B
TBE	Tris Borate-EDTA
TGN	Trans-Golgi Network
TPB	Tryptose Phosphate Broth
ULT	Ultra-low temperature
UTR	Un-translated region
ZIKV	ZIKA virus

CHAPTER 1

LITERATURE REVIEW

1.1 Disease burden of dengue

Dengue is a vector-borne infectious disease caused by dengue virus (DENV) which is transmitted into human host by *Aedes* mosquito bites. The earliest record of dengue-like illness dates back to 265-420 AD where the disease was referred to as 'water poison' in a Chinese medical encyclopedia (Murray et al., 2013). Between 1779 and 1780, dengue epidemics were first reported in the continents of Asia, Africa and North America (Gubler & Clark, 1995; Mayer et al., 2017). Gradually, multiple DENV serotypes spread to many regions of Southeast Asia after World War II and this caused epidemics of dengue hemorrhagic fever (DHF) since 1950s (Bostan et al., 2017). By mid of 1970s, DHF served as a major cause of hospitalization and death among children in Southeast Asia (Bostan et al., 2017).

In the last 50 years, the disease has spread widely to cause remarkable morbidity and mortality globally (Guzman, 2011). Prior to the 1970s, dengue epidemics were only found in 9 countries. However, the disease was endemic in more than 125 countries up to 2016 (as illustrated in Figure 1.1) mainly in the tropical and sub-tropical regions (Cheah et al., 2014; Liew et al., 2016; Guo et al., 2017). It was estimated about two-fifth of the world's populations are living in areas with risk of contracting dengue (Murray et al., 2013; Woon et al., 2016; Guo et al., 2017). This rapid global expansion of dengue disease is believed to be facilitated by a complex set of factors including global urbanization, increased international travel, climate change and inadequate public health measures (Hii et al., 2011; Lee et al., 2012; Ong, 2016).

Rapid global urbanization can lead to increased breeding habitats for mosquito in artificial container whilst climate change with higher temperature impacts greater pupation and higher survival rate of vector (Hii et al., 2011; Ong, 2016). Previous study found that the extrinsic incubation period of dengue viruses shortens from 12 days at 30 °C to 7 days at 32-35 °C (Watts et al., 1987; Hii et al., 2011). Increased international travel and trade contribute to large scale expansion of mosquito vector which this creates chances for dengue viruses to circulate to previously non-endemic areas (Rezsa, 2012).

In a study by Bhatt et al. (2013) it was estimated that an annual average of 390 million dengue cases occurred globally, of which 96 million were symptomatic infections (Bhatt et al., 2013; Cheah et al., 2014) and several hundred thousand infections develop into severe DHF (Wahala & De Silva, 2011). Southeast Asia and the Western Pacific regions are most critically affected by dengue (Mia et al., 2013; Liew et al., 2016). Around 2.9 million dengue episodes and 5906 deaths were estimated per annum in Southeast Asia during 2001 to 2010 (Shepard et al., 2013; Guzman & Harris, 2015). Moreover, Southeast Asia contributed the largest number of 82 (31.3 %) out of a total of 262 dengue outbreaks reported from six World Health Organization regions between 1990 and 2015 (Guo et al., 2017). Alarmingly, it was predicted that dengue is poised to affect about 50 % to 60 % of the world population by 2080 (Hales et al., 2002; Pang & Loh, 2016).

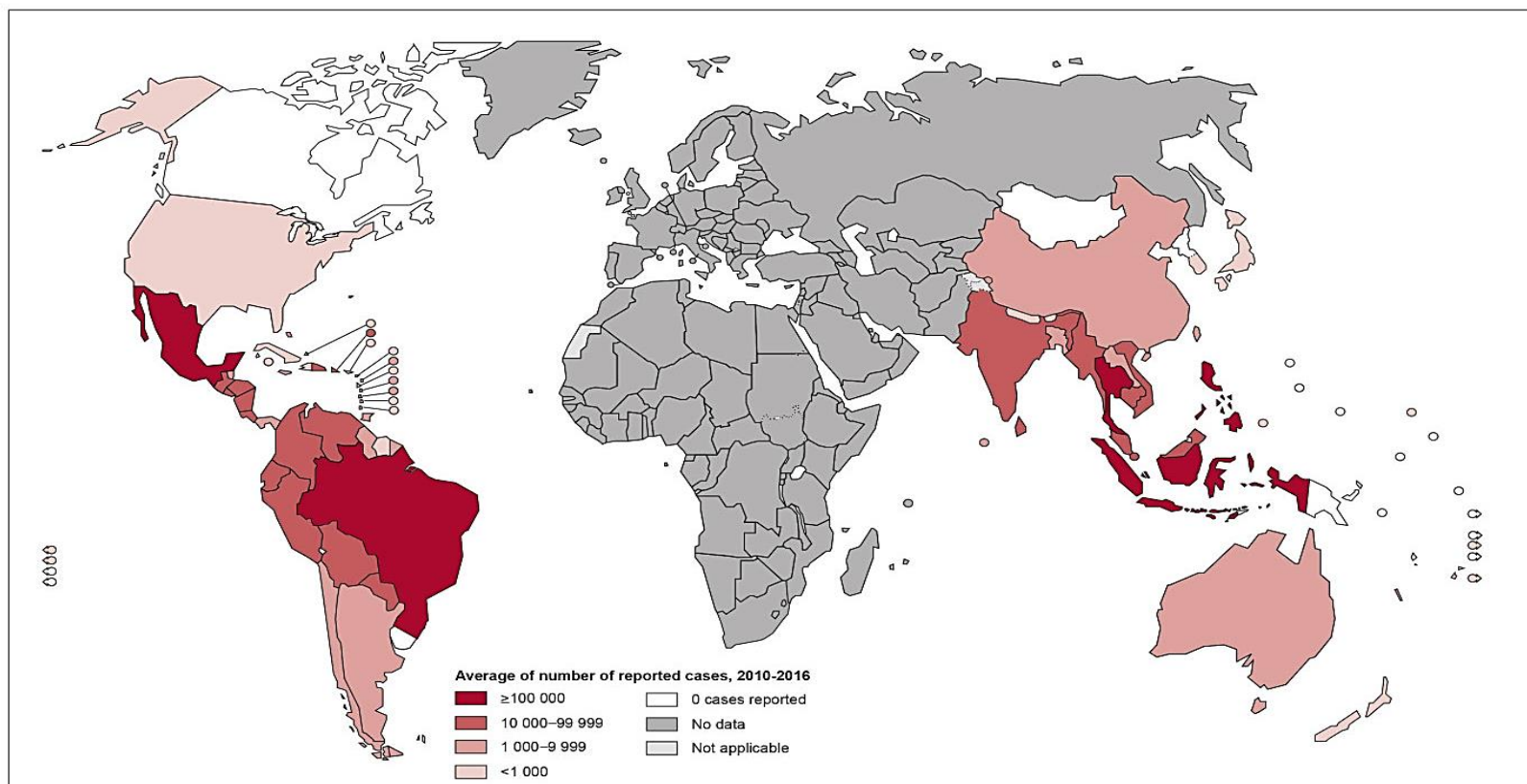


Figure 1.1: Worldwide distribution of dengue from 2010-2016. Image was taken from World Health Organization (n.d.).

In Malaysia, dengue infection was first reported in 1902 following the virus transmission from Singapore to Penang (Skae, 1902; Pang & Loh, 2016). Thereafter the first major dengue outbreak occurred in 1973 when almost the whole nation was involved (Cheah et al., 2014; Nani, 2015; Pang & Loh, 2016). Following that, the disease continued to spread across the country at an alarming pace. Dengue disease is presently hyperendemic in Malaysia, such that all the four DENV serotypes have been detected to circulate in the country (Cheong et al., 2014; Mallhi et al., 2015; Pang & Loh, 2016). Figure 1.2 shows that dengue cases and deaths in Malaysia have increased approximately eighteen-fold and eight-fold respectively over the past 2 decades. An unprecedented upsurge of dengue incidence (250 %) was observed from 43346 cases in 2013 to 108698 cases in 2014. In fact, the actual dengue situation is likely worse than that the statistics recorded due to misclassification and underreporting of the disease.

The impact of dengue is formidable, imposing a profound health and economic burden on society. In Malaysia, annual economic burden of dengue was appraised at RM196 million between 2001 and 2010 (Shepard et al., 2012). In a larger picture, overall management cost of dengue in Malaysia was estimated at RM1.082 billion in 2015 (Packierisamy et al., 2015). Substantial burdens elicited by dengue disease have underlined the needs for vaccine and effective control alternatives.

Thus far, there is no specific treatment for dengue but patients often recover upon appropriate medical care. The first licensed dengue vaccine (Dengvaxia) by Sanofi Pasteur has not been approved yet for use in Malaysia although the vaccine has been employed in several other endemic countries (Liu, 2017. para. 1; Godói et al., 2017). The vaccine was found to increase risk of severe dengue symptoms in people who have never been infected and that led to

permanent ban of the vaccine in Philippines (Dyer, 2017). Hence, the Ministry of Health of Malaysia insists to review the vaccine and gather more safety data from clinical studies.

In the absence of specific medication, efforts for dengue management mainly rely on vector control measures including chemical fogging, removal of potential larval breeding sources and field release of genetically modified (GM) mosquitoes (Ong, 2016). However, these regimes in practice have had mild impacts on dengue containment. Fogging is the typical measure that will be carried out based on dengue cases reported. Despite the insecticides used are chemically safe for humans, previous study in Peninsular Malaysia found that chemical fogging was ineffective in eliminating gravid female *Aedes* mosquitoes (Chua et al., 2005). Moreover, *Aedes* mosquitoes may develop resistance to conventional applied insecticides and reduce effectiveness of chemical fogging (Ong, 2016).

Open field release of GM sterile male *Aedes aegypti* in an uninhabited forest in Malaysia was carried out in December 2010 (Subramaniam et al., 2012; Ong, 2016). This laboratory-modified male mosquitoes carry a late-acting lethality transgene that could result in elimination of offspring when they mate with wild-type female mosquitoes (Phuc et al., 2007; Lacroix et al., 2012; Ong, 2016). The GM mosquito is basically short-lived and the release does not cause any nuisance biting, make it an implementable alternative (McGraw & O'Neill, 2013, Ong, 2016). However, the cost for the synthesis of GM mosquito and the post-maintenance could be heavy and genetic engineering itself may have negative impact on the killing effect of GM male mosquito on female mosquito (Lacroix et al., 2012; Ong, 2016).

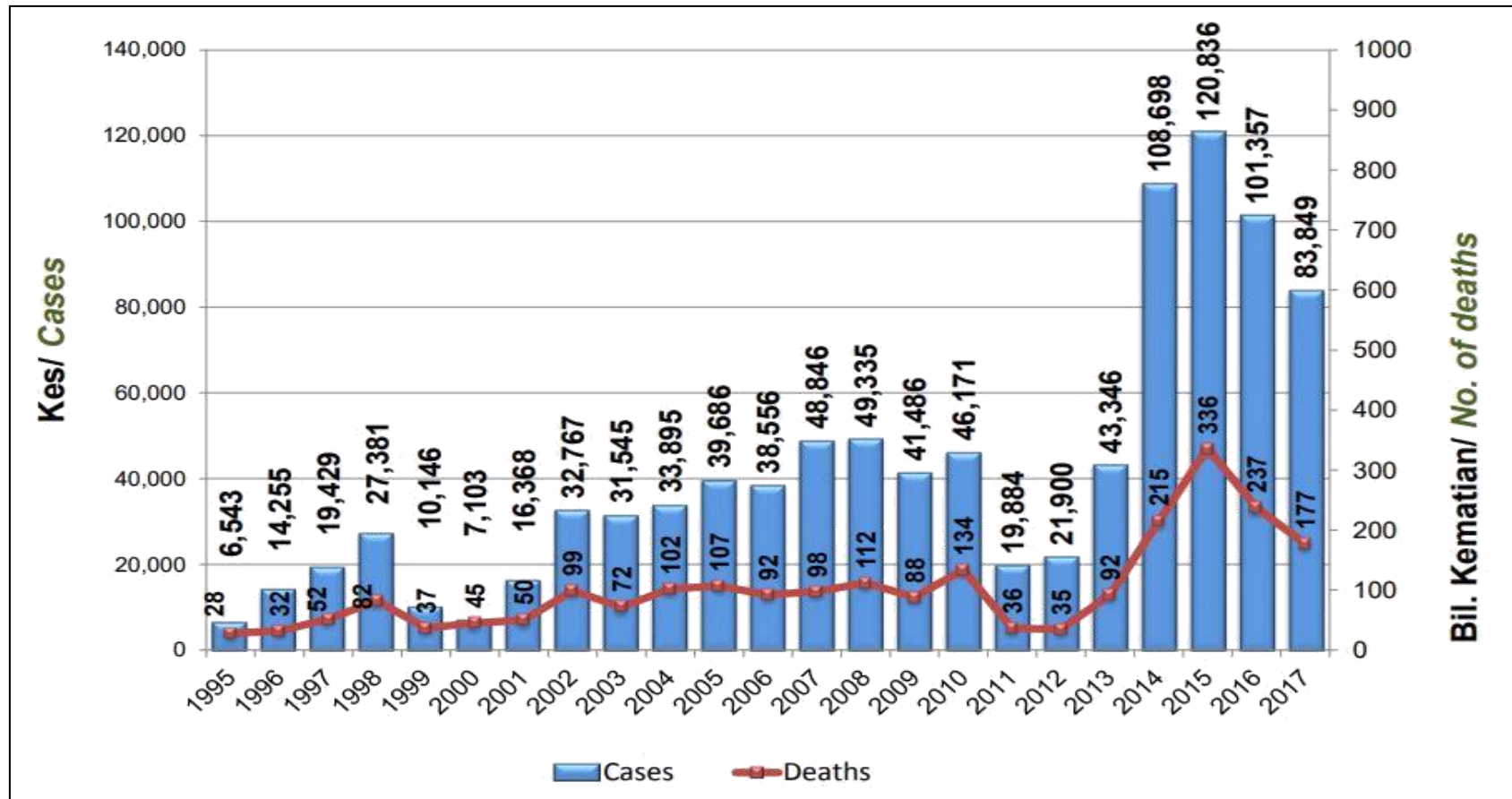


Figure 1.2: Annual dengue cases and deaths in Malaysia from 1995 to 2017. Data were adapted from <http://idengue.remotesensing.gov.my/idengue/page2.php?kandungan=content/statistik.pdf>.

1.2 The dengue virus

Dengue virus (DENV) is a member of the genus *Flavivirus*, pertaining to the family *Flaviviridae* (Westaway & Blok, 1997). The virus was previously known to manifest as four genetically related but antigenically distinct serotypes designated as DENV-1, DENV-2, DENV-3 and DENV-4, which they share 65 % to 70 % sequence homology (Rico-Hesse, 1990; Pang & Loh, 2016). These DENV1-4 evolved from a common sylvatic ancestor and eventually they escaped into human urban transmission cycle to cause epidemic (Halstead, 2007). It was then in 2013 a fifth serotype (DENV-5) was identified in Sarawak state of Malaysia. The DENV-5 isolate was validated by infecting rhesus monkeys who were pre-infected with other four dengue serotypes and had already recovered from the infection. Eventually, the monkeys produced a significantly different set of antibodies in response to DENV-5 infection and this evidenced that the new virus was indeed a new serotype (Mustafa et al., 2015). Nevertheless, the study of this new DENV-5 has not been published.

DENV-1 and DENV-2 of respective Hawaii and New Guinea strains were only first designated by Sabin and Schlesinger in 1940s (Gubler, 2006). Later in 1956 during an epidemic in Manila, DENV-3 and DENV-4 of Philippines strain were isolated and defined by Hammon et al. (1960). Eventually, each DENV serotype evolved allopatrically into distinct genetic lineages or genotypes (Twiddy et al., 2002; King et al., 2008; Kyle & Harris, 2008; Lee et al., 2012; Mishra et al., 2015) that show 5 % to 6 % of genomic sequence dissimilarity (Hesse, 2007).

Dengue virion is spherical in shape and 40 to 60 nm in diameter. It has a linear positive-sense RNA genome of approximately 10,700 nucleotides in length (as shown in Figure 1.3). Its RNA genome consists of an open reading frame (ORF) which is flanked by 5'

untranslated region (UTR) that has a type 1 cap (m7GpppAmp) structure and 3' UTR that lacks a polyadenosine tail (Gebhard et al., 2011). The ORF encodes a large polypeptide which is co- and post-translationally cleaved into three structural proteins: capsid protein (C), membrane protein (precursor as prM) and envelope protein (E); and seven non-structural proteins: NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5. These non-structural proteins have individual roles in virus replication (Leyssen et al., 2000; Anoop et al., 2012; Lee et al., 2012; Rathakrishnan & Sekaran, 2013; Mishra et al., 2015). The 45 kDa NS1 protein is crucial for dengue diagnostic on the basis of viral haemagglutination and neutralization activity (World Health Organization, 2014).

Within a dengue virion, the C protein associates with the viral genome to form an inner nucleocapsid core of about 30 nm in diameter. The nucleocapsid is covered with a well-organized lipid membrane containing E glycoprotein and prM protein that forms an outer shell of dengue virion. The peplomer E glycoprotein comprises 180 E monomers that form 90 E homodimers on the virion surface (Henchal & Putnak, 1990; Kuhn et al., 2002; Mukhopadhyay et al., 2005; Perera & Kuhn, 2008; Wahala & De Silva, 2011). Each E monomer (as shown in Figure 1.4) is a complex of three beta (β)-barrel domains; domain I (EDI), domain II (EDII) and domain III (EDIII). These protein domains are crucial for viral infectivity. For instance, EDIII that protrudes from the viral surface allows viral binding to cellular receptors and EDII has a fusion peptide at distal end to aid membrane fusion (Mukhopadhyay et al., 2005; Wahala & De Silva, 2011). Meanwhile, viral prM protein functions to modulate folding of E glycoprotein (Ma et al., 2004; Pang & Loh, 2016).

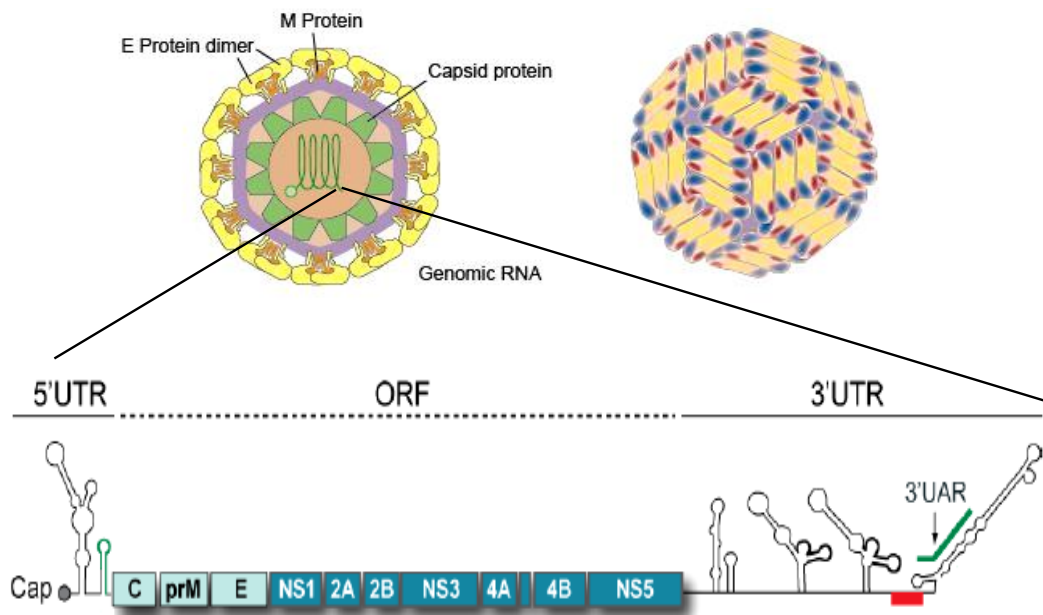


Figure 1.3: Schematic representation of dengue virion and its RNA genome. Images were taken from Gebhard et al. (2011).

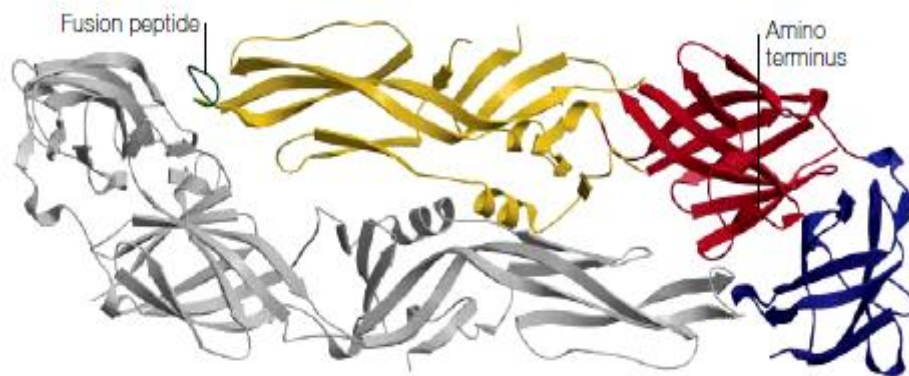


Figure 1.4: Schematic representation of E dimer structure. E monomer comprises domain I (red colour), domain II (yellow colour) and domain III (blue colour). The grey colour polypeptide represents another E monomer. Image was taken from Mukhopadhyay et al. (2005).

1.2.1 Transmission and vectors of dengue virus

The widespread of DENV relies on its complex interaction with mosquito vector, host and environmental factors. Located near to the equator, the climate in Malaysia is hot and humid with copious rainfall throughout the year providing a favourable environment for mosquito breeding (e.g. in householders with accumulated rainwater) (Johansson et al., 2009). Presently, dengue infection is no longer confined to urban areas but also rural settings owing to improved accessibility and growth of agricultural settlements that enables DENV transmission among the groups of people that live in farm settlements (Guo et al., 2017).

DENV is transmitted via the mechanisms of blood meal ingestion by mosquitoes from an infected host and subsequent virus deposition to a second susceptible host. DENV can invade egg cells and be present in progenies when DENV replication occurs concurrently with oviposition by female mosquito (Monath, 1994).

Transmission of DENV is categorized into two cycles (as shown in Figure 1.5): enzootic cycle of which sylvatic DENV is transmitted between non-human primates and arboreal *Aedes* species mosquitoes; and urban cycle of which endemic DENV is transmitted between humans and peri-domestic *Aedes* species (Vasilakis et al., 2007; Chen & Vasilakis, 2011; Franco et al., 2011; Pang & Loh, 2016).

Enzootic cycle transmission of dengue viruses mainly occurs in the forest of Asia and Africa. A previous study in Asia had identified sylvatic DEN-1, DENV-2 and DENV-4 transmission between sentinel monkeys and primary vector arboreal *Ae. niveus* mosquitoes (Vasilakis et al., 2007). Besides, Diallo and colleagues (2003) isolated sylvatic DENV-2 from *Ae. luteocephalus*, *Ae. taylori*, *Ae. furcifer* and *Ae. aegypti* mosquitoes in Africa. Previous studies underscored that sylvatic dengue strains may transiently escape from the enzootic

cycle (spill over as shown in Figure 1.5) and cause infection in human primate. This was evidenced by the first isolation of sylvatic DENV-2 from human blood in 1970 in Senegal and subsequently in 1983 and 1990 (Vasilakis et al., 2007; Franco et al., 2011). In Malaysia, the first DHF case caused by a sylvatic DENV-2 was reported in 2008 of which the strain showed close phylogenetic relationship with the 1970s Asian sylvatic strains detected from a sentinel monkey (Cardosa et al., 2009; Franco et al., 2011). Despite low risk of human infection by sylvatic strains, the cases are very likely underreported owing to indiscernible clinical presentations from the infection by endemic strains (Franco et al., 2011).

Urban transmission of DENV involves two crucial arthropod vectors (as shown in Figure 1.6) which have distributed across the globe: *Aedes aegypti* and *Aedes albopictus*, aside from other least important species of the genus *Aedes* (Ramos et al., 2008; Chen & Vasilakis, 2011; Pang & Loh, 2016). Meanwhile, these *Aedes* species are also the vectors for other arboviruses such as chikungunya virus (CHIKV) and zika virus (ZIKV). *Ae. aegypti* is known as the primary vector for DENV in most regions of the world due to its proximal association with human population in urban habitats, high viral duplication capability and highly anthropophilic (World Health Organization, 2009; Gubler, 2011; Guzman & Harris, 2015; Ebi & Nealon, 2016; Guo et al., 2017). Although initially known as a forest species from Africa, *Ae. aegypti* has evolved into a highly domesticated mosquito that poses huge threat to mankind (Monath, 1994).

In contrast, *Ae. albopictus* is known as a less efficient vector that is associated with milder dengue epidemics (Issack et al., 2010; Lambrechts et al., 2010; Zhang et al., 2014; Guo et al., 2017). This is supported by the study of Lambrechts and colleagues (2010) that determined lower rates of virus dissemination from mid gut to salivary glands of *Ae. albopictus*. Nonetheless, *Ae. albopictus* is highly reproductive and adaptable to diverse

environmental conditions. More often this species is found outdoors and has less contact with humans (Lambrechts et al., 2010). *Ae. albopictus* originated from Asia and has spread to Africa, America and parts of Europe in recent decades as a result of increased travel and trade across the boundaries (Monath, 1994; Lambrechts et al., 2010).

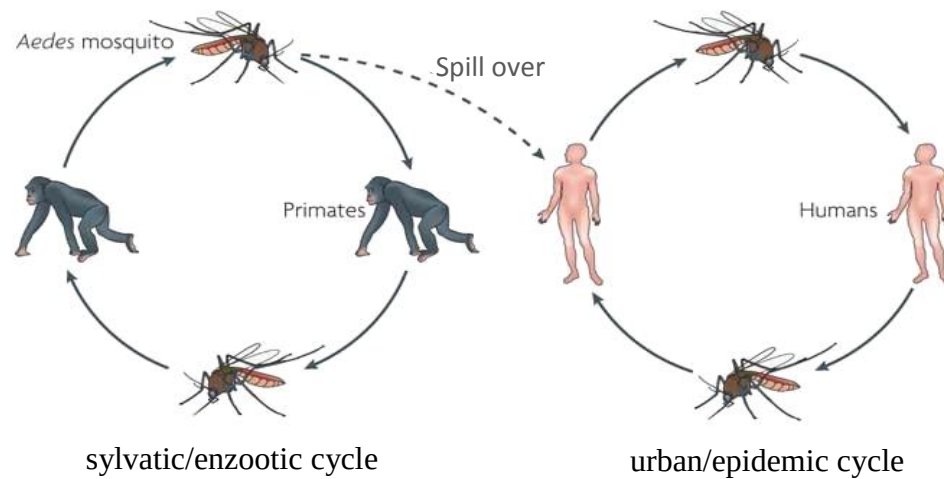


Figure 1.5: Transmission cycles of DENV. Image was taken from Whitehead et al. (2007).

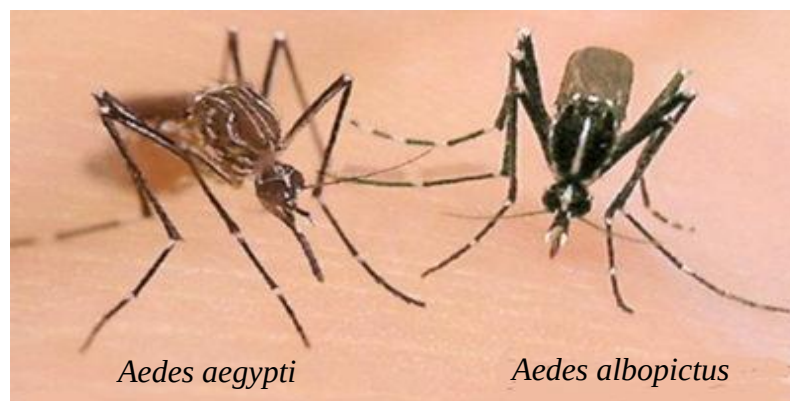


Figure 1.6: *Ae. aegypti* and *Ae. albopictus* mosquitoes, the DENV-transmitting vectors. Image was adapted from Florida Medical Entomology Laboratory (n.d.).

1.2.2 Mechanisms of DENV replication

After DENV binds to complementary receptor on the host's cell surface, membrane fusion allows it to enter the host cell by receptor-mediated endocytosis. Thereafter, viral RNA is released into the cytoplasm from the endosomal vesicle and acts as a template for viral genomic replication and protein synthesis (Gebhard et al., 2011). A negative-sense RNA complementary to DENV genome is first synthesized (Sekaran et al., 2014) which acts as template for amplification of DENV genome as catalyzed by RNA-dependent RNA polymerase (RdRp) activity of the viral NS5 protein (Gebhard et al., 2011). Each amplified RNA copy forms complex with C protein (encapsidation) and then incorporate with a lipid bilayer, E protein and prM protein during transition through the lumen of endoplasmic reticulum. The nascent virus particles then matures and are released from the host cell (Allison et al., 2003; Mukhopadhyay et al., 2005; Gebhard et al., 2011).

Other than the coding sequences in genomic ORF, there are various RNA elements in the 5' and 3' UTR of DENV genome (as shown in Figure 1.7) which serve to regulate its genomic replication. The 5' UTR region of roughly 95 to 101 nucleotides, contains two RNA domains; SLA and SLB which are interconnected with an oligo(U) sequence. SLA is the promoter for viral RdRp whereas SLB facilitates long range RNA-RNA interaction and genome replication (Gebhard et al., 2011). In addition, there is a hairpin (cHP) element right after the translation start codon of the coding sequence which it is essential for viral RNA replication (Clyde et al., 2008; Gebhard et al., 2011).

In contrast, the 3' UTR is approximately 450 nucleotides in length and this nucleotide region contains three domains; I, II and III. Domain I significantly vary in size between DENV serotypes. Domain II has a dumbbell (DB) structure which duplicated in tandem (Shurtleff

et al., 2001; Gebhard et al., 2011). Two pseudoknot structures of the DB element, CS2 and repeated CS2 (RCS2) conserved sequences, are required for viral RNA translation and synthesis (Gebhard et al., 2011; Manzano et al., 2011). Domain III has a CS1 element and a terminal stem-loop structure (3'SL). The CS1 sequence facilitates interaction between both ends of the genomic RNA (Gebhard et al., 2011).

In spite of an efficient genomic replication mechanism within the infected host, DENV readily mutates due to the intrinsically error prone RNA polymerase that lacks the 3' exonuclease proofreading activity (Mishra et al., 2015; Sessions et al., 2015). Such genetic changes may bring about the emergence of new genotype or fitter viruses with possibly an altered virulence.

Intracellular assembled immature virions have a rough and spiky surface due to protrusions of 60 trimers of prM-E heterodimers above the viral envelope. During virion maturation in Trans-Golgi network (TGN), cellular protease enzyme cleaves the pr peptide from the prM protein. This results in rearrangement of E dimer to lie flat, forming smooth virion surface. In this stage, the pr peptide remains attached on E dimer to 'cap' the fusion peptide and it only detaches when the mature viral particle is released extra-cellularly into neutral pH environment. Upon membrane fusion with the host cell, DENV particle undergoes another rearrangement of E proteins (as shown in Figure 1.8) to form an intermediate structure of $T=3$ icosahedral symmetry. Such rearrangements of viral E proteins were suggested to be induced by acidic pH condition of cellular environment (Kuhn et al., 2002; Mukhopadhyay et al., 2005; Perera & Kuhn, 2008; Wahala & De Silva, 2011).

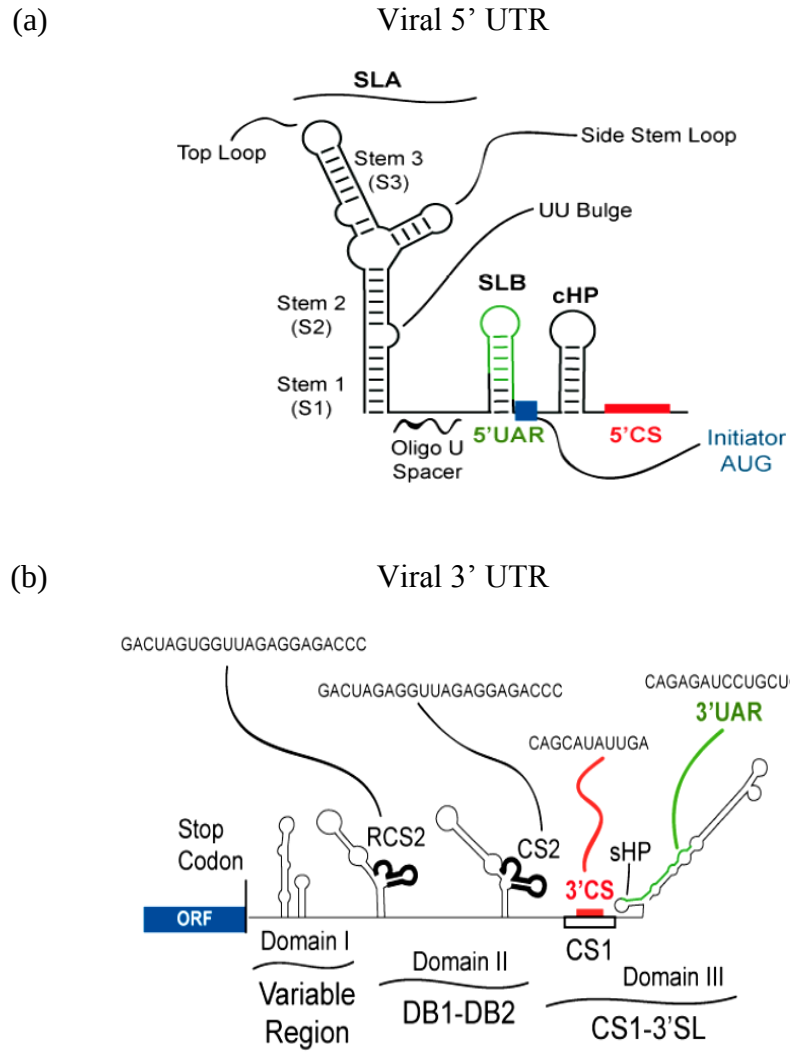


Figure 1.7: Predicted secondary structure of (a) 5' UTR and (b) 3' UTR of DENV genomic RNA with various RNA elements. Images were taken from Gebhard et al. (2011).

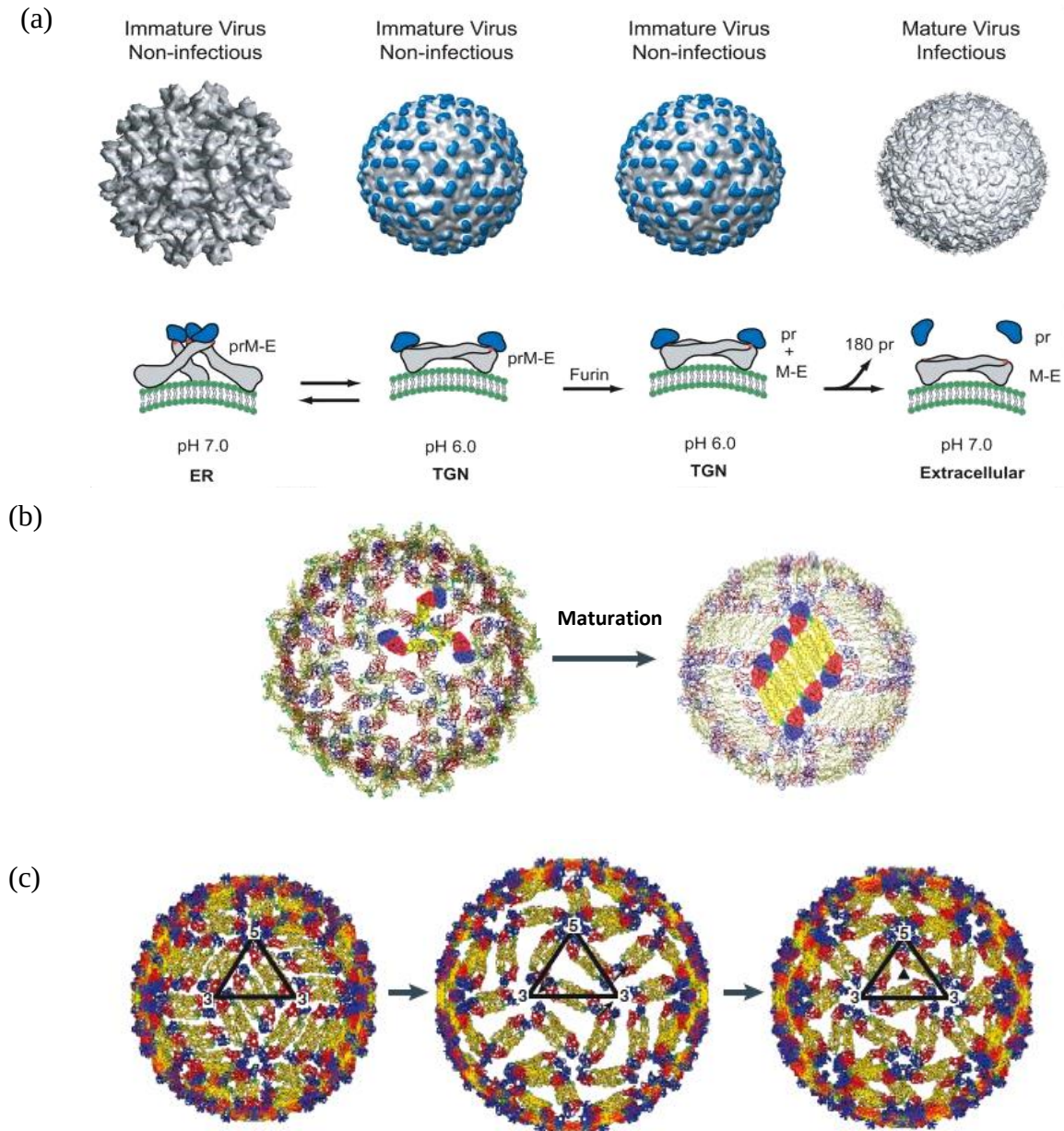


Figure 1.8: Rearrangement of E proteins during maturation of dengue viral particle. (a) Changes of cryo-EM reconstructed dengue viral structure in parallel with conformational change of E proteins and release of pr peptides (blue colour) from the mature viral particle. (b) Change of the E proteins layout from irregularly arranged to tightly packed sets of three parallel E homodimers during maturation of dengue viral particle. (c) Rearrangement of E homodimers post viral fusion with host cell to form an intermediate structure with $T=3$ icosahedral symmetry. The black arrows show the orientation that E homodimers rotate. Images were taken from Mukhopadhyay et al. (2005) and Perera and Kuhn (2008).

1.2.3 Pathogenesis and clinical manifestations of dengue disease

Dengue infections are generally self-limiting and asymptomatic but may elicit undifferentiated dengue fever (DF), severe dengue hemorrhagic fever (DHF), or dengue shock syndrome (DSS) (as shown in Figure 1.9). In particular, different host profile (age, host's genetic background and immune status) and infecting virus strain may lead to unequal clinical presentations (World Health Organization, 2014; Halsey et al., 2012; Bhatt et al., 2013; Pang & Loh, 2016).

Dengue infection comprises of three phases: febrile, critical and recovery phase (as shown in Figure 1.10). During the febrile phase, patients may present a wide spectrum of symptoms including fever, arthralgia, myalgia, headache, nausea and transient rashes which last for 2 to 7 days (Halsey et al., 2012; Rathakrishnan et al., 2014). Abrupt DENV replications during incubation period bring about viraemia. This causes sharp decrease in blood platelets (thrombocytopenia), white blood cells (leucopenia), but hematocrit increases by 5 % to 10 % (Kalayanarooj, 2011; World Health Organization, 2014). Most of the patients recover from this phase without complications while others may develop DHF along transition to critical phase.

DHF causes plasma leakage with increased vascular permeability, severe abdominal pain, organ failure and worst still, death. Concomitant circulatory failure on top of DHF may lead to DSS (Sam et al., 2013; Sekaran et al., 2014; Bhattacharjee et al., 2017). Additionally, dengue patients with prolonged shock, co-infections, or comorbidities may experience expanded atypical manifestations that could be fatal (World Health Organization, 2014). The critical phase lasts for few days, after which patients may recover with the aid of proper

clinical treatment and immune responses to stop plasma leakage and reabsorb extravascular fluid (Academy of Medicine of Malaysia, 2010).

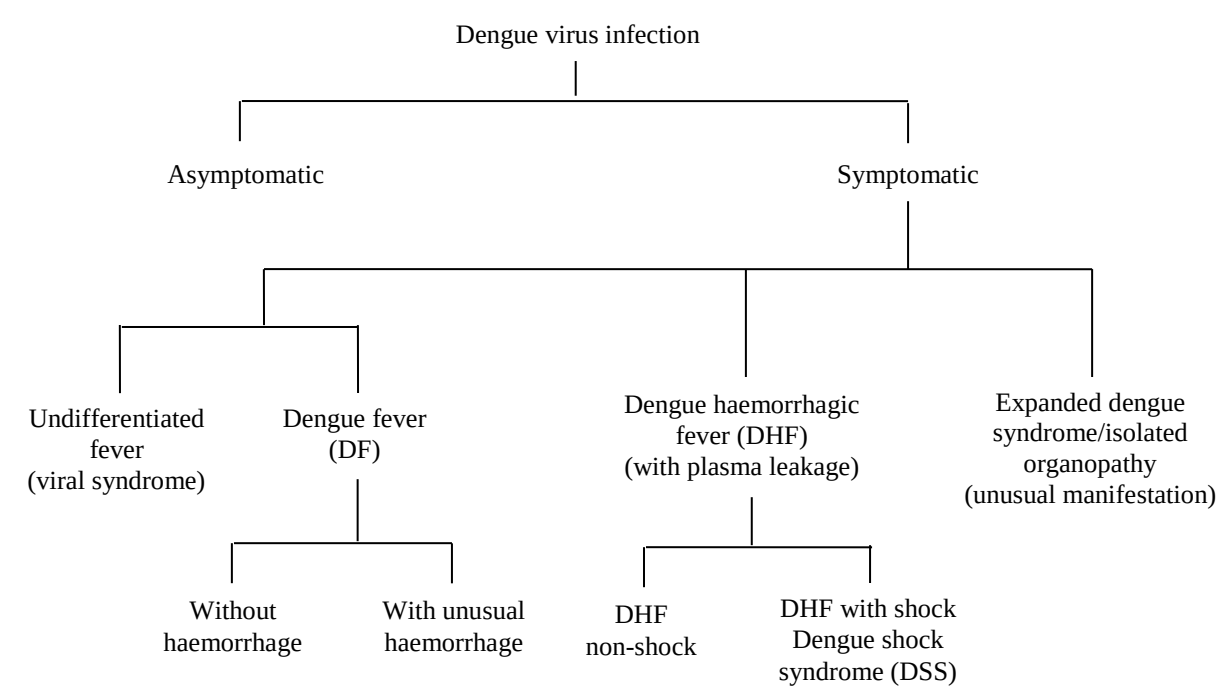


Figure 1.9: Clinical manifestations of dengue infection. Flow chart was adapted from World Health Organization (2014).

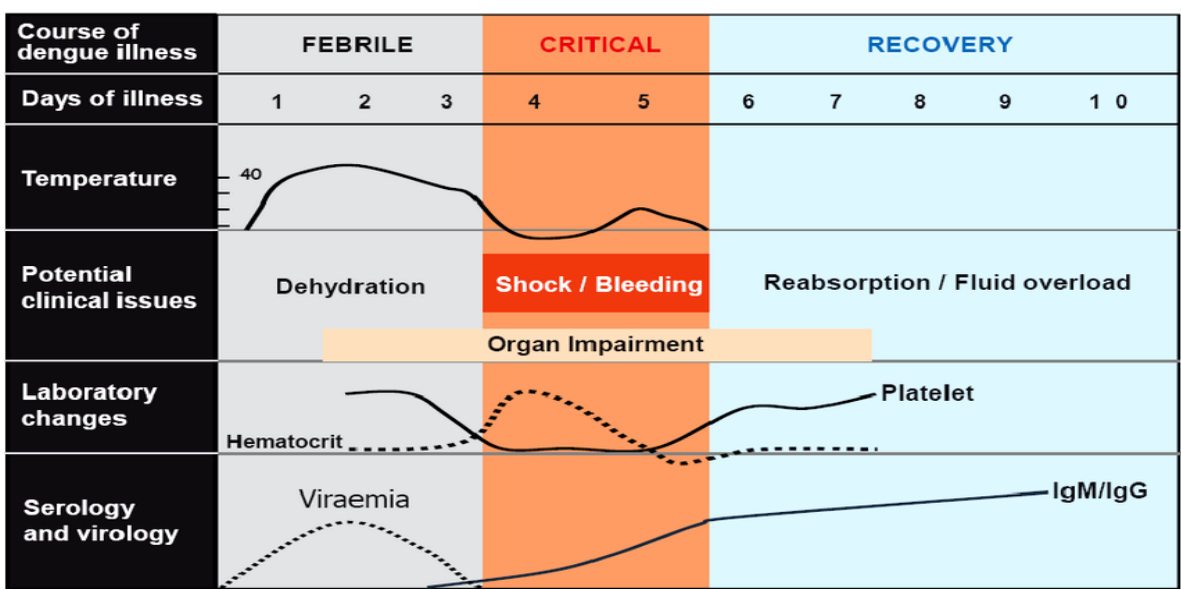


Figure 1.10: Clinical courses of dengue infection. Image was taken from Academy of Medicine of Malaysia (2010).

1.2.4 Immunological responses during DENV infection

The human immune system spontaneously produces IgM anti-dengue antibodies in response to recognition of DENV about 2 days after onset of infection. IgM antibody levels continues to elevate until day 10. Otherwise, IgG antibody levels only begins to elevate near to the end of defervescence. In subsequent dengue infections, both IgM and IgG antibodies will be mounted to confer protection but IgM level remains low as shown in Figure 1.11 (Rathakrishnan & Sekaran, 2013).

Primary infection with either one of the four dengue serotypes will trigger the immune system to confer life-long immunity against subsequent homotypic infections but only brief protection from heterotypic infections (World Health Organization, 2014; Bhatt et al., 2013). Patients' exposures to secondary dengue infection by heterologous serotypes tend to have higher propensity of developing severe dengue or DHF on account of the antibody-dependent enhancement (ADE) phenomena (Gubler, 2002; Jiang et al., 2012).

ADE is an *in vivo* mechanism of which sub-neutralizing antiviral antibodies facilitate viral entry into susceptible cells (Takada & Kawaoka, 2003). Pre-existing anti-dengue antibodies triggered from primary dengue infection or maternal-fetal transfer are unable to neutralize the secondary infecting heterotypic DENVs. Instead, the antibodies form complexes with DENVs and augment viral infection in Fc receptor-bearing myeloid cells such as macrophages and monocytes (Takada & Kawaoka, 2003; Wahala & De Silva, 2011). Responding immunological cascade up-regulates production of cytokines that mediate increased vascular permeability and cause DHF in acute phase (Halstead et al., 2010; Guzman et al., 2013). Hence, immunity against DENV infection not only provides protection but also can exacerbate disease severity in case of secondary heterotypic dengue infection.

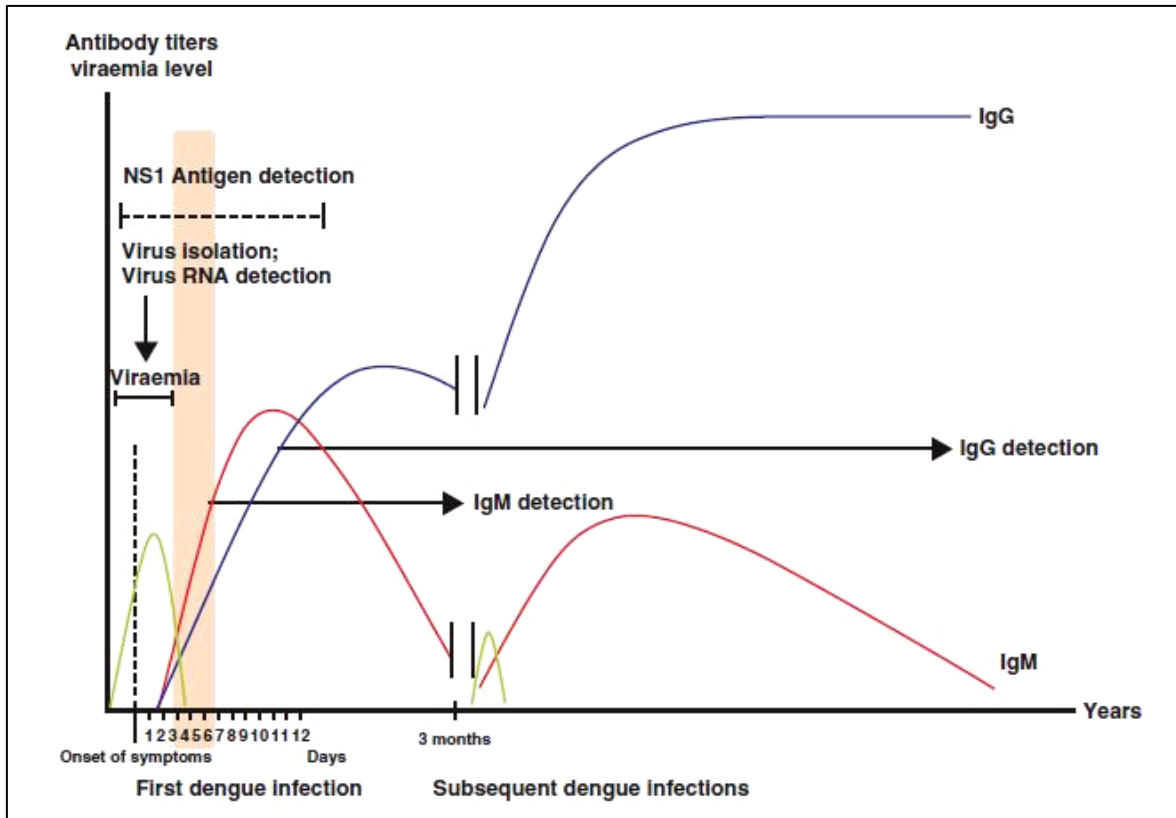


Figure 1.11: Anti-dengue immunoglobulin level in response to dengue infection. Image was taken from Rathakrishnan and Sekaran (2013).

1.3 DENV molecular typing approaches

DENV typing is important for disease management and public health surveillance. Plaque reduction neutralization technique and PCR amplification of viral RNA are the common approaches for viral typing. Nevertheless, plaque reduction neutralization using paired sera can be difficult for serotyping due to cross-reactivity among the flaviviruses and different dengue serotypes (Bhatnagar et al., 2012).

The RNA genome of DENV comprised of ten genes and individual gene varies in length (Rathakrishnan & Sekaran, 2013; Mishra et al., 2015). Majority of dengue studies use PCR amplification approach by amplifying viral E gene sequence for DENV diagnosis and

molecular typing. Predominant neutralizing epitopes reside in E protein of DENV and hence the E gene sequence may provide immunological pertinent information. In a study by Chew and colleagues (2015), the PCR-amplified viral E sequences enabled them to identify genogroups of DENV-1 and DENV-2 among the DENVs that found to circulate in Klang Valley areas, Malaysia.

Nonetheless, there were dengue studies that managed to identify DENV serotype and genotype by analysing other gene sequences including NS5 gene sequence, E-NS1 gene junction sequence and C-prM gene junction sequence. A study by Bhatnagar and colleagues (2012) used PCR amplification of partial NS5 gene sequences to serotype DENV 1-4. In contrast, a study in Brazil amplified viral E-NS1 sequences of approximately 240 bp, which then enabled genotyping of 15 DENV-2 isolates by means of phylogenetic analysis (Miagostovich et al., 2003).

The publication of Lanciotti and colleagues (1992) documented a rapid assay for DENV detection and typing by using a pair of universal primers and amplification of 511 bp C-prM sequence. Thereafter several dengue studies used the same approach for DENV typing. Recently, Ahmad and colleagues (2018) successfully serotyped DENVs that were detected in mosquito by PCR amplification of C-prM sequence. Another study by Shrivastava and colleagues (2018) used the same approach of C-prM sequence and managed to identify both serotype and genotype of the DENVs circulated in Pune city of India. These studies collectively indicated that dengue viral C-prM sequence is useful for molecular typing beside the typical viral E sequence.

1.4 Chikungunya virus

Chikungunya virus (CHIKV) of the genus *Alphavirus* and family *Togaviridae*, is relatively small enveloped virus (approximately 70 nm in diameter) with a RNA genome surrounded by inner nucleocapsid core and outer lipid membrane that facilitates its entry into target host cells (Solignat et al., 2009; Galán-Huerta et al., 2015; Murugan & Sathishkumar, 2016). CHIKV has a positive-sense single stranded RNA genome of 11,800 nucleotides embedded between a cap-structure at 5' end and poly-adenosine tail at 3' end. The genome comprised two open reading frames (ORFs) flanking an untranslated junction region of 65 nucleotide in length. Out of the two ORFs, N-terminal ORF (7422 nucleotides) encodes four non-structural proteins (nsP1, nsP2, nsP3 and nsP4) while the C-terminal ORF (3744 nucleotides) encodes 5 structural proteins (C, E3, E2, 6K and E1) (Solignat et al., 2009; Li et al., 2012; Murugan & Sathishkumar, 2016).

CHIKV was first reported in Tanzania in 1952 and being isolated in 1953 (Ross, 1956; Murugan & Sathishkumar, 2016). Eventually, CHIKV has spread widely to tropical regions of Africa, Southeast Asia, the Indian subcontinent, the Pacific region and Americas (Carletti et al., 2017). In December 2013, two autochthonous CHIKV infections were confirmed in St Martin, France (Galán-Huerta et al., 2015). Thereafter, local transmission has been reported in over 43 countries, remarkably the re-emergence of CHIKV in the Americas in the late 2013 had led to 1.1 million infections (Yactayo et al., 2016).

Similar to dengue, CHIKV is transmitted via the bites of infected *Aedes* mosquitoes, primarily *A. aegypti* and *A. albopictus* (Murugan & Sathishkumar, 2016). Owing to similar ecology and transmitting vector, areas with outbreaks of other arboviruses usually have higher risk of concomitant CHIKV outbreaks. CHIKV infection is generally not fatal and

infected humans may develop dengue-like symptoms including fever, rash, myalgia and arthralgia (Li et al., 2012; Murugan & Sathishkumar, 2016). These similar clinical manifestations often cause difficulty in distinguishing chikungunya from dengue infection.

First CHIKV outbreak in Malaysia was traced back to 1998 in Port Klang, Selangor with total 51 cases reported (AbuBakar et al., 2007). The second outbreak happened in 2006 in Bagan Panchor, Perak and caused 200 infection cases with no death. Sam and colleagues (2009) found that the infecting strain was closely related to the previous Malaysian strain identified from 1998 outbreak, suggesting CHIKV is endemic in the country. Eventually in last decade, CHIKV has spread all over the country with relatively more cases identified in Kuala Lumpur, Malacca, Johor and Selangor (Apandi et al., 2009). A large outbreak happened in Sarawak, Malaysia in 2009 with 348 cases reported (Liew & Yung, 2012). This outbreak led to the first fatal case of chikungunya infection as reported by Chua and colleague (2010).

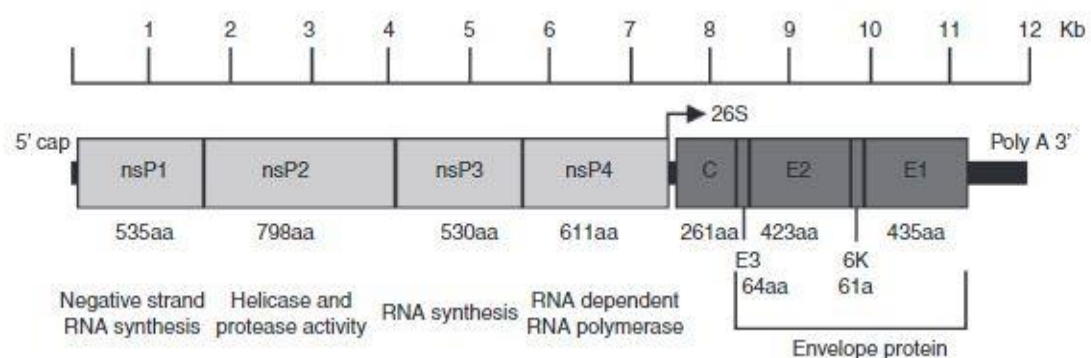


Figure 1.12: Genome organisation of chikungunya virus.

1.5 Zika virus

Zika is a mosquito-borne disease caused by zika virus (ZIKV) that likely spread from East Africa to West Africa and Asia. In recent years, the re-emergence of zika has created global alarm that prompted the World Health Organisation to issue a Public Health Emergency of International Concern on 2016 (Chang et al., 2016; Jamal et al., 2016; Petersen et al., 2016)

ZIKV belongs to the same genus *Flavivirus* and family *Flaviviridae*. It is an enveloped virus of 40-60 nm in diameter and spherical in shape. ZIKV has similar genomic and structural characteristics with DENV that belongs to the same genus. Likewise, its outer lipid membrane contains E proteins that facilitate receptor binding and membrane fusion with host cells (Haddow et al., 2012; Charrel et al., 2016; Moghadam et al., 2016).

Historically, ZIKV was first isolated from a sentinel monkey in Uganda in 1947. Thereafter, human infection with ZIKV was first identified in Nigeria in 1953 (Macnamara, 1954; Chang et al., 2016; Jamal et al., 2016; Petersen et al., 2016). First major zika outbreak causing 5000 infections was identified on the island of Yap (Federal States of Micronesia) in 2007, which was followed by a few outbreaks in French Polynesia and other countries in 2013 (Duffy et al., 2009; Chang et al., 2016; Jamal et al., 2016; Petersen et al., 2016). It was then in 2015 ZIKV infection aggravated and caused explosive epidemics across the globe, with estimated 1.5 million cases in Brazil alone (Jamal et al., 2016; Moghadam et al., 2016).

ZIKV infection is generally asymptomatic, or it may trigger including fever, rash, myalgia, arthralgia, headache and conjunctivitis (Duffy et al., 2009; Jamal et al., 2016). Some patients may develop digestive symptoms such as nausea, vomiting, diarrhoea, abdominal pain and aphthous ulcers (Moghadam et al., 2016). Noting that these similar disease symptoms usually pose difficulty in differentiating DENV, CHIKV and ZIKV infections. Nevertheless,

there are some distinguishable presentations of ZIKV infection such as more eminent oedema of the extremities, less severe headache and milder thrombocytopenia (Chan et al., 2016; Gautret & Simon, 2016; Moghadam et al., 2016).

ZIKV is primarily transmitted by *Aedes* mosquito vector, mainly *Ae. aegypti*. Other than that, ZIKV is also likely to be transmitted via sexual route or trans-placental route during pregnancy (Haddow et al., 2012; Petersen et al., 2016). A drastic rise in the incidence of microcephaly had been recorded in Brazil during the zika outbreak in 2015 and this had raised the question as to whether ZIKV is correlated with such condition. Eventually, a few studies have strengthened the association between ZIKV infection and microcephaly in neonates. For instance, Calvet and colleagues (2016) identified ZIKV RNA from the amniotic fluid of two pregnant mothers whose foetuses had been diagnosed with microcephaly and the result was consistent with intrauterine transmission of ZIKV.

In Malaysia, ZIKV was first isolated from *Ae. aegypti* that were sampled from Bentong, Pahang in 1966 (Marchette et al., 1969; Jamal et al., 2016). Meanwhile, a few studies of arthropod-borne viruses identified 30 % ZIKV seropositivity in human sera that were collected from East and Peninsular Malaysia in 1950s and 1990s (Tappe et al., 2015; Jamal et al., 2016). Such findings prompt us to the supposition that whether the ZIKV seropositivity is due to cross-reactivity with other endemic *flaviviruses* or previous human cases in Malaysia have been misdiagnosed. It was not until 2014 that an acute ZIKV infection was reported in a German traveller returning from Sabah, Malaysian Borneo and thereafter in 2016 two autochthonous ZIKV infections were detected in Sabah (Tappe et al., 2015; Jeffree et al., 2018).

1.6 Statement of research problem and objectives

Prevalence of dengue transmission in Malaysia is hyperendemic and causes high morbidity. Co-existence of all the four DENV serotypes exacerbates the likelihood of secondary dengue infections which can lead to DHF or DSS. In a worst case scenario, introduction of exogenous dengue strains and prolonged dengue epidemic may bring up natural selection of fitter strains with altered virulence. These apprehensive factors support the need for dengue epidemiology study and surveillance.

In this study, there were 467 serum samples negative for dengue serological test done with commercial dengue kit. These 467 patients showed dengue-like symptoms but DENV was not detected from their sera. This poses possibility of chikungunya or zika infection as CHIKV and ZIKV share similar vector transmission cycle and clinical manifestations with DENV. In fact, CHIKV and ZIKV have had existed in Sarawak and neighbouring states in previous years (Yusof et al., 2011; Liew & Yung, 2012; Dony et al., 2018). Hence it is also important to screen for CHIKV and ZIKV for this particular group of sera in order to identify the actual infecting agents.

In Sarawak, there is a gap of dengue transmission data owing to discontinuous dengue epidemiology studies. Despite a relatively lower rate of dengue incidence in Sarawak, comprehensive dengue epidemiology data remains lacking to provide insights into transmission of DENVs in the region and the identities of circulating DENVs. In accordance with that, this study was initiated with the following objectives:

- i) To identify DENVs circulating in Kuching, Sarawak and monthly distribution pattern of DENVs from September 2014 to June 2016.

- ii) Determine genetic diversity (serotype and genotype) and evolutionary relationships of the circulating DENVs.
- iii) To compare E and C-prM gene sequences for dengue molecular typing by means of phylogenetic analysis

CHAPTER 2

MATERIALS AND METHODS

2.1 Collection of patient sera

Patient serum specimens were collected from Sarawak General Hospital (SGH) and Borneo Medical Centre (BMC) within the duration of September 2014 to June 2016. SGH has been the main referral hospital for dengue cases from Kuching division. Patient sera were collected with ethical approval from hospital. These serum samples were initially tested by the hospital-based laboratory technologists using commercial immunochromatographic SD Bioline Dengue NS1 Ag + Ab combo kit (Abbott, Santa Clara, USA) that detects dengue NS1 antigen, IgM and IgG antibodies. Sera were kept in ice box during the transfer and then kept at -80 °C in an ultra-low temperature (ULT) freezer (Thermo Scientific, Massachusetts, USA) to preserve the viability of virus.

Figure 2.1 shows the summarized methodology applied in this study. Collected patient sera were separated into two groups: serologically dengue positive sera (positive for NS1 and/or IgM and/or IgG) and serologically dengue negative sera (negative for all NS1, IgM, and IgG) tested with commercial dengue kit.

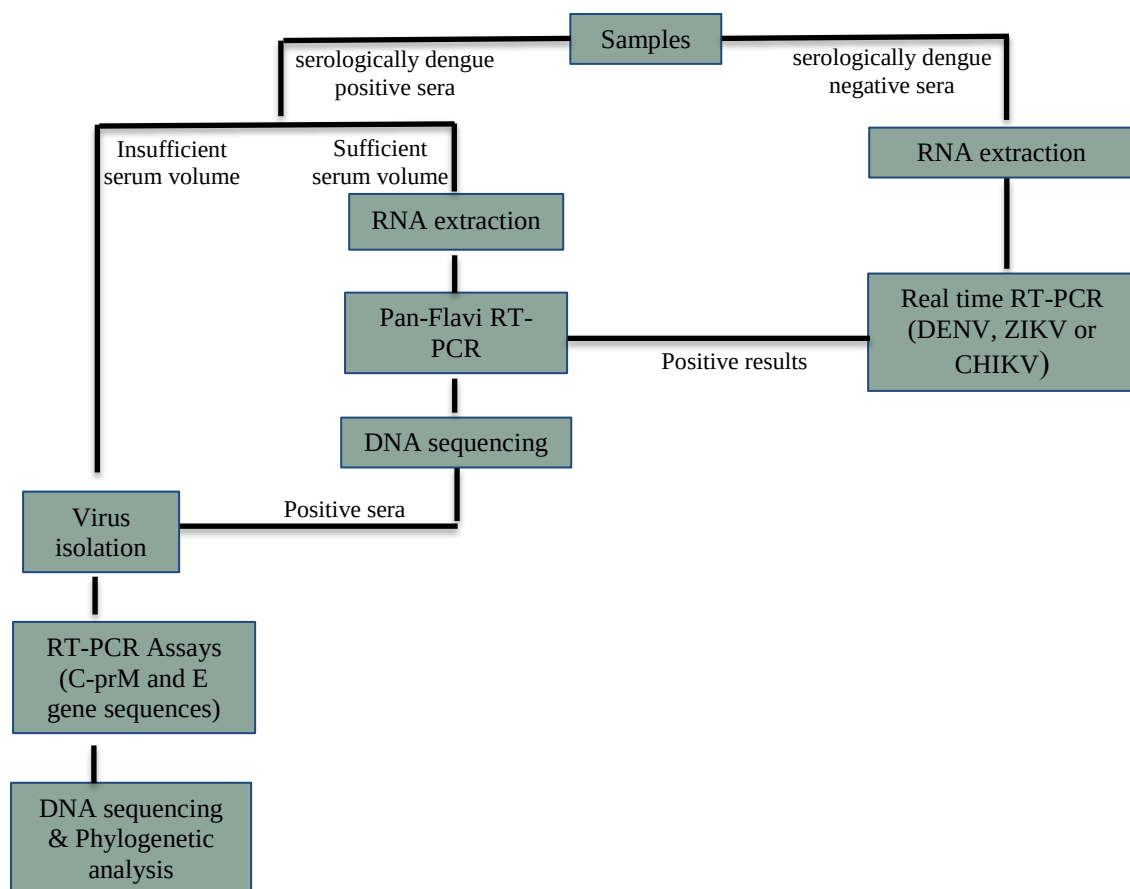


Figure 2.1: Summary of methodology for the present study

2.2 Nucleic acid extraction

Majority of the patient sera were subjected to viral RNA extraction with the exception of those with insufficient serum volume. Serum was thawed and aliquot into 1.5 ml microcentrifuge tubes of 200 µl each. Viral RNA extraction was done by using High Pure Viral Nucleic Acid Kit (Roche Diagnostics, Mannheim, Germany) with slight modifications to the manufacturer's protocol. At first, the Inhibitor Removal Buffer and Wash Buffer were added with recommended volume of absolute ethanol respectively. Poly(A) and Proteinase K powders provided in the kit were dissolved in Elution Buffer and mixed accordingly.

Extraction process was conducted in a sterile laminar flow hood (Esco Technologies, Inc., Pennsylvania, USA) to preclude contamination. First, each tube of 200 µl serum specimen was added with 200 µl Binding Buffer (6 M guanidine-HCl, 10 mM Tris-HCl, 20 % Tripton® X-100 [w/v], pH 4.4 [+25 °C]) and 55 µl of pre-mixed poly(A) and proteinase K. After a brief vortex, each tube was incubated in a 72 °C water bath for 10 minutes. Afterward, each tube was added with 100 µl isopropanol, mixed and centrifuged briefly. The content of each tube was transferred to the upper reservoir of the filter tube which was pre-assembled into a collection tube. Following that, the tube was centrifuged using benchtop Mini Spin® (Eppendorf AG, Hamburg, Germany) with 12 x 1.5/2.0 ml capacity fixed-angle rotor (rotor model F-45-12-11) at maximum speed of 13,400 rotations per minute (rpm) [or $12,100 \times g$ relative centrifugal force (RCF)] for 1 minute. Then, the collection tube containing flow-through was discarded whereas the filter tube was combined with a new collection tube. Thereafter, 500 µl Inhibitor Removal Buffer (5 M guanidine-HCl, 20 mM Tris-HCl, pH 6.6 [+25 °C]) was added to the upper reservoir of the filter tube followed by 1 minute centrifugation at maximum speed.

The collection tube containing flow-through was discarded and replaced with a new collection tube following the centrifugation. Afterward during the washing stage, 450 µl Wash Buffer (20 mM NaCl, 2 mM Tris-HCl, pH 7.5 [+25 °C]) was added into the filter tube followed by centrifugation at 13,400 rpm or 12,100 x g RCF for 1 minute. After that, the collection tube was disposed and the remaining filter tube was assembled into a new collection tube. Washing procedures were repeated for a total of two washes. Upon 1 minute centrifugation of second wash, flow-through was discarded and collection tube was reassembled to the filter tube for additional 1 minute centrifugation at 13,400 rpm to get rid of any residual Wash Buffer.

After the washing step, the filter tube was inserted into a sterile 1.5 ml microcentrifuge tube and dried at 80 °C in an oven (BINDER GmbH, Tuttlingen, Germany) for 5 minutes. Following that, the tube was left to cool in laminar flow hood for 2 minutes. Next, 40 µl Elution Buffer was pipetted into the upper reservoir of filter tube, left to stand for 2 minutes, followed by 2 minutes centrifugation at maximum speed 13,400 rpm for viral RNA elution. Eluted viral RNA was then analysed for its purity (260/280 ratio 1.8-2.0) and concentration using Nanodrop™ 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Massachusetts, USA). Remaining eluted RNA was kept at -20 °C for subsequent molecular diagnoses.

2.3 Screening of patient sera

Two approaches of reverse transcription-polymerase chain reaction (RT-PCR) involving two different sets of previously published flavivirus-universal primers were employed for virus detection from patient sera (Scaramozzino *et al.*, 2001; Moureau *et al.*, 2007). Nucleases-free primers of 100 µmol (as shown in Table 2.1) were ordered and synthesized

by First BASE Laboratories (Selangor, Malaysia). These primers targeted the N terminal end of flaviviral non-structural protein 5 (NS5) gene and generated PCR products of approximately 250bp, which the nucleotide sequence allowed taxonomic identification of *flavivirus* species.

Table 2.1: List of primers for PCR screening of patient sera

Primer	Orientation	Primer sequence (5'-3')	Genome position ^a
MAMD	Forward	AAC ATG ATG GGR AAR AGR GAR AA	8918-8940
cFD2	Reverse	GTG TCC CAG CCG GCG GTG TCA TCA GC	9180-9155
PF1S	Forward	TGY RTB TAY AAC ATG ATG GG	8909-8928
PF2R-bis	Reverse	GTG TCC CAI CCN GCN GTR TC	9180-9161

^aThe given genome position of primers are relative to the partial NS5 gene of DENV-3 Sri Lanka strain (GenBank accession no. NC_002640).

2.3.1 First pan-flavivirus RT-PCR

All pipettes and tube racks were pre-cleaned with DNAzap (Ambion, Austin, TX, USA) to avoid contamination. Extracted viral RNA of serologically dengue positive sera were first tested with the published consensus primers MAMD and cFD2 (Scaramozzino *et al.*, 2001). Reverse transcription of RNA was performed by adding 1 µl of 10 pmol/µl reverse cFD2 primer to 5.5 µl (or 80-120 ng for accurate sequence data) of each RNA in PCR tube. The mixtures were mixed by flicking and centrifuged briefly using Mini Spin® (Eppendorf, Hamburg, Germany), followed by incubation at 70 °C for 10 minutes to denature RNA secondary structure. Afterward, reaction tubes were immediately placed on ice for 2 minutes followed by a brief spin. Each reaction was then added with 2 µl of 5X RT buffer, 0.5 µl of 10 mM dNTPs mix and 1 µl of 20 U/µl M-MuLV reverse transcriptase (Fermentas Life

Sciences, Fermentas International Inc., Ontario, Canada). Reaction mixtures were incubated for 1 hour at 37 °C and 10 minutes at 70 °C to complete cDNA synthesis.

PCR was conducted in a reaction volume of 50 µl containing 5 µl (or 50-100 ng) of cDNA template, 31 µl ultrapure water, 5 µl of 10X Taq buffer, 3 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTP mix, 2 µl of each 10 pmol/µl cFD2 and MAMD primers and 1 µl of 5 U/µl *Taq* DNA polymerase (Thermo Scientific). Final reaction mixtures were incubated under following in-house thermal cycling conditions: Initial 5 minutes denaturation at 94 °C, 35 cycles of 30 seconds denaturation at 94 °C, 30 seconds primer annealing at 53 °C and 30 seconds extension at 72 °C, followed by a final nucleotides extension at 72 °C for 5 minutes and eventually chilling at 4 °C for reaction termination. All RT-PCR assays included a non-template control (NTC) and a positive control of RNA from DENV-2 (DENV-2 strain New Guinea C) were performed using programmable Mastercycler nexus (Eppendorf, Hamburg, Germany).

2.3.2 Second pan-flavivirus RT-PCR

Second pan-flavi RT-PCR assay using published degenerate primers PF1S and PF2R-bis (Moureau et al., 2007) as performed for those RNA which were tested negative for the first pan-flavi RT-PCR protocol. For RT, 5 µl (or 80-120 ng) of each RNA was added to 1 µl of 10 pmol/µl reverse primer PF2R-bis in PCR tube. Thereafter, the mixtures were flicked and centrifuged briefly, followed by incubation at 70 °C for 10 minutes. Then, PCR tubes were directly placed on ice for 2 minutes followed by a brief centrifugation. Each reaction mixture was then added with 2 µl of 5X RT buffer, 1 µl of 10 mM dNTPs mix, 0.5 µl of 20 U/µl M-MuLV reverse transcriptase (Fermentas Life Sciences, Fermentas International Inc.,

Ontario, Canada) and 0.5 µl of ultrapure water. Following that, reaction mixtures were incubated at 37 °C for 1 hour and 70 °C for 10 minutes for cDNA synthesis.

PCR amplification reaction was performed in 50 µl reaction volume containing 10 µl (or 50-100 ng) of cDNA template, 26 µl of sterile ultra-pure water, 5 µl of 10X Taq buffer, 3 µl of 25 mM MgCl₂, 2 µl of each 10 pmol/µl PF1S and PF2R-bis primers, 1 µl of 10 mM dNTPs mix and 1 µl of 5U/µl *Taq* DNA polymerase (Thermo Scientific). PCR thermal cycling incubations were set as follows: initial template denaturation at 94 °C for 5 minutes, 35 cycles of 15 seconds denaturation at 94 °C, 30 seconds primer annealing at 53 °C and 45 seconds extension at 72 °C with a 5 minutes final elongation step at 72 °C, followed by chilling at 4 °C.

2.3.3 Agarose Gel Electrophoresis (AGE)

PCR amplified product was analyzed by agarose gel electrophoresis (AGE). Agarose gel of 2 % concentration (w/v) was prepared by adding 1.8 g agarose powder to 90 ml of 1X Tris Borate-EDTA (TBE) buffer, heated in microwave to dissolve the agarose powder and added with 3 µl Ethidium Bromide (EtBr) before the solution was poured into casting tray. PCR products in tubes were respectively added with 10 µl of 6X loading dye [0.25 % bromophenol blue (w/v), 0.25 % xylene cyanol FF (w/v), 30 % glycerol (v/v)], flicked and centrifuged for 1 minute.

1X TBE buffer was added into the electrophoretic chamber to cover the agarose gel. Thereafter, each PCR product was slowly loaded into separate sample wells of the 2 % agarose gel. One of the wells was loaded with 100bp DNA ladder (Thermo Scientific, CA, USA) to serve as a marker. Then, a voltage of 170 V was applied for approximately 40

minutes or until the dye line has migrated through half of the gel. Afterward, the agarose gel was checked by using gel imaging system Molecular Imager® Gel Doc™ XR+ with Image Lab™ software (Bio-Rad, CA, USA). The separated DNA fragments were stained with ethidium bromide (EtBr) which caused the fragments to be visible under UV light. Digital visual of the gel as shown by the imaging software was saved with relevant labelling. Then, the DNA fragments of interest were excised from the agarose gel and transferred into 1.5 ml microcentrifuge tube for subsequent depuration.

2.3.4 Purification of DNA fragments from agarose gel

Desired DNA fragments were recovered from agarose gel by using QIAquick Gel Extraction Kit (QIAGEN GmbH, Germany) according to the manufacturer's protocol. Each microcentrifuge tube containing gel slices of DNA fragment was first added with 450 µl (3 volume) of Buffer QG and incubated at 50 °C water bath for 10 minutes or until the gel slices had fully dissolved. During incubation, each microcentrifuge tube was flicked for 5 minutes to help dissolution. Then, each tube was briefly centrifuged and added with 150 µl (1 volume) isopropanol. Thereafter, the mixture was transferred into a preassembled QIAquick spin column in 2 ml collection tube and centrifuged for 1 minute at 13,400 rpm using Mini Spin® (Eppendorf, Hamburg, Germany). Flow-through was discarded after the spin and spin column was reassembled into the collection tube followed by addition of 500 µl Buffer QG. Then, each tube was centrifuged again for 1 minute at 13,400 rpm followed by discard of flow-through and reassemble of the spin column.

Following that, 750 µl of Buffer PE was added to each tube. The column was left to stand for 2 minutes followed by 1 minute centrifugation. Flow-through was discarded and each

tube was centrifuged for another minute at 13,400 rpm to get rid of any residual wash buffer. After that, the spin column was placed into a sterile 1.5 ml microcentrifuge tube, dried in oven for 5 minutes and subsequently cooled for 5 minutes at room temperature. DNA elution was done by adding 40 µl of ultrapure water to the upper reservoir of spin column. Then, the column was left to stand for 2 minutes followed by 3 minutes centrifugation at 13,400 rpm. The flow-through containing eluted DNA in the microcentrifuge tube was kept at -20 °C for subsequent DNA sequencing.

2.3.5 DNA sequencing and analysis

Nucleotide sequencing was done by using a 4-capillary sequencer (ABI PRISM 3130, Applied Biosystems). DNA products were sequenced in both directions with the use of respective PCR primers.

2.3.5.1 Sequencing PCR

The DNA fragments purified from agarose gel were checked with NanoDrop 1000 (Thermo Scientific) and sequencing reactions were prepared in 96-well plate according to the recommendations of approximately 100 ng to 150 ng of template DNA and 1.7 to 2.0 DNA purity. Higher DNA concentration will result in higher signal intensity. Meanwhile, sequencing primers were prepared by diluting the relevant 10 pmol/µl PCR primers to a lower concentration 3.2 pmol/µl. Sequencing PCR was done by using BigDye® Terminator v3.1 Cycle Sequencing kit (Applied Biosystems, CA, USA) with slight modifications to the manufacturer's protocol. At first, appropriate volume of each purified DNA sample was

aliquot into two adjoining wells which served respectively for forward and reverse sequencing reaction. Then, sterile ultra-pure water was added to the sample to reach a final volume of 10 μ l. Next, each sample well was added with 3.5 μ l 5X Big Dye sequencing buffer, 1.0 μ l of each 3.2 pmol/ μ l forward and reverse primer, 1.0 μ l Big Dye v3.1 and 4.5 μ l sterile ultra-pure water. The final 20 μ l reaction mixtures were briefly mixed by vortex and centrifuged. After that, reaction mixtures were subjected to 5 minutes thermal incubation at 94 °C, 40 cycles of 30 seconds denaturation at 94 °C, 30 seconds primers annealing at 50 °C and final 2 minutes nucleotides extension at 60 °C.

2.3.5.2 Sequencing clean-up

After sequencing PCR, sequencing reactions were purified by using BigDye® Sequencing Cleanup kit (MCLAB, CA, USA) to remove salts and unincorporated dye terminators from the previous sequencing PCR step. This was done with some adjustments were made to the manufacturer's protocol.

The 96-well plate containing sequencing reactions was centrifuged shortly using plate centrifuge to settle all the reaction mixtures down to the bottom area of the wells. BigDye Cleaning Bead (BCB) in bottle was swirled to re-suspend the beads. Then, 20 μ l of BCB was added to each well, mixed thoroughly by pipetting and incubated at room temperature for 5 minutes. After that, each reaction was added with 84 μ l of 85 % ethanol and mixed by pipetting. The plate was then placed onto a 96-well magnetic plate (DynaMagTM-96 Side Magnet) for 5 minutes until the reaction mixture was clear. Without removing the well plate from the magnetic plate, the clear supernatant was fully aspirated out of each well.

During washing step, 100 µl of 85 % ethanol was added into each well while the well plate remained attached to the magnetic plate. No mixing was needed for this step. The plate was left to stand at room temperature for 5 minutes and then the clear supernatant was totally aspirated out of each well. Washing step was repeated for a total of two washes. Following that, the well plate was detached from the magnetic plate and dried in an oven at 70 °C for 5 minutes.

Later, the plate was left at room temperature for 2 minutes. For DNA elution, 15 µl of elution buffer was added into each well and mixed thoroughly by pipetting until the beads were fully re-suspended. Then, the well plate was left to stand at room temperature for 5 minutes, followed by attachment to the magnetic plate for another 5 minutes until all reaction solutions turned to clear. Thereafter, 10 µl of the clear solution containing eluted DNA was transferred from each well onto a new plate and incubated at 94 °C for 2 minutes. The plate was then centrifuged to settle down the reaction mixtures and loaded into the sequencer for sequencing process.

2.3.5.3 Nucleotide sequence analysis

Flaviviral partial NS5 gene sequences generated by sequencer were trimmed and assembled into consensus sequences by using SeqMan program (DNASTAR). Resulting consensus sequences data were saved as GCG Pileup (*.msf*) files which were then converted into FASTA format (*.fas*) with the use of Clustal X (Version 2.1) program. Following that, the FASTA files of the partial NS5 consensus sequences were appended to the web-based Basic Local Alignment Search Tool (BLAST) program (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) for determination of flaviviral species and serotype.

2.4 Virus isolation

DENV isolation was performed for sera with low volume and those tested positive for pan-flavi RT-PCR screening. In-house maintained *Aedes albopictus* clone C6/36 cell line (ATCC, Virginia, US) was used for DENV replication in a 24-well plate. The whole processes of inoculation, cell feeding and virus harvest were carried out in a biological safety cabinet with proper aseptic practices to avoid infectious splashes and contamination of cell culture.

2.4.1 Inoculation

C6/36 cells were first seeded in the 24-well culture plate and added with 500 µl Leibovitz 15 (L-15) medium (Gibco, Life Technologies, USA) supplemented with 10 % heat inactivated foetal bovine serum (FBS; Gibco, Life Technologies, USA) and 10 % tryptose phosphate broth (TPB). Cell monolayer was formed after incubation at 30 °C. Up to this point, cell supernatant was removed and refilled with 500 µl fresh L-15 medium containing 1 % FBS and 10 % TPB. After then, each serum sample was added in volume of 10 µl to the cell culture.

The culture was incubated at 30 °C and examined microscopically on daily basis. Medium was replenished every 4 to 5 days until the cytopathic effect (CPE) was observed in the culture. CPE of dengue replication in the C6/36 cells was characterized by cell lysis. Blind passage was performed up to passage 3 in basis of 14 days for each passage. In order to propagate dengue virus isolates, C6/36 cells were seeded in a T-25 culture flask and maintained with 5 ml L-15 medium containing 10 % FBS and 10 % TPB. The medium was then fully aspirated and replaced with 1 ml L-15 medium supplemented with 1 % FBS and

10 % TPB upon approximately 70 % confluency of the cell culture. Then, supernatant of the cell culture with expressed CPE was aliquot in volume of 100 μ l, into the T-25 flask containing C6/36 cells. Afterward, the culture flask was gently rocked for 10 minutes on tilt shaker (Edmund Bühler GmbH, Germany) to allow even distribution of virus. After the swing, the culture flask was added with additional 4 ml of fresh L-15 medium containing 1 % FBS and 10 % TPB. The culture flask was then incubated at 30 °C and observed daily. Culture supernatant was removed and replaced with fresh medium in the interval of 5 days until CPE expression in the culture.

2.4.2 Recovery of dengue virus particles

Upon more than 80% of the C6/36 cells experiencing CPE, infected cell culture in T25 flask was subjected to a single freeze thaw at -80 °C. Resulting cell lysate was transferred into a 10 ml falcon tube and centrifuged at 1100 rpm (or $146 \times g$ RCF) and 4 °C for 10 minutes by using Beckman Avanti® J-25 High Speed Centrifuge (Beckman Coulter, Fullerton, CA, USA) with rotor model JS 7.5. Then, culture supernatant was aliquot in volume of 200 μ l into a 1.5ml microcentrifuge tube for RNA extraction similarly as described in Section 2.2. Remaining supernatant was aliquot into cryogenic tube and stored at -80 °C in an ultra-low temperature (ULT) freezer (Thermo Scientific, Thermo Fisher Scientific Inc., Massachusetts, USA).

2.5 Molecular typing of dengue isolates

RNA extracted from the DENV isolates were subjected to two different RT-PCR assays by using primers that targeted viral capsid-premembrane (C-prM) gene junction and entire

envelope (E) gene respectively. Nucleotide sequences data of these PCR amplified genomic regions were utilized for phylogenetic analysis which determined the genotype grouping of the DENV isolates.

2.5.1 Molecular typing based on C-prM gene junction sequence

Published PCR primers (Lanciotti et al., 1992) targeting the DENV genomic C-prM gene junction region were ordered from manufacturer IDT and used for initial molecular typing of the DENV isolate. The consensus D1 and D2 primers are universal for all dengue serotypes and amplified a short fragment product of 511bp during PCR. Details of the primers are listed in Table 2.2.

Table 2.2: Primers for PCR amplification and sequencing of viral C-prM gene junction

Primer	Orientation	Primer sequence (5'-3')	Genome position ^a
D1	Forward	TCA ATA TGC TGA AAC GCG CGA GAA ACC G	134-161
D2	Reverse	TTG CAC CAA CAG TCA ATG TCT TCA GGT TC	644-616

^aThe given genome position of primers are relative to the C-prM gene junction of DENV-2 strain ThNH81/93 (GenBank accession no. AF169688).

2.5.1.1 RT-PCR amplification

Reverse transcription of isolate RNA was performed with similar steps as described in Section 2.3.1 whereby 1 µl of 10 pmol/µl D2 primer was used in place of the reverse primer for cDNA template synthesis. Straightway after the RT incubation, PCR was carried out in 50 µl of reaction mixture containing 3 µl of cDNA template, 5 µl of 10X Taq buffer, 3 µl of

25 mM MgCl₂, 1 µl of 10 mM dNTP mix, 2 µl of each 10 pmol/µl primer D1 and D2, 1 µl of 5 U/µl *Taq* DNA polymerase (Thermo Scientific) and 33 µl of sterile ultra-pure water. The reaction mixture in PCR tube was then flicked, centrifuged and incubated for amplification by using Mastercycler nexus (Eppendorf, Hamburg, Germany). The incubation conditions comprised template denaturation at 94 °C for 5 minutes, 35 cycles of denaturation at 94 °C for 15 seconds, primer annealing at 53°C for 30 seconds and nucleotides extension at 72 °C for 45 seconds, followed by a final extension at 72 °C for 5 min then reaction termination at 4 °C.

2.5.1.2 Gel electrophoresis and purification of DNA fragments

PCR products were analyzed with electrophoresis by using 2 % agarose gel similarly as described in Section 2.3.3. Amplicons of viral C-prM gene junction were cut and purified from the agarose gel similarly as described in Section 2.3.4.

2.5.1.3 DNA sequencing and analysis

The 511bp DNA fragments purified from agarose gel were sequenced in both directions with the use of D1 and D2 primers. The protocols used were similarly as described in Section 2.3.5.1 and Section 2.3.5.2. Resulting sequence data were analyzed similarly as described in Section 2.3.5.3 to re-confirm DENV serotype.

2.5.2 Molecular typing based on E gene sequence

A total of 27 DENV isolates (10 DENV-1 isolates, 10 DENV-2 isolates and 7 DENV-3 isolates) were subjected to PCR amplification of the E gene. Specific E gene primers were designed in-house with respect to different serotypes and genotypes of DENV isolates.

2.5.2.1 Generation of dengue genotype-specific primers

Reference genome sequences of respective DENV genotype were downloaded from GenBank and aligned with sequence alignment software MegAlign (version 5.07; DNASTAR Inc.). The aligned data in FASTA format (*.fas*) was appended to Primaclade, a primer design software (<http://primaclade.org/cgi-bin/primaclade.cgi>). Primer sets spanning entire E gene and at least 100bp flanking the E gene were selected for each genotype. Table 2.3 listed the primers for PCR and sequencing of DENV envelope gene. These primers were validated by testing against an isolate RNA of the corresponding DENV genotype.

Table 2.3: Primers for PCR amplification and sequencing of dengue viral envelope gene

Genogroup	Primer ^a	Primer sequence (5' – 3')	Genome Position ^b
DENV-1 Asia genotype	D1E-1F	ACA GAT ACA AAG AGT GGA GAC TTG G	791-815
	D1E-1R	ACA CAC TTR AAC TTW GCA CAC GTT A	1300-1276
	D1E-2F	AAC ACC ACC ACY GAY TCA A	1134-1152
	D1E-2R	TGA TGT TGA AGC TCC CGA G	1616-1598
	D1E-3F	GAC TAC GGA GCT CTT ACA YTG GAT	1464-1487
	D1E-3R	ATT CTG GGT TAC TCC TTT TTC ATC T	1979-1955
	D1E-4F	AAA AGG GAT GTC ATA TGT GAT GTG	1817-1840
	D1E-4R	ACA CCG CTG AAC AAA ACT CC	2287-2268
	D1E-5F	GAG GCA ACY GCC AGA GGA	2142-2159
	D1E-5R	GAA ATT TGT ATT GCT CTG TCC AAG T	2523-2499

Table 2.3 continued

DENV-2 Cosmopoli -tan genotype	D2E-1F	ATA ATG GCA GCA ATC YTG GC	842-861
	D2E-1R	CAC AAT GGT GTA YTC CAR GTT TT	1357-1335
	D2E-2F	AAC CYA GCC TAA ATG AAG ARC A	1173-1194
	D2E-2R	CTG TTT CTT YGC ATG RGG AT	1681-1662
	D2E-3F	TGG TGT TGC TGC AAA TGG	1524-1541
	D2E-3R	TTG ACT GTR ATC ARG CGA CCT AAG	2001-1978
	D2E-4F	CAG ARA CAC AAC AYG GAA CAA TA	1875-1897
	D2E-4R	TGC TCC AAA RAC TTG GTG G	2263-2245
	D2E-5F	AAG CAG AAC CYC CAT TYG G	2040-2058
	D2E-5R	TTC TGG TTG GAA TTT GTA TTG TTC T	2533-2509
DENV-3 Genotype I	D3EgI-1F	TTA GGC AYC CAG GGT TCA	820-837
	D3EgI-1R	AAC CRT TTC CCC AGC CTC	1248-1231
	D3EgI-2F	AAY GTR ACA ACC GAC TCA AGG	1134-1154
	D3EgI-2R	GTT GTR GCC CCT GAT GTC	1606-1589
	D3EgI-3F	ACC TGA ATA TGG AAC CCT TGG	1454-1474
	D3EgI-3R	TTT CCC TTG TCC ATC CTC C	1964-1946
	D3EgI-4F	AGA CTC AAG ATG GAC AAA TTG GA	1785-1807
	D3EgI-4R	CCG TRT AAG CAC TTC CAA ATA TY	2265-2243
	D3EgI-5F	ATG TTY GAG GCM ACT GCC	2130-2147
	D3EgI-5R	ACA CTC CAT TCT CCC ARG C	2577-2559
DENV-3 Genotype III	D3EgIII-1F	GTC GAG AAG GTA GAG ACA TGG G	795-816
	D3EgIII-1R	CTT ACA CAC GTA GTT CTG GTC CTG	1214-1191
	D3EgIII-2F	GGA YAT AGA GCT TCA GAA GAC CGA	1058-1081
	D3EgIII-2R	TCC AAA CCT GTC CGT GGT	1504-1487
	D3EgIII-3F	AGG AGA YCA ACA CCA GGT RG	1370-1389
	D3EgIII-3R	CTT TCT TCA ACA CAA AGG TAT TYG T	1860-1836
	D3EgIII-4F	CAY TGA CAG GAG CYA CAG AA	1717-1736
	D3EgIII-4R	AGT TCA GAA CAC CAC CYA CTG	2220-2200
	D3EgIII-5F	AYG CCT TGA AAA TCA ACT GG	2083-2102
	D3EgIII-5R	AGA TTC TCC ATT CTG GTT GTT GA	2611-2589

^aAlphabet F and R in the abbreviations of primers define respective forward and reverse primer.

^bThe given genome position of primers are relative to the E gene region of multiple reference DENV strains.

2.5.2.2 RT-PCR amplification of viral E gene

Representative DENV isolates were selected for full E gene amplification based on temporal criteria. One or two representative isolates of individual serotype were selected from different months where respective serotype was identified. Isolate RNA was aliquot into 5 PCR tubes of 5.5 µl each for reaction with 5 different reverse primers of each genotype. RT was performed similarly as described in Section 2.5.1.1. After RT step, PCR was carried out in 50 µl of reaction volume comprising 5.0 µl of each cDNA template, 31 µl ultrapure water, 5 µl of 10X Taq buffer, 3 µl of 25 mM MgCl₂, 1 µl of 10 mM dNTP mix, 2 µl of corresponding forward and reverse primers at 10 pmol/µl and 1 µl of 5 U/µl *Taq* DNA polymerase (Thermo Scientific). The final reaction mixtures were mixed by flicking and centrifuged briefly. Thereafter, reaction mixtures were subjected to thermal incubation at 94 °C for 5 minutes, 35 cycles 30 seconds denaturation at 94 °C, 45 seconds primer annealing at 55 °C and 1 minute extension at 72 °C, followed by a final extension step at 72 °C for 5 minutes and reaction termination at 4 °C.

2.5.2.3 Gel electrophoresis and purification of DNA fragments

PCR products were analysed with electrophoresis similarly as described in Section 2.3.3. DNA fragments of envelope gene with respect to each isolate were purified from agarose gel similarly as described in Section 2.3.4.

2.5.2.4 DNA sequencing and analysis

DNA fragments of E gene were sequenced similarly as described in Section 2.3.5.1 and Section 2.3.5.2, with the use of PCR primers particular for respective DNA fragment.

2.5.3 Phylogenetic analysis

Fragment sequences of respective E gene and C-prM junction region were aligned with reference DENV full genome sequences downloaded from GenBank using MAFFT sequence alignment software. Generated readouts were saved in FASTA format (*.fas*) and then appended to Molecular Evolutionary Genetics Analysis (MEGA, Version 6) program (<http://www.megasoftware.net/>) for phylogenetic analyses using maximum likelihood statistical method with 1000 bootstrap replications. Preliminary phylogenetic trees constructed allow identification of clusters corresponding with known lineages of global reference sequences. After that, those highly dissimilar reference sequences were eliminated and only best related reference sequences were included to construct the final simpler phylogenetic trees.

2.6 Real-time RT-PCR screening of dengue serological negative sera

Serologically dengue negative (negative for NS1, IgM and IgG) sera were tested with an in-house one-step qualitative real-time RT-PCR (unpublished) (qRT-PCR) using TaqMan[®] Fast Virus One-Step Master Mix (ABI, CA, USA). Individual pair of PCR primers and DNA probe was used for respective assay that diagnosed for dengue virus (DENV), zika

virus (ZIKV) and chikungunya virus (CHIKV) according to a standard thermal program as shown in Table 2.4.

Table 2.4: Thermal cycling profile of real-time RT-PCR diagnoses of primary samples

Stage 1	RT	50 °C	30 min	
Stage 2	RT inactivation/ initial denaturation	95 °C	60 sec	
Stage 3	Amplification	95 °C	15 sec	40 cycles
		60 °C	60 sec	

2.6.1 Screening for dengue virus

Primers and probe (as shown in Table 2.5) for one-step pan-DENV qRT-PCR target the 3' UTR of dengue viral genome. The TaqMan probe was labelled at the 5' end with the 5(6)-carboxyfluorescein (FAM) reporter dye and at the 3' end with tetramethylrhodamine (TAMRA) quencher.

The assay was carried out in 20 µl of reaction mixture containing 5 µl (or 80-120 ng) of isolate RNA template, 5 µl of 4X Taqman® Fast Virus 1-Step Master Mix (Life Technologies, USA), 0.5 µl of each 20 pmol/µl forward and reverse primer, 0.5 µl of 10pmol/µl probe and 8.5 µl of sterile ultrapure water. The reaction mixtures in 96 well plate were briefly vortex and centrifuged, followed by thermal incubation as described in Table 2.4 using 7500 Real-Time PCR System (Applied Biosystems, Singapore). A non-template

control and a positive control of RNA extracted from prototype DENV-2 strain New Guinea C were included in this assay.

During readouts analysis, the baseline and threshold were set using the auto-baseline and threshold features in the StepOne software. Samples were considered negative for Ct (cycle threshold) value greater than 30 or not determined.

Table 2.5: Primers and fluorogenic probe used for qRT-PCR detection of DENV

Amplimer or probe	Sequence (5'-3')	Genome position ^a
pDENV-UTMB-F	CAT ATT GAC GCT GGG ARA GAC	10640-10660
pDENV-UTMB-R	WTC SAT YTY SYG GCG YTC TGT G	10717-10696
pDENV-UTMB-P	AGA TCC TGC TGT CTC YDC AR	10664-10683

^aThe given genome position of amplimers and probe are based on the genomic sequence of DENV-2 China strain (GenBank accession no. KY937190).

2.6.2 Screening for chikungunya virus

The published primers and probe (as shown in Figure 2.6) for one-step CHIKV qRT-PCR assay target partial capsid (C) gene of CHIKV (Cecilia et al., 2015). The fluorescent probe was labelled with 4,7,2'-trichloro-7'-phenyl-6-carboxyfluorescein (VIC) at the 5' end and a Black Hole Quencher (BHQ-1) at the 3' end.

The assay was performed in 20 µl of reaction mixture containing 5 µl of isolate RNA template, 5 µl of 4X Taqman[®] Fast Virus 1-Step Master Mix (Life Technologies, USA), 0.5 µl of each 20 pmol/µl forward and reverse primer, 0.5 µl of 10pmol/µl probe and 8.5 µl of sterile ultrapure water. Meanwhile, a non-template control and a positive control of CHIKV strain from Sibu were included in this qRT-PCR assay. Reaction mixtures were mixed and

incubated according to the parameters described in Table 2.4. Samples that gave a Ct value greater than 30 were considered negative.

Table 2.6: Primers and probes for one-step qRT-PCR diagnosis of CHIKV

Amplimer or probe	Sequence (5'-3')	Genome position ^a
CHIKV-F	CGA AAA RGA RCC GGA GRA A	8401-8419
CHIKV-R	GAT AGT ACC CRG GKC TCA TGA CGT T	8465-8441
CHIKV-P	CCC TRC GCA TGC TTG A	8421-8436

^aThe given genome position of amplimers and probe are relative to the genomic sequence of CHIKV India strain (GenBank accession no. JN558836).

2.6.3 Screening for zika virus

An in-house one-step ZIKV qRT-PCR assay was designed (unpublished) wherein the primers and probe target partial viral NS5 gene. Information of the primers and probe are shown in Table 2.7. The probe was designed to include a 5(6) carboxyfluorescein (FAM) reporter dye at the 5' end and a Black Hole Quencher (BHQ-1) at the 3' end. The assay was validated for its specificity against ZIKV prior to its use for screening of patient sera.

The assay was carried out in 20 µl of reaction mixture containing 5 µl of isolate RNA template, 5 µl of 4X Taqman[®] Fast Virus 1-Step Master Mix (Life Technologies, USA), 0.5 µl of each 40 pmol/µl forward and reverse primer, 0.5 µl of 10 pmol/µl probe and 8.5 µl of sterile ultrapure water. A non-template control and three positive controls of ZIKV strain Cambodia, strain Uganda and strain Malaysia were included. Similarly, reaction mixtures were mixed and incubated as described in Section 2.6.2. Same cut-off of Ct value (30) was chosen to determine the outcomes of the assay.

Table 2.7: Primers and probes for one-step qRT-PCR diagnosis of ZIKV

Amplimer or probe	Sequence (5'-3')	Genome position ^a
ZIKV-NS5-F	GAY GGK AGR TCC ATT GTG G	9818-9836
ZIKV-NS5-R	GCA GTC TCM CGR ATG CTC	9915-9898
ZIKV-NS5-P	CGC CAC CAA GAT GAA YTG ATT GGC	9845-9868

^aThe given genome position of amplimers and probe are based on the genomic sequence of ZIKV Uganda strain (GenBank accession no. NC_012532).

CHAPTER 3

RESULTS

3.1 Detection of dengue viruses

Table 3.1 shows the molecular-based and culture-based diagnosis results of serologically dengue positive sera. Patient sera were grouped into different categories based on the serological test results of SD Bioline commercial dengue NS1 Ag + Ab combo kit. Pan-flavi RT-PCR protocols were employed for virus detection because serological detections of anti-dengue IgM and IgG antibodies do not reflect a definitive dengue infection. Instead, cross-reactions with other flaviviral infections are possible in certain extent. In this context, the use of pan-flavi primers will allow detection of infecting *flaviviruses* other than DENV.

There were 323 serologically dengue positive sera collected within the study period. Out of those, 275 sera were subjected to preliminary molecular typing by PCR whereas another 48 sera were subjected to virus isolation directly due to low serum volume. The first pan-flavi RT-PCR using cFD2 and MAMD primers yielded 59 positive results. An additional 9 positive results were generated from the second pan-flavi RT-PCR using degenerate primers PF1S and PF2R-bis. In sum, 68 (24.7 %) positive results were yielded from those two pan-flavi RT-PCR assays of primary sample diagnosis.

Agarose gel electrophoresis analyses of PCR products are illustrated in Figure 3.1. Sequence data of amplicons were generated from DNA sequencing and then assembled using SeqMan program (DNASTAR).

Table 3.1: Virus detection from primary serologically dengue positive sera

Patient sample	Total sample collected (n)	Pan-flavi RT-PCR Positive (x) /Total sample tested with pan-flavi RT-PCR		DENV isolate recovered (y) /Total of low volume sample inoculated	Overall detection rate ^c
		First ^a	Second ^b		
NS1+, IgM-, IgG-	136	50/103	5/53	17/33	53 %
NS1+, IgM+, IgG-	44	3/39	3/36	1/5	16 %
NS1+, IgM-, IgG+	13	4/12	0/8	0/1	31 %
NS1+, IgM+, IgG+	26	1/19	1/18	0/7	8 %
NS1-, IgM+, IgG+	39	0/38	0/38	0/1	0 %
NS1-, IgM+, IgG-	20	0/20	0/20	0/0	0 %
NS1-, IgM-, IgG+	45	1/44	0/43	0/1	2 %
Total	323	59/275	9/216	18/48	27 %

^a First pan-flavi RT-PCR assay using cFD2 and MAMD primers.

^b Second pan-flavi RT-PCR assay using PF1S and PF2R-bis primers.

^c Overall detection rate is calculated according to the equation $[\frac{(x+y)}{n} \times 100 \text{ \%}]$.

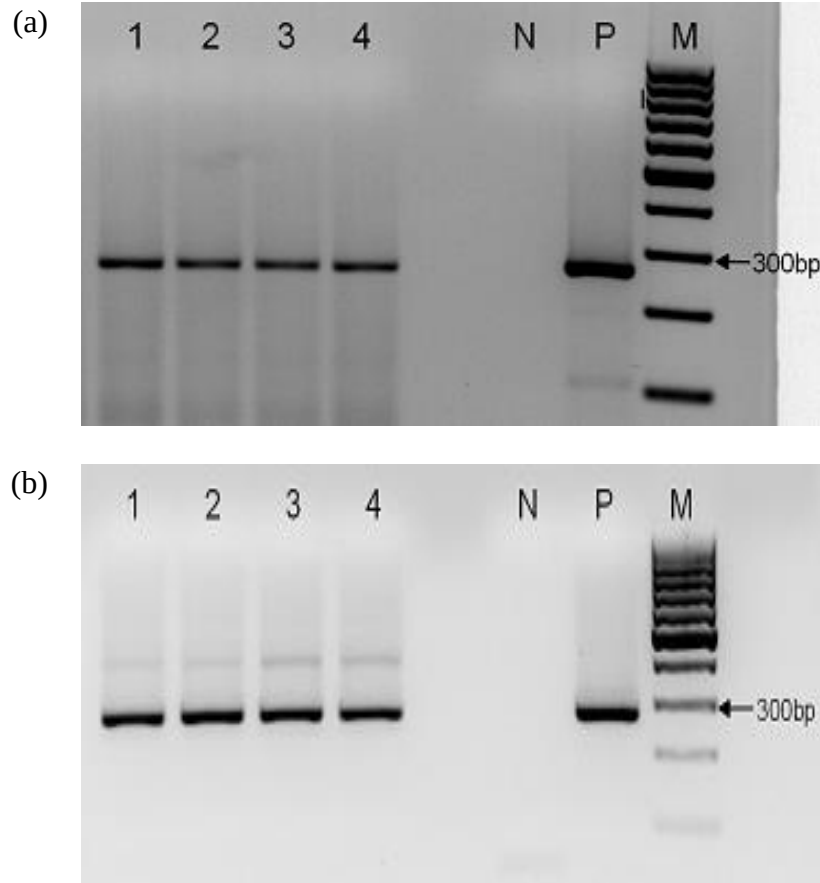


Figure 3.1: Example of agarose gel electrophoresis of PCR-amplified products. Lane M is 100bp DNA ladder (Thermo Scientific, California, USA). Lanes N and P are non-template control and positive control (DENV-2, strain New Guinea C). (a) Lanes 1-4 are PCR products of a dengue RNA that was amplified by the first pan-flavi RT-PCR assay. (b) Lanes 1-4 are PCR products of a RNA that was amplified by the second pan-flavi RT-PCR assay.

Table 3.2: Virus detection from serologically dengue negative sera by qRT-PCR

Patient sample	Total sample collected	qRT-PCR positive result ^a		
		DENV	CHIKV	ZIKV
NS1-, IgM-, IgG-	467	4	0	0

^aPositive result yielded from respective qRT-PCR of DENV, CHIKV and ZIKV

Table 3.3: Virus detection from serologically dengue negative sera by pan-flavi RT-PCR

Patient sample	Pan-flavi RT-PCR positive/total sera tested with pan-flavi RT-PCR
NS1-, IgM-, IgG-	4/46

Table 3.2 shows the results of one-step real-time RT-PCR (qRT-PCR) conducted for a total 467 serologically dengue negative sera which were tested negative for NS1 antigen, IgM and IgG antibodies by using commercial dengue kit. All these sera were tested with three qRT-PCR assays for DENV, CHIKV and ZIKV. In this context, qRT-PCR approaches enable efficient screening of large number of samples and minimize risk of sample contamination. Four (0.9 %) dengue positive results were yielded from qRT-PCR but no positive detection for CHIKV and ZIKV. These 4 dengue-positive sera were further confirmed with application of pan-flavi RT-PCR (as shown in Table 3.3). Sequencing data of resulted PCR products enabled identification of DENV-2 from these 4 sera. In this case, the PCR generated results of these 4 sera were inconsistent with the presumptive clinical serological test results which detected no dengue NS1 antigen and anti-dengue immunoglobulin. Despite high sensitivity and specificity of commercial dengue rapid test kit, a negative diagnostic result does not definitely rule out dengue infection (Osorio et al., 2010; Wang & Sekaran 2010). Furthermore, false negative may result from lower sensitivity

of the test kit against blood sera which are taken after 3 days of fever onset (Osorio et al., 2010).

A sum of 42 representative serologically dengue negative sera then were selected for each month spanning the entire period of which total sera collected. These 42 selected sera together with the other 4 serologically dengue negative sera (which were pre-tested positive for dengue qRT-PCR) were then subjected to pan-flavi RT-PCR screening using published primer cFD2 and MAMD (Scaramozzino et al., 2001). Noting that only 46 out of 467 serologically dengue negative sera were tested with pan-flavi RT-PCR in consideration of tedious work to screen the huge number of sera using conventional PCR approach and these sera had been tested with qRT-PCR in earlier stage.

Table 3.3 shows the results of pan-flavi RT-PCR for wherein negative results were generated for the 42 randomly selected serologically dengue negative sera. In contrast, positive PCR amplicons were generated for the 4 sera which were pre-tested positive for dengue qRT-PCR. Subsequent DNA sequencing identified that these 4 positive cases were DENV-2.

Higher virus detection and recovery rates were recognized from patient sera which were positive for dengue NS1 antigen. These serum samples were likely taken during early viremia within first 5 days of the course of infection before seroconversion of anti-dengue IgM and IgG antibodies (Bessoff et al., 2008; Muñoz-Jordán et al., 2009; Kassim et al., 2011). In contrary, virus detection rate was significantly lower for patient sera that did not contain dengue antigen NS1. These sera were mostly taken approximately 6 or more days post infection during which increased dengue specific IgM and IgG antibodies had suppressed the viremia in patients and hence resulted in negative RT-PCR results (Kassim et al., 2011).

Classical culture-based approach of virus isolation was employed to replicate the detected DENV from sera in order to have sufficient virus stock for subsequent analysis. The progress of C6/36 cells after inoculation is shown in Figure 3.3. The uninfected control C6/36 cells were more spherical in shape. After DENV invasion, C6/36 cells started to change morphologically and eventually lysed for DENV release. A total of 120 patient sera (68 sera positive for pan-flavi RT-PCR, 48 sera with low volume and 4 sera positive for dengue qRT-PCR) were subjected to virus isolation. Out of 72 PCR positive inoculums, 43 (59.7 %) have DENV isolated. On the other hand, DENV was isolated from 18 (37.5 %) out of 48 inoculums with low volume insufficient for PCR screening. Some of the cell cultures developed cytopathic effect (CPE) earlier within first passage whereas others were slower in later passages.

This study successfully identified 90 dengue positive results of which 72 positive results generated from overall PCR approaches and 18 positive results generated from culture-based virus isolation. Out of the 90 positive results, 12 of them with undetermined serotype group due to very low concentration of amplicons not applicable for nucleotide sequencing. The other 78 positive results with determined serotype groups as below:

- 34 (37.8 %) were DENV-1
- 27 (30.0 %) were DENV-2
- 17 (18.9 %) were DENV-3

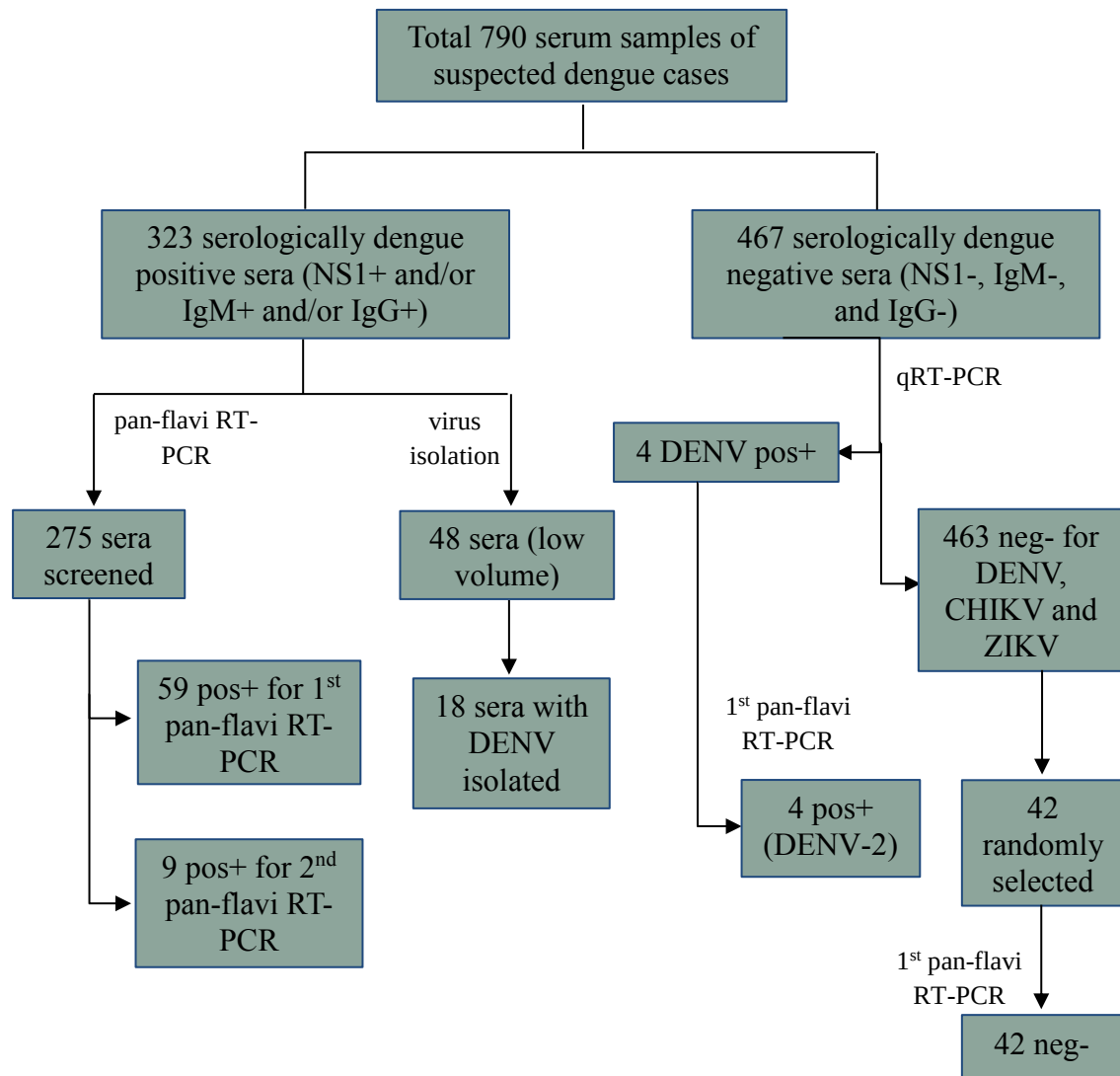
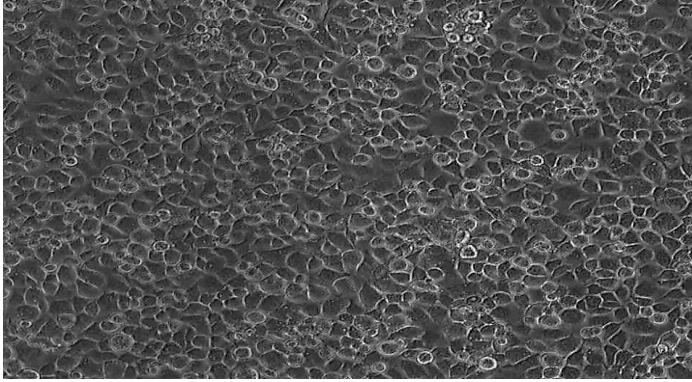
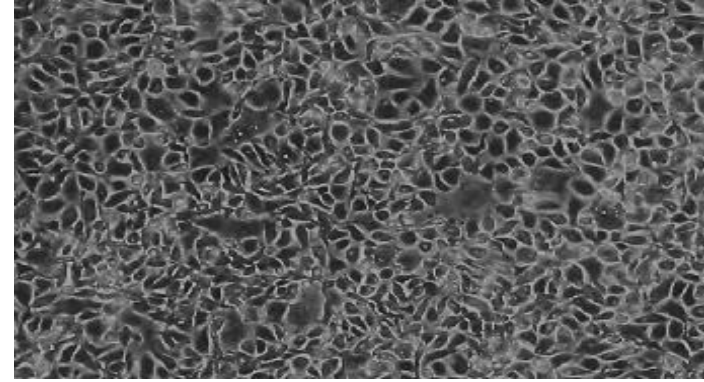


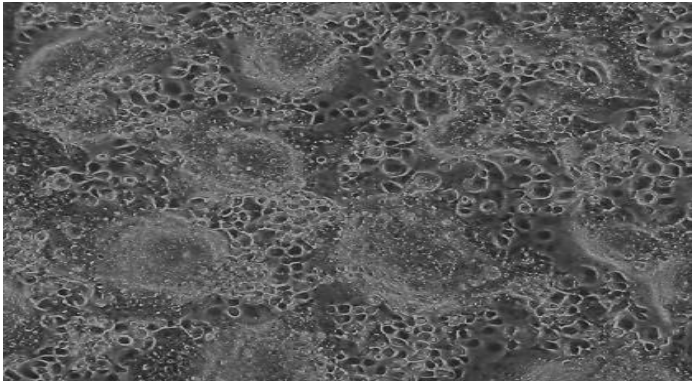
Figure 3.2: Summary of dengue screenings for patient sera collected.



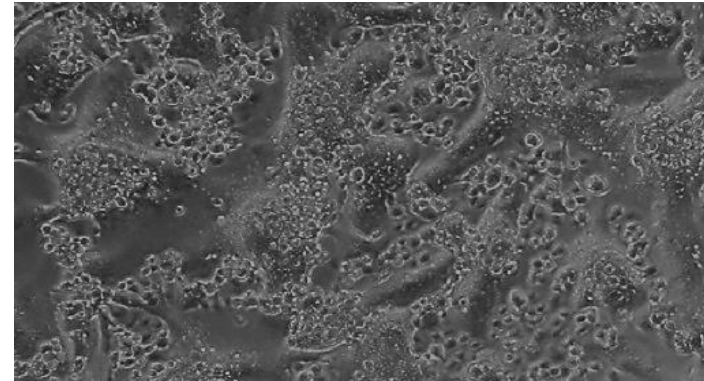
I. Uninfected C6/36 cells



II. Preliminary sign of infection



III. Day 4-6 of infection



IV. Day 7-9 of infection

Figure 3.3: Progress of DENV infected C6/36 cells. Images were taken at 10 × magnification of light inverted microscope. (I) uninfected C6/36 cells were round in shape. (II) C6/36 cells began to change morphologically (elongate) and formed cellular syncytia. (III) C6/36 cells started to lyse (CPE) by day 4-6 of infection depending on virus concentration. (IV) C6/36 cell lysis was further enhanced day 7-9 of infection after replenishment of culture media.

3.2 Age-wise distribution of dengue cases

Table 3.4 shows that the biggest cohort of dengue infected patients comprised young adults aged between 21 to 30 years. This age group contributed 32.8 % of total serologically dengue positive sera recruited for this study whilst 43 % of them were confirmed for presence of dengue viral RNA via laboratory PCR screenings. Patients aged 13-20 years old were also highly susceptible to dengue infection, contributing 20.1 % of total serologically dengue positive sera. In contrast, children below 12 years old were least affected and only contributed 5.9 % of total 323 serologically dengue positive sera collected.

Table 3.4: Age-specific incidence of dengue infection

Age group (year)	Serologically dengue positive case (%)		Laboratory confirmed case ^a (%)	
≤12	19	(5.9 %)	5	(5.9 %)
13-20	65	(20.1 %)	19	(22.1 %)
21-30	106	(32.8 %)	37	(43.0 %)
31-40	43	(13.3 %)	7	(8.1 %)
41-50	33	(10.2 %)	7	(8.1 %)
51-60	36	(11.2 %)	5	(5.8 %)
≥61	21	(6.5 %)	6	(7.0 %)
Total	323	(100 %)	86	(100 %)

^aDengue cases confirmed by pan-flavi RT-PCR and virus isolation.

3.3 Typing of DENV isolates

3.3.1 C-prM gene junction sequence of dengue isolates

The D1 and D2 primers have been shown to be sensitive and specific for detection of all the four DENV serotypes by amplifying C-prM gene junction fragment of approximately 511bp (Lanciotti *et al.*, 1992; Gupta *et al.*, 2008). Only a single common pair of primers was used to detect all 4 DENV serotypes without the need for additional serotype-specific primers (1992).

In this study, a total of 61 DENV isolates (32 DENV-1 isolates, 21 DENV-2 isolates and 8 DENV-3 isolates) were subjected to RT-PCR amplification of C-prM sequence. Agarose gel electrophoresis of the PCR amplicons is shown in Figure 3.4. Fragment sequences data of amplicons were generated, assembled and subsequently used to confirm the identity of DENV isolates by performing BLAST searches. Results showed that all isolates matched with reference DENV sequences available in the GenBank database with nucleotide similarity index from 99 % to 100 % [as shown in Appendix C (I)]

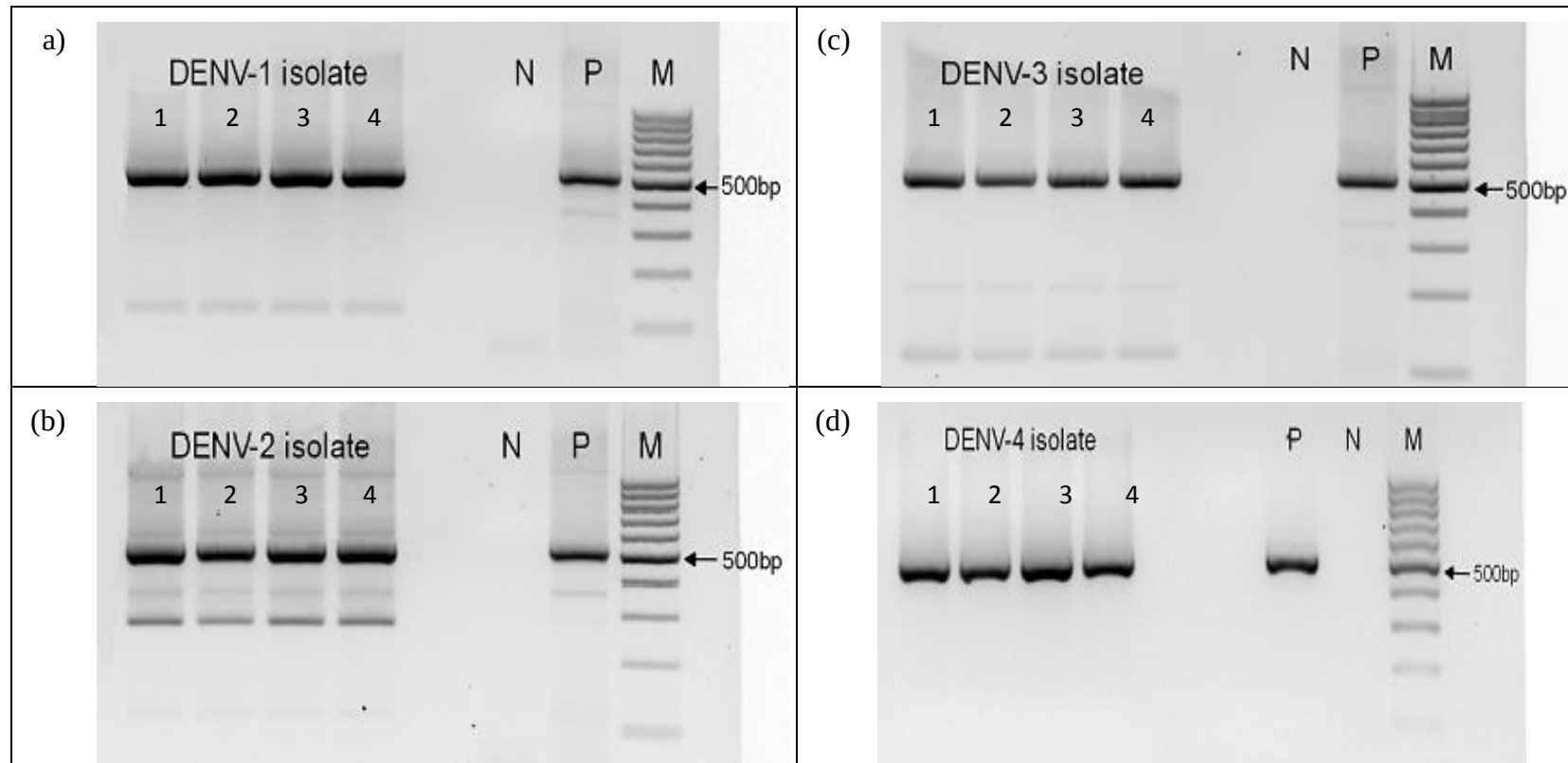


Figure 3.4: Example of gel electrophoresis of PCR-amplified C-prM junction fragments. Lane M is 100bp DNA ladder (Thermo Scientific, California, USA). Lanes N and P are negative non-template control and positive control (DENV-2, strain New Guinea C) respectively. Lanes 1 to 4 in respective gel image are PCR products of individual RNA of (a) DENV-1, (b) DENV-2, (c) DENV-3 and (d) DENV-4 respectively.

3.3.2 Full E gene sequences of dengue isolates

Genotype-specific consensus E gene primers were designed with reference to genotype grouping of DENV isolates from the phylogeny of C-prM gene junction sequences. There were 5 pairs of overlapping primers for each DENV genotype, in which they anneal to distinctive sites of viral E gene for generation of nucleotide fragments. A total of 37 DENV isolates (10 DENV-1 isolates, 10 DENV-2 isolates and 7 DENV-3 isolates) of this study were subjected to E gene PCR amplification. These 37 DENV isolates were actually selected from the total 61 DENV isolates which were amplified for their C-prM sequences in earlier analysis.

Figure 3.5 illustrates the PCR generated amplicons of E gene of a DENV-1 isolate. Nucleotide sequencing of each PCR amplicons recovered from agarose gel generated clean and reliable nucleotide sequences. Subsequently, sequence data were utilized for BLAST search confirmation of DENV isolates. BLAST search results reveal that all the selected DENV isolates matched with reference sequences of dengue viruses available in GenBank with high nucleotide homology [as shown in Appendix C (ii)]. Thereafter, E gene sequence data were used for phylogenetic analysis which will be discussed in Section 3.5.

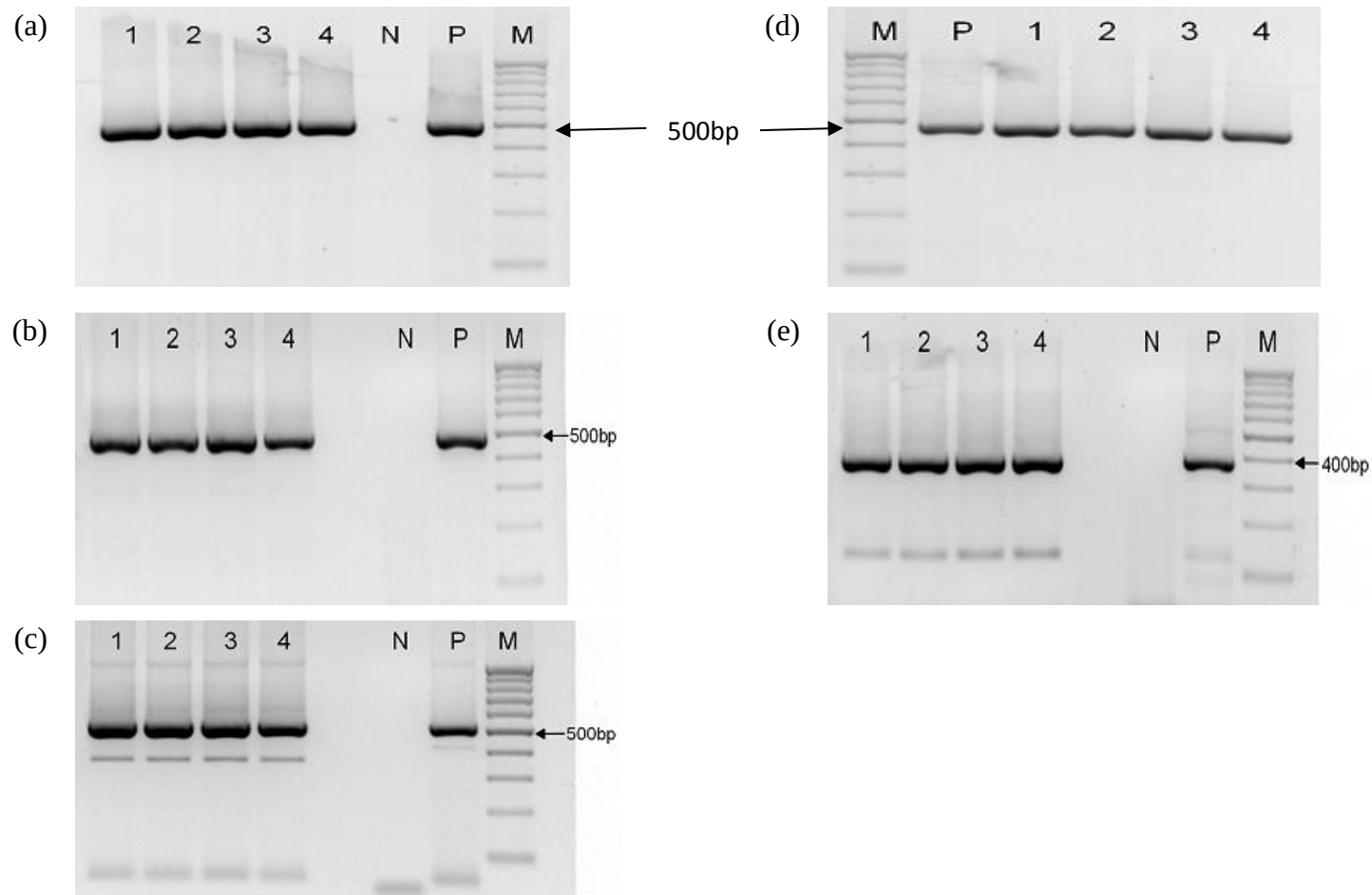


Figure 3.5: Examples of gel electrophoresis of PCR-amplified DENV-1 E gene fragments which were amplified by (a) primers D1E-1F and D1E-1R, (b) primers D1E-2F and D1E-2R, (c) primers D1E-3F and D1E-3R, (d) primers D1E-4F and D1E-4R and (e) primers D1E-5F and D1E-5R.. Lanes 1 to 4 are PCR product duplicates of a DENV-1 isolate RNA. Lane M is 100bp DNA ladder (Thermo Scientific, California, USA). Lanes N and P are negative non-template control and positive control (DENV-1, strain Hawaii) respectively.

3.4 Distribution of dengue viruses

Figure 3.6 shows the temporal distribution (September 2014 to June 2016) of different serotypes of DENV out of total serologically dengue positive sera (positive for NS1 and/or IgM and/or IgG as tested with commercial dengue kit) recruited from September 2014 to July 2016. Spatial distribution was not presented due to unavailability of geographical data on where the patients residing in.

In spite of seasonal changes, dengue infection has evolved to persist throughout the study period with an exception on September 2015 during which there was no DENV detected from the relatively low number of solely 4 patient sera. More cases of DENV-3 infection were detected from September to December 2014, suggesting probable predominance of DENV-3. A shift of predominant serotype from DENV-3 to DENV-2 likely occurred in December 2014. DENV-2 continued to circulate simultaneously with DENV-1. DENV-3 was least detected only in March and December for 2015.

Another switch of serotype from DENV-2 to DENV-1 was likely eventuated in November 2015 during which there was a surge of DENV-1 detection. Sharp increases in dengue cases were observed during transitions from February to March 2015 and December 2015 to January 2016, in conjunction with relatively greater rainfall from January to February 2015 and January to February 2016 in Kuching as shown in figure 3.7 (World Weather Online, n.d.).

Overall data show co-circulation of DENV-1, DENV-2 and DENV-3 in absence of DENV-4. DENV-1 and DENV-2 are the predominant serotypes within the study period with minor circulating DENV-3.

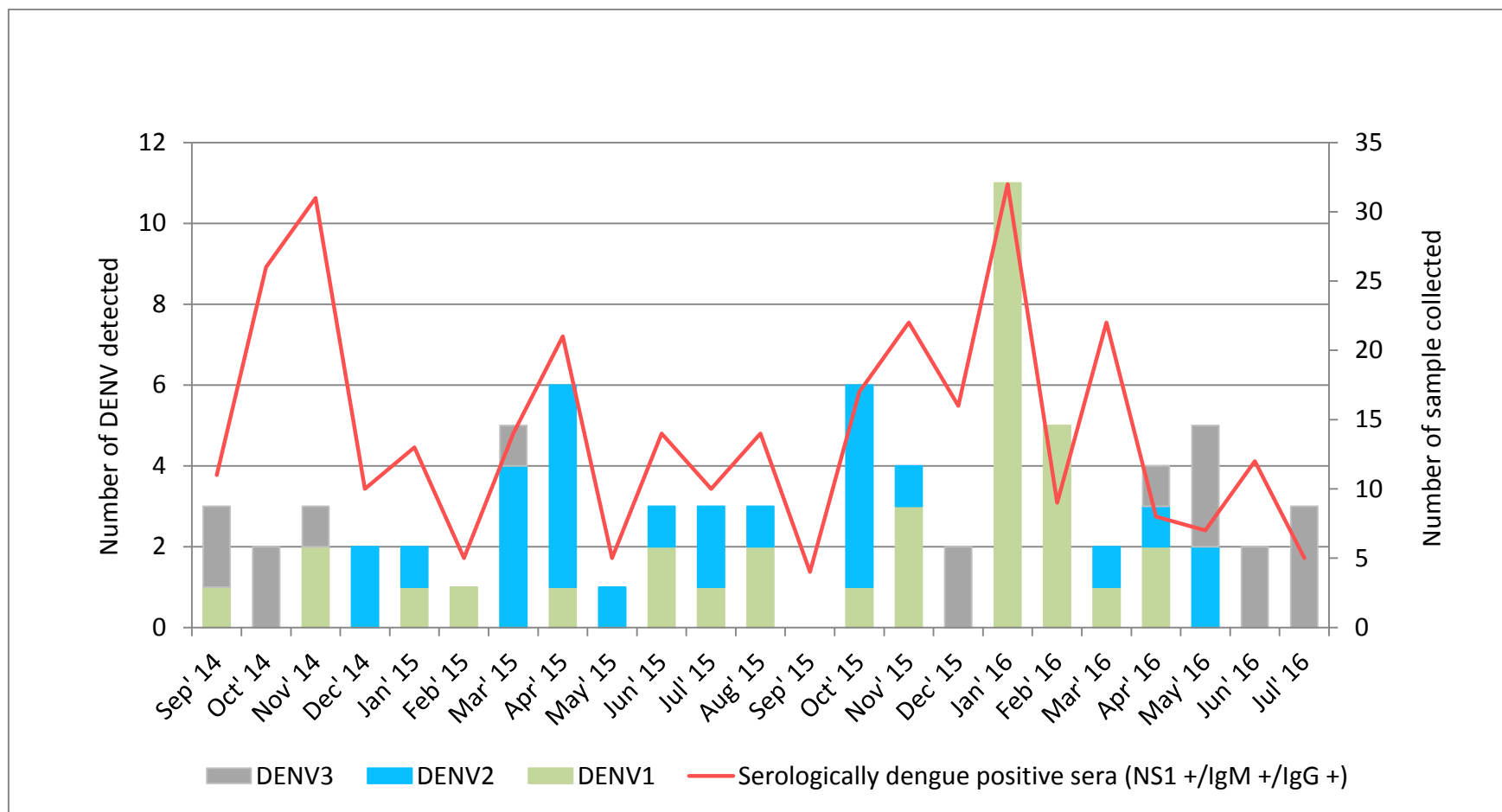


Figure 3.6: Monthly distribution of detected dengue viruses corresponding to number of serologically dengue positive sera.

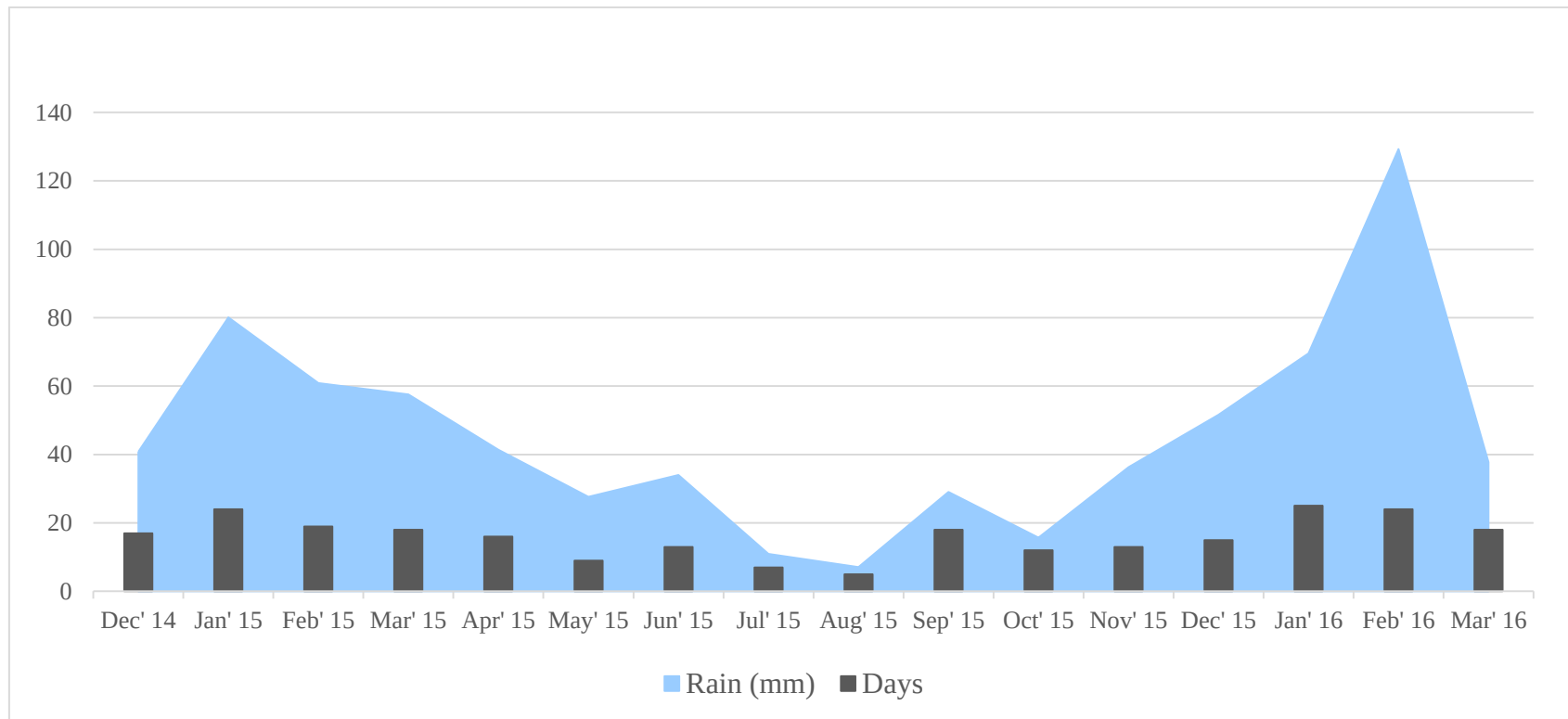


Figure 3.7: Average rainfall amount (mm) and rainy days in Kuching, Sarawak. Adapted from World Weather Online (n.d.).

3.5 Phylogenetic analyses

Phylogenetic analyses were performed in order to determine genotype abundance and provide insights into relationship among DENV isolates and strains from other geographical regions.

The final data set used for phylogenetic analyses on C-prM gene junction sequences included: 60 DENV-1 C-prM sequences (418 nt); 55 DENV-2 C-prM sequences (415 nt); 34 DENV-3 C-prM sequences (385 nt). Otherwise, final data sets for phylogenetic analyses on E gene sequences comprised following size: 42 DENV-1 E sequences (1485 nt); 51 DENV-2 E sequences (1485 nt); 39 DENV-3 E sequences (1479 nt).

Figures 3.8 shows the maximum likelihood phylogenetic analyses on both C-prM and E sequences of 9 representative isolates and 27 reference strains of different DENV serotypes. The sequences of known serotypes grouped accordingly within distinctive clusters with strong bootstrap supports. Representative isolate sequences also grouped precisely within corresponding serotype clusters in consistent with their serotype identities determined from BLAST analyses of their gene sequences. In addition, phylogenetic analyses were further performed for each DENV serotype in order to identify genotype identity of DENV isolates in a clearer illustration of phylogenetic trees.

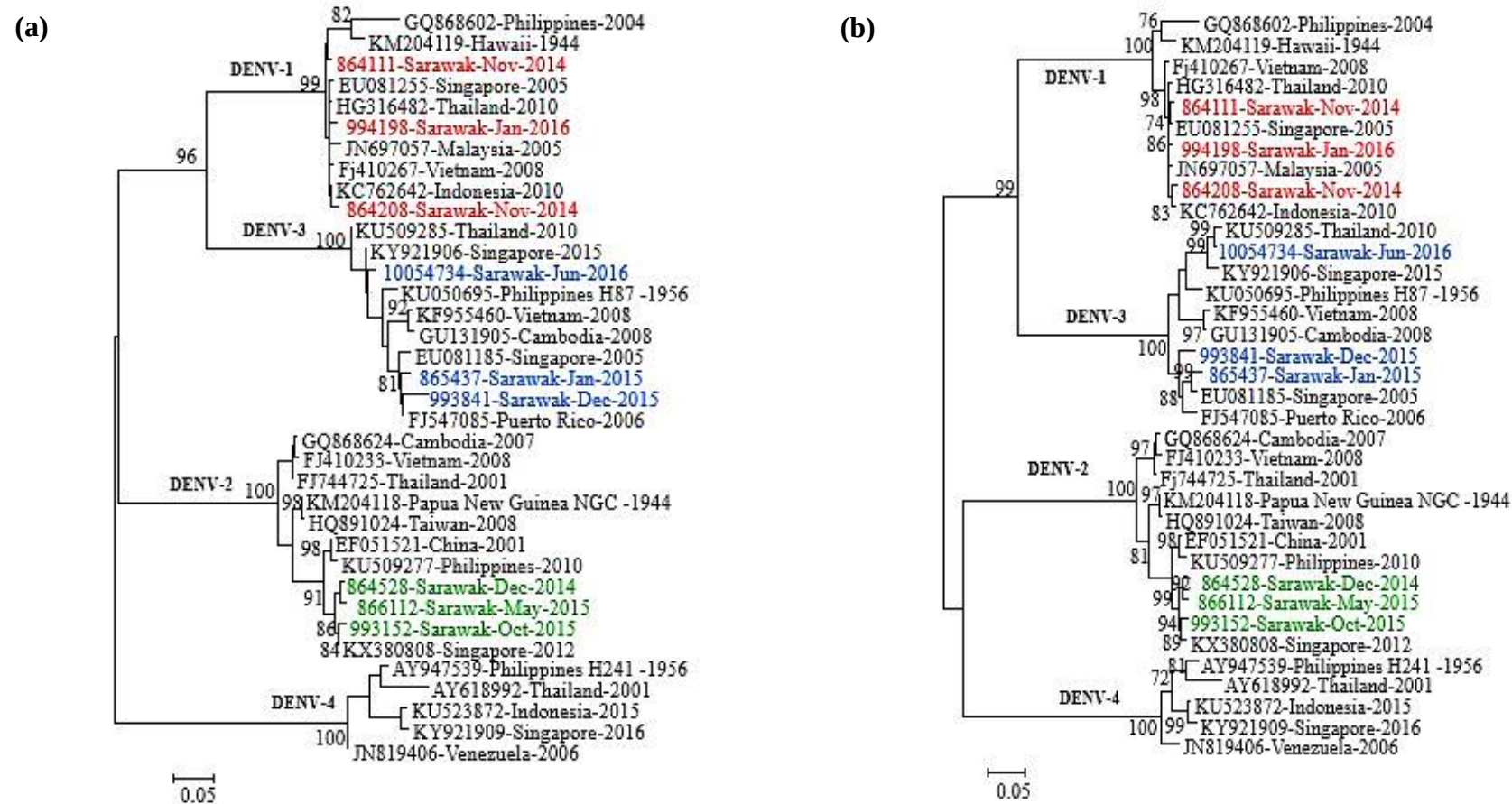


Figure 3.8: Phylogenetic trees of four DENV serotypes based on (a) partial C-prM sequences and (b) E sequences. The trees were constructed by maximum likelihood method with bootstrap 1000 replications. DENV isolates sequences from this study are designated in red for DENV-1, green for DENV-2 and blue for DENV-3. The reference sequences are in abbreviations of GenBank accession number/location/year of isolation. The scale bar on the left indicates nucleotide substitutions per site.

3.5.1 Phylogenies of DENV-1 isolates

Figure 3.9 (a) presents the phylogenetic analysis result on 60 C-prM gene junction sequences including 27 geographically diverse DENV-1 reference strains, 1 outgroup DENV-3 strain H87 and 32 DENV-1 isolates of this study. In contrast, Figure 3.9 (b) shows the phylogenetic tree based on entire DENV-1 E gene sequences of selected 31 DENV-1 reference strains, 10 DENV-1 isolate and 1 outgroup DENV-3 strain H87.

Referring to genogroups classification as proposed by Li and colleagues (2017), DENV-1 strains segregated into five genogroups namely I, II, III, IV and V. Genotype I consisted of strains from Southeast Asia, China and Australia. Genotype II comprised strains from Thailand during the 1960s, while genotype III was composed of 1972 and 2005 Malaysian strains. Genotype IV included strains from Southeast Asia and East Asia whereas genotype V included strains from American continent and the Caribbean.

Both C-prM and E gene sequences phylogenies of DENV-1 revealed that all the DENV-1 isolates sequences fell within genotype I. Figures 3.9 (a) and (b) show an integrate finding that the DENV-1 isolate sequences of a distinct cluster (994197, 994783, 994773, 994506, 994482, 994330, 994316, 994314, 01087742, 01032528, 994793, 955913, 993546, 994312, 994722, 993335, 955496, 865153, 864111 and 864712) are closely related to strains from Peninsular Malaysia (KJ806835 and KU666940), Singapore (KJ806962) and a Japanese strain (LC011945). Such phylogenetic connections suggest the likelihood of regional transmission of DENV in Asia.

A single DENV-1 isolate sequence (864208) was intimately related to strains from Singapore (GQ398255) and Indonesia (KC762642 and KU509253), suggesting importation from these two localities due to geographical proximity. Another 10 DENV-1 isolate

sequences (992545, 993456, 955376, 955357, 993533, 994313, 01087973, 994774, 865973 and 994198) grouped with reference strain sequences from China (KX621253 and KX225493), Singapore (KJ806951), Taiwan (KU365900) and Peninsular Malaysia (KT175084), indicating wide expansion of these viral strains probably due to increased tourist travel and trade within the Asia region.

3.5.2 Phylogenies of DENV-2 isolates

Figure 3.10 (a) presents the phylogenetic analysis on C-prM gene junction sequences of 21 DENV-2 isolates from this study, 33 DENV-2 reference strains from different geographical regions and 1 outgroup DENV-4 strain. Meanwhile, Figure 3.10 (b) reveals the phylogenetic analysis on E gene sequences of 10 DENV-2 isolates, 40 DENV-2 reference strains and 1 outgroup DENV-4 strain.

Phylogeny of DENV-2 formed into 6 distinct genogroups based on the classification as proposed by Twiddy and colleagues (2002). Asian I genotype of DENV-2 comprised strains sampled from Southeast Asia whereas Asian II genotype consisted of strains from East Asia and America. DENV-2 strains from Pacific Islands and Latin America composed the American genotype. Asian/American genotype included strains from Latin America, the Caribbean and China while sylvatic genotype comprised strains from Africa. Moreover, Cosmopolitan genotype comprised strains from dispersed geographical regions including Australia, Southeast Asia and East Asia.

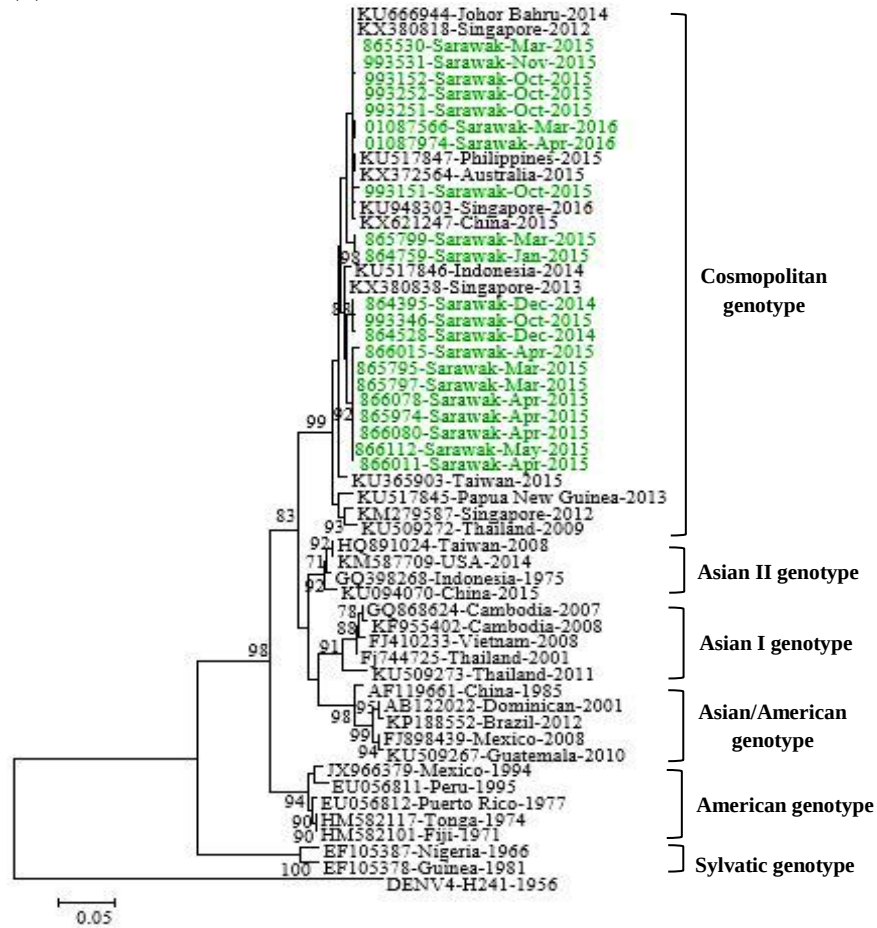
Both C-prM and E sequences phylogenies unanimously grouped all the DENV-2 isolate sequences within the Cosmopolitan genotype of which 2 phylogenetically distinct lineages were observed. This suggests independent entries of non-native DENV-2 Cosmopolitan strains in Kuching city.

Figure 3.10 (a) and (b) show an integrate finding that the DENV-2 isolate sequences (864395, 993346, 864528, 866015, 865795, 865797, 866078, 865974, 866080, 866112 and 866011) of Cosmopolitan genotype shared high homology with reference strains sampled from Singapore (KX380838), Selangor (KJ806931), Sabah (KJ806883) and Sarawak (KJ806880). Such labelled topology linked to previous Sarawak and Selangor strains (as

shown in E sequence phylogeny) indicates that these 11 isolates likely stemmed from local transmission or introduced from Peninsular Malaysia across the South China Sea.

On the other hand, 10 DENV-2 Cosmopolitan isolate sequences (865530, 993531, 993152, 993252, 993251, 01087566, 01087974, 993151, 865799 and 864759) clustered with strains from neighbouring geographical regions, notably Peninsular Malaysia (KJ806900, KJ666944 and KT175120), Singapore (KU948303 and KX380818), China (KX621247) and Philippines (KU517847). In this case, these isolates might be introduced from neighbouring localities in Southeast Asia.

(a)



(b)

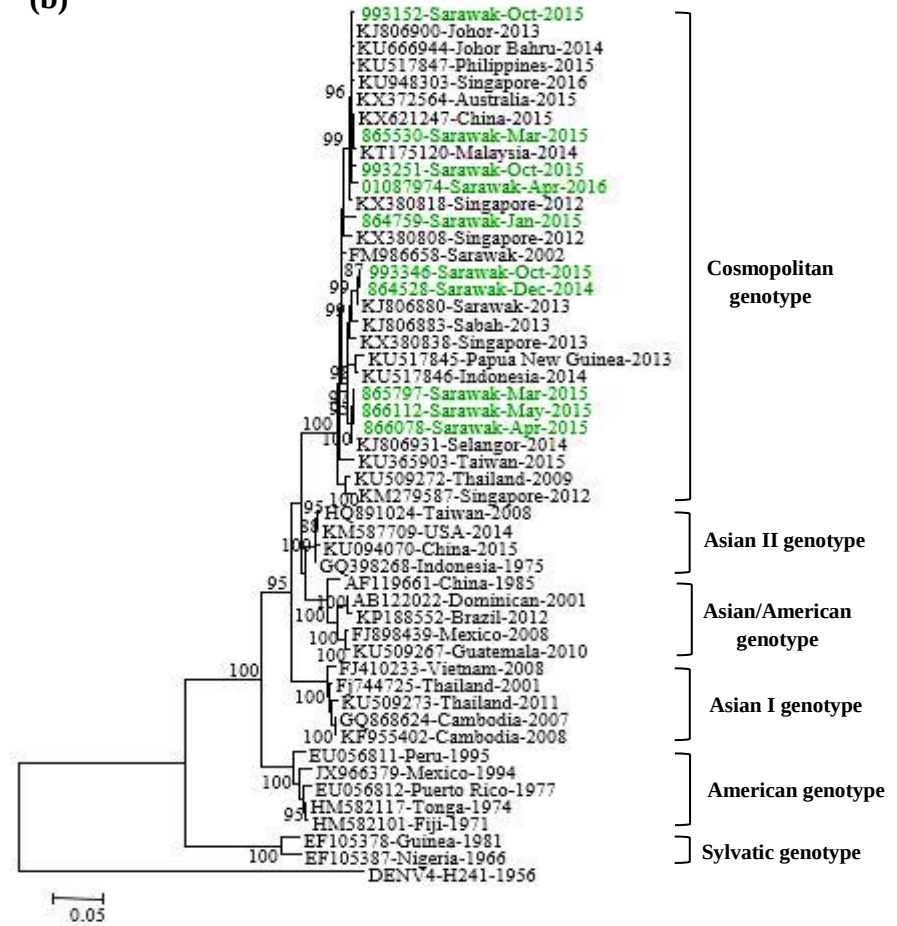


Figure 3.10: Phylogenetic trees of DENV-2 based on (a) partial C-prM sequences and (b) E gene sequences of selected strains. The tree was constructed by maximum likelihood method with bootstrap 1000 replications and rooted with an out-group DENV-4 Philippines strain (accession number AY947539). DENV-2 sequences of this study are designated in green. The reference strain sequences are in abbreviations of GenBank accession number/location/year of isolation. The scale bar on the left indicates nucleotide substitutions per site.

3.5.3 Phylogenies of DENV-3 isolates

Figure 3.11 (a) reveals the phylogenetic tree based on partial C-prM gene junction sequences of 25 DENV-3 global reference strains, 8 DENV-3 isolates of present study and 1 outgroup DENV-1 Hawaii strain. On the other hand, Figure 3.11 (b) shows the phylogenetic analysis on full E gene sequences of 7 DENV-3 isolates, 31 DENV-3 reference strains and 1 outgroup DENV-1 Hawaii strain.

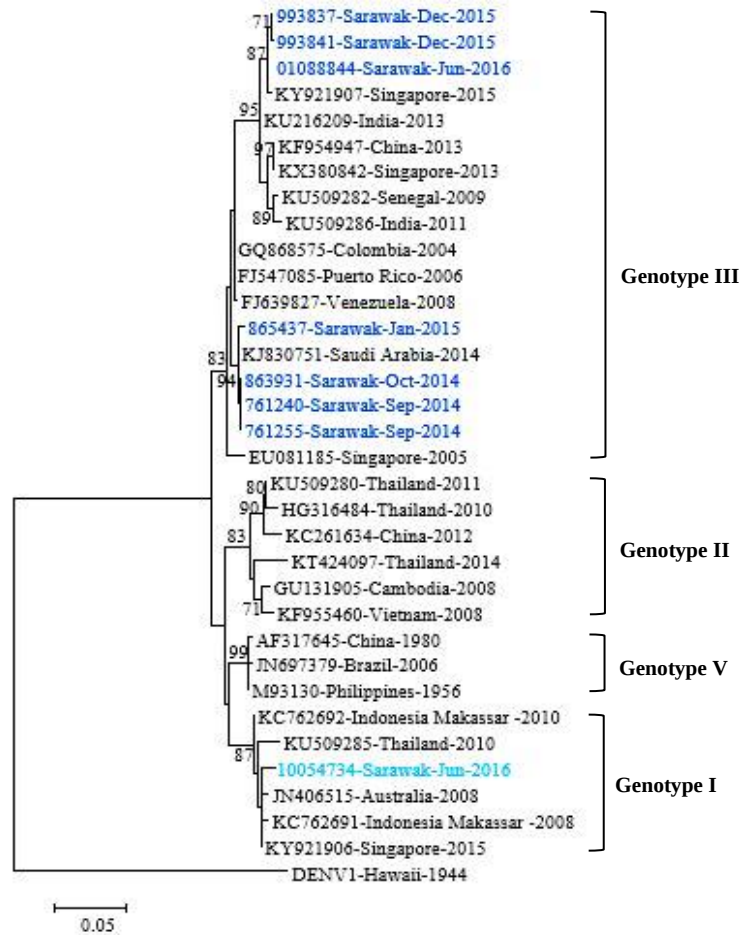
There were 5 discrete genogroups of DENV-3 determined based on the classification as proposed by Wang and colleagues (2016) and Sharma and colleagues (2011). DENV-3 strains from Southeast Asia and Australia built up the genotype I while genotype II was composed of strains from Southeast Asian countries especially Thailand. Genotype III included strains from wide geographical distribution of Southeast Asia, Latin America, the Caribbean, East Asia and South Asia. Furthermore, genotype IV comprised strains from Puerto Rico during the 1960s to 1970s and genotype V included sylvatic strains from China, Philippines and Brazil.

Both phylogenies of DENV-3 grouped 7 isolate sequences within genotype III and a single isolate sequence within genotype I. Notably, isolates sequences pertaining to genotype III segregated into two distinct clusters. The single DENV-3 genotype I (10054723-Sarawak-Jun-2016) isolate is most likely imported from Singapore as supported by its strong phylogenetic link (99 % E gene nucleotide homology) to Singapore strain and patient's travel history to Singapore before fever onset when she arrived in Kuching.

Three DENV-3 genotype III isolate sequences of present study (993837, 993841 and 01088844) were phylogenetically linked to a 2015 Singapore strain (KY921907) with 100 % bootstrap value as shown in the E gene phylogeny. The strong phylogenetic relationships

suggest that these 3 isolates are probably introduced from Singapore due to geographical proximity. The other 4 DENV-3 genotype III isolate sequences (865437, 761240, 761255 and 863931) clustered with sequences of strains from neighbouring Peninsular Malaysia (KP176709), Thailand (JF968106) and Saudi Arabia (KJ830751). These 4 isolates likely share a common ancestor of Malaysia origin.

(a)



(b)

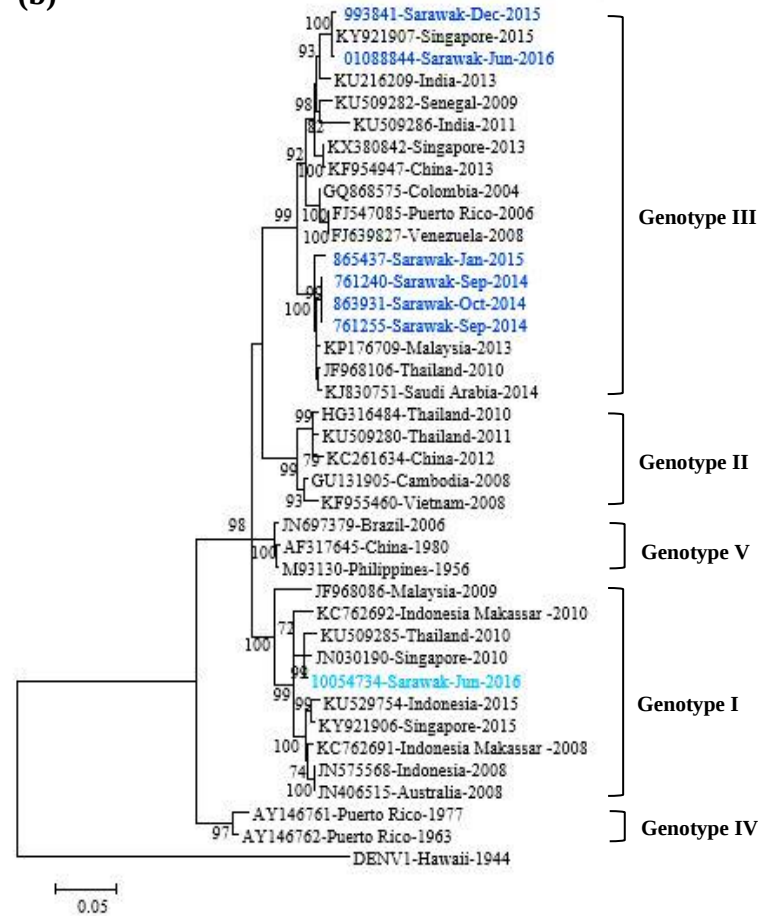


Figure 3.11: Phylogenetic trees of DENV-3 based on (a) partial C-prM sequences and (b) E gene sequences of selected strains. The tree was constructed by maximum likelihood method with bootstrap 1000 replications and rooted with an out-group DENV-1 Hawaii strain (accession number EU848545). DENV-3 sequences of present study are denoted in blue. The reference strain sequences are in abbreviations of GenBank accession number/location/year of isolation. The scale bar on the left indicates nucleotide substitutions per site.

CHAPTER 4

DISCUSSION

4.1 Detection of DENV, CHIKV and ZIKV

Malaysia has an equatorial climate which is always hot and humid, providing an optimal environment for breeding of *Aedes* mosquitoes and transmission of DENV. Similar situation is observed in Sarawak which is located in the northwest of the Borneo Island and bordered by the Sabah state, Kalimantan of Indonesia and Brunei. Dengue disease has persisted in Sarawak with relatively higher number of dengue cases in Kuching division according to the record in iDengue online platform (Ministry of Health Malaysia, n.d.).

In this study, all the collected dengue-suspected serum samples were subjected to at least two separate published pan-flavi RT-PCR (Scaramozzino et al., 2001; Moureau et al., 2007) as a confirmatory step to the clinical serology test results of each sample. Three different dengue serotypes (DENV-1, DENV-2 and DENV-3) were detected from the preliminary molecular testing approaches. Apart from detecting DENV, the dengue-serologically-negative sera were screened for CHIKV and ZIKV as these viruses share similar ecology, vector transmission cycle and clinical presentations. No CHIKV or ZIKV was detected from both the pan-flavi RT-PCR and qRT-PCR approaches. Nevertheless, the negative results raised the question as to which agent that actually caused the febrile-like symptoms among patients. The main CHIKV outbreak in the state traced back to 2009 and thereafter the disease has become less prevalent. On the other hand, ZIKA cases began to surge globally since 2015 and a few cases were actually identified in Malaysia (Dony et al., 2018).

Surprisingly, 4 serologically dengue negative sera (negative for NS1, IgM and IgG) were tested positive by both qRT-PCR and conventional PCR. This result highlights the issue that

the dengue serology rapid test kit used by hospitals could potentially lead to a false negative conclusion and as such, a negative serological diagnostic result (negative for NS1, IgM and IgG) does not definitely rule out dengue infection (Osorio et al., 2010; Wang & Sekaran., 2010). In fact, false negative may result from lower sensitivity of the test kit against blood sera which are taken after 3 days of fever onset (Osorio et al., 2010).

Higher virus detection and recovery rates were seen among the sera which were serologically tested with the presence of dengue NS1 positive and absence of IgM and IgG against the SD Bioline commercial dengue kit. This is likely because the serum samples were collected from suspected patients during early viremia within first 5 days of the course of infection before seroconversion of IgM and IgG antibodies (Bessoff et al., 2008; Muñoz-Jordán et al., 2009; Kassim et al., 2011). Infecting DENVs remained intact when the patient blood were withdrew in hospital and hence viral RNA could be detected by PCR amplification.

In contrast, virus detection was extremely low among serum samples with absence of NS1 but IgM and/or IgG detected. These samples were collected approximately 6 or more days post infection during which immune B cells have started to produced IgM and IgG antibodies to suppress viremia in patients (Kassim et al., 2011). Infecting DENVs would have been eliminated by the host's immune system whilst the IgM and IgG antibodies persisted for longer period.

From the preliminary screening results, it is likely that there were more primary dengue infection cases as illustrated by a relatively larger number of serum samples without anti-dengue IgM detected. In contrast, secondary infection likely comprised 38 % of the total cases as represented by the sera with detectable anti-dengue IgM antibody irrespective of NS1 and IgG detections.

Other than RT-PCR, culture-based virus isolation is also important to prepare sufficient virus stock for analyses and provide insight into dengue epidemiology. After inoculation, some of the cell cultures developed cytopathic effect (CPE) earlier within first passage whereas others were slower in later passages. These observations could be due to different plausible factors such as day of illness and presence of anti-dengue antibodies.

Meanwhile, delayed blood collection from the time of symptoms onset will drastically reduce the rate of virus isolation in accordance with low viremia (Jarman et al., 2011). The idea is supported by two studies which observed higher rate of virus isolation for blood sample that was collected within first 3 days of illness while the patient was febrile (Yamada et al., 2002; Klungthong et al., 2007). Furthermore, existing IgM or IgG will bind to DENV and block it from invading C6/36 cells without any effect on PCR detection of DENV RNA (Jarman et al., 2011). This explains the observation that some sera did not have DENV isolated from the cell culture although they were PCR positive for dengue.

4.2 Age-wise prevalence of dengue cases

In the past, dengue infection was typically acknowledged to be more prevalent among children and was a major cause of paediatric hospitalisation in Southeast Asia (Martina et al., 2009; Bhatia et al., 2013; Pang & Loh, 2016). However in this study young adults aged between 21 to 30 years contributed the most or both dengue suspected and laboratory confirmed dengue cases. In contrast, less dengue suspected and laboratory confirmed cases were seen among children aged 12 years or below.

In fact, several recent dengue studies have reported an age shift of dengue epidemiology inclining towards adults in many dengue endemic countries (Bhatia et al., 2013; Mohd-Zaki

et al., 2014; Pang & Loh, 2016; Woon et al., 2016;). In Malaysia, previous studies reported similar trend in which age group between 13 to 35 years was most affected (Pang & Loh, 2016). Likewise in neighbouring country Thailand, the study of Kalayanaroj and Nimmannitya (2004) found increasing incidences of severe dengue infection among adults. Nevertheless, there is no genetic evidence to our knowledge relating vulnerability of specific age group to dengue infection. Some studies proposed that the changing trend of demographic was facilitated by different health seeking behaviour and time interval between sequential infections (Gubler, 2004; Gupta et al., 2006; Bhatia et al., 2013).

4.3 Molecular typing of DENV isolates

Different DENV genomic regions may vary in evolution or mutation rate and researchers have utilized different genomic sequences to generate dengue epidemiology pertinent information (Rico-Hesse, 1990; Mishra et al., 2015). In this study, two different PCR approaches targeting viral C-prM (Lanciotti et al., 1992) and E gene (in-house design) were used for molecular characterization of DENV isolates. Among these two gene regions, the E gene is most commonly targeted for molecular typing of DENV. Despite an earlier description of the C-prM to target DENV, it is only recently that this region has been ‘re-discovered’ as target region for DENV detection. Sequences generated from targeted DENV genome regions allow the study of phylogenetic relationships of DENV strains that serve to shed light on the epidemiological aspect of DENV circulation.

The main advantage of using the C-prM gene junction sequence is that a single common pair of primers could be used for amplification and sequencing of all the four DENV serotypes. This method designed by Lanciotti and colleagues (1992) also requires shorter time to

amplify/sequence the C-prM sequence of 511bp (as shown in Figure 3.4) compared to viral E gene sequence. Taking into account of these advantages, the C-prM sequence analysis could have great implications for dengue epidemiology. To date, there is no study that compare the specificity of C-prM sequence for DENV typing. Hence, this study was intended to compare the specificity of C-prM sequence against E sequence for DENV typing.

4.4 Monthly distribution of dengue viruses

Generally, three DENV serotypes (DENV1-3) were identified from the study. DENV-1 and DENV-2 were predominantly detected during the study period while DENV-3 was less frequently detected. No DENV-4 was detected although the serotype was found to be circulating in Sarawak from 1997-1999 (Holmes et al., 2009).

DENV-2 was the predominant serotype in 2015 and co-circulated with DENV-1 and DENV-3 before getting replaced by DENV-1 in the subsequent year. DENV-3 remained rare and DENV-4 was not detected. There was a huge increase in cases in January 2016 probably due to a sudden switch of predominant strain from DENV-2 to DENV-1. Interestingly it shows that in Sarawak, different serotypes co-circulated but the dominant strain changes from time to time. Similarly, the study by Holmes and colleagues (2009) noted the same trend of which DENV-2, DENV-3 and DENV-4 co-existed in Sarawak during 1997-2002. This is especially crucial as the populations are more prone to secondary infection with not only different serotypes but also different genotypes in light with genotype shift, which attributed to more severe clinical complications. On rare occasions, this epidemiological dynamic situation could lead to inter-serotypic recombination and emergence of fitter DENV that would be better able to evade host immunity (Craig et al., 2003; Aaskov et al., 2007).

Previously, similar changing trends of dominant serotype were observed in Malaysia. Dengue surveillance done by Ministry of Health (MOH) Malaysia combining dengue data from different states showed predominant serotype shifts from DENV-2 in 2000, to DENV-3 in 2001-2002, DENV-1 in 2003-2005, DENV-2 in 2006-2009, DENV-1 in 2010-2011, DENV-3 in 2012 and DENV2 in 2013-2014 (Mohd-Zaki et al., 2014; Nani, 2015). Moreover, another study of dengue cases from East Coast of Peninsular Malaysia highlighted the switches of the predominant serotype from DENV-1 in 2005, DENV-1 and DENV-2 in 2007 and DENV-3 from 2008 to 2009 (Vinomarlini et al, 2011). This indicates that the predominant serotype varies between different regions or states of Malaysia.

In Sarawak, the highest number of dengue cases was reported in January 2016. This could be due to the relatively higher rainfall and rainy days in January 2016. The average rainfall amount was 69.6 mm and total 25 rainy days in January 2016 (World Weather Online, n.d.). This caused stagnant water (in cans, buckets, discarded tires and other artificial containers) that serve as breeding site for *Aedes* mosquito and eventually higher chances of DENV transmission to human hosts.

Referring to the DENV distribution as shown in Figure 3.5, DENV-1 and DENV-2 are considered the major infecting serotypes in Kuching division. These findings are similar to the national data that described co-circulation of all the four dengue serotypes and predominance of DENV-1 and DENV-2 in recent years (Nani, 2015; Woon et al., 2016). DENV-4 strain has been least prevalent in Malaysia as well as other countries in Southeast Asia (Huang et al., 2012; Nani, 2015).

4.5 Phylogenetic relationships of dengue isolates

Phylogenetic analysis serves as a useful approach to generate meaningful data including the evolutionary genetic change, transmission routes of virus and nature of selection pressures acting across the DENV genome. In this study, phylogenetic analyses were performed to determine genotype abundance and provide insights into the relationships among DENV isolates and strains from other geographical regions.

Previously, a few dengue studies managed to identify DENV serotype based on C-prM gene nucleotide sequence. Nevertheless, there is no study to compare the preciseness of C-prM sequence against E gene sequence for molecular typing. The phylogenetic analyses results of this study showed that both C-prM and E sequences gave similar clustering topologies. As such, the C-prM PCR would be a more useful tool for preliminary DENV typing compared to the traditional use of E gene PCR as the former uses a common primer pair that detects all four DENV serotypes which is not the case for E gene PCR.

4.5.1 Phylogenetic relationships of DENV-1

DENV-1 genotype I strains commonly predominate in Southeast Asian countries among the other lineages of the serotype (Huang et al., 2012). Previous studies showed extensive circulation of DENV-1 genotype I strains from Thailand and Indonesia during 2008 to 2010 (Huang et al., 2012; Chew et al., 2015). Other studies also described circulation of DENV-1 genotype I strains in Singapore (2008-2010), China (2001-2010) and Laos (2007-2010) (Lee et al., 2012; Dubot-Pérès et al., 2013; Jiang et al., 2013). Widespread expansion eventually led to importation and local evolution of genotype I strains in Malaysia to displace

pre-existing genotype III as the predominant lineage (Shu et al., 2009; Huang et al., 2012; Teoh et al., 2013).

The DENV-1 isolate (864208-Sarawak-Nov-2014) exhibits high degree of C-prM sequence homology to a 2008 Australia strain (KR919821) whereas the Australia strain shares a 99 % genomic sequence similarity with a 2008 Singapore strain (GQ398255). Such strong phylogenetic relationship suggests the origin of the strain (864208-Sarawak-Nov-2014) from Singapore due to recent importation.

Overall, the reference strains that showed intimate relationships with DENV-1 isolates were majorly sampled from Southeast Asia regions except a strain (LC011945) from Japan. Previous study of Churrotin and her colleagues (2016) suggested that this Japanese strain is linked to other genotype I strains from Southeast Asia in 2010, particularly Singapore and Malaysia, and they likely shared a common ancestor of Indonesia strain which previously caused an epidemic with highest peak of DHF in 2010.

In conjunction with that, the 20 DENV-1 genotype I isolates (994197, 994783, 994773, 994506, 994482, 994330, 994316, 994314, 01087742, 01032528, 994793, 955913, 993546, 994312, 994722, 993335, 955496, 865153, 864111 and 864712) are probably locally circulating strains with trace of expansion to other countries in Asia.

4.5.2 Phylogenetic relationships of DENV-2

From the previous studies, it has been known that DENV-2 Cosmopolitan genotype predominantly circulating in Malaysia (Chew et al., 2015). This is supported by reported active distribution of DENV-2 Cosmopolitan strains in Klang valley (1989-2000) and

Sarawak (1997-2002) (Holmes et al., 2009; Chew et al., 2015). Altogether the data from previous and present studies illustrated consistent genotype distribution of DENV-2 in Sarawak for the last 20 years. Similarly, DENV-2 Cosmopolitan genotype had predominated in Singapore and frequently exchanged with Malaysia (Lee et al., 2012; Chew et al., 2015).

Important to note that DENV-2 viruses (FM986658-Sarawak-2002 and KJ806880-Sarawak-2013) sampled earlier from Sarawak also belong to the same Cosmopolitan genogroup. These two strains could be from different origins (as they were grouped in distinct clusters) and eventually evolved to present circulating strain in Sarawak.

It is known that DENV-2 tend to trigger more severe form of dengue infection upon secondary infection (Soo et al., 2016). In conjunction with that, detection of DENV-2 which share sequence homology with strains from neighbouring countries create an alert of possible future outbreak of severe DENV-2 in the region.

4.5.3 Phylogenetic relationships of DENV-3

DENV-3 comprises five genotypes that are mainly detected in the Americas and Asia (Monteil et al., 2016). The DENV-3 isolates detected from this project were clustered in two different genotypes I and III. In the past, DENV-3 genotype I was endemic in the Malay archipelago especially in Indonesia (Schreiber et al., 2009; Herman et al., 2016). The study of Holmes et al. (2009) described distribution of DENV-3 genotype I in Sarawak during 1997. However, recent studies indicated a shift of predominance to DENV-3 genotype III in Malaysia and Thailand (Huang et al., 2012). Likewise, this study identified mainly DENV-3 genotype III isolates from the total cases collected.

The earliest DENV-3 genotype III strains originated from Indian subcontinent in 1960s. Since then, this genotype has spread to geographically diverse regions of the world and predominated against other genotypes which likely confined to specific geographical regions (Schreiber et al., 2009; Huang et al., 2012). Grouping of the DENV-3 genotype III isolates within phylogenetically distinct clusters implies there have been independent entries of viruses into this region.

Noted that an unusual Saudi Arabian strain (KJ830751) clustered in the same lineage with 4 DENV-3 isolates (865437, 761240, 761255 and 863931) of this study. This Saudi Arabian strain was sampled in 2016 and it was found to share 99.2 % genomic sequence similarity with a 2011 Malaysian strain (LT898451) (Monteil et al., 2016). Such phylogenetic relationship suggests importation of DENV-3 to Saudi Arabia probably during the pilgrimage season, similar to multiple introduction of DENV-2 strains as reported by El-Kafrawy and colleagues (2016). Thence, likely the 4 DENV-3 isolates are locally transmitted strains.

CHAPTER 5

CONCLUSION

There have been gaps of dengue epidemiological data in Sarawak owing to discontinuous study and surveillance. Worst still, the epidemiological data available may not truly reflect the actual disease situation due to underreporting.

This study provides an important insight into frequent and multiple importations of DENV into Kuching, Sarawak. This is supported by the observations that not only do multiple DENV serotypes co-circulate, but in the case of DENV-3 different genotypes also circulate simultaneously in this region. Moreover, there was remarkable co-circulation of different sub-lineages for each serotype identified. These observations prompt the vigilance of heterologous secondary infection which can lead to severe dengue haemorrhagic fever (DHF).

In contrast, there was no chikungunya or zika viruses detected from the qRT-PCR screening of the serum samples negative for dengue serology test (absence of NS1 antigen, IgM and IgG antibodies). It is unusual that the patients presented to hospital with dengue-like symptoms but molecularly tested negative for dengue, chikungunya and zika infection. This urges for future study with more sensitive molecular methods to identify the real infecting agent for this particular group of sera.

The findings of this study ascertain predominant serotype shift which is also observed in other states of the country. Likely this trend will continue with alternative predominance between DENV-1 and DENV-2 in Kuching, Sarawak. Owing to co-circulation of multiple serotypes and frequent strain exchanges, it is predicted that dengue disease will continue to hog nation headline although there were ups and downs in the trend of dengue incidence in

recent decade. This situation demands us to have contemporary dengue surveillance and dengue epidemiological study in order to mitigate the impact of dengue disease.

Attentive study of phylogenies provide a picture of which DENV isolates are intimately related to strains from Southeast Asia and some Asian localities, particularly Peninsular Malaysia, Indonesia, Thailand and Singapore. Such phylogenetic relationships suggest that there is recurrent traffic of dengue viruses in Southeast Asian regions. This underlines the need to prioritise airports fever screening among arriving passengers from Southeast Asia countries in order to impede DENV importation.

The generated C-prM and E phylogenies of each DENV serotype show similar clustering patterns. This evidences that viral C-prM sequence is comparable to E gene sequence for laboratory diagnosis and typing of dengue viruses. Thence, the C-prM sequence amplification approach can have important implications for dengue epidemiology studies provided its high sensitivity, specificity and advantages in term of time and cost.

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APPENDICES

Appendix A

Preparation of 6X loading dye – 10 ml

Note: 0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water

1. 25 mg of bromophenol blue was weighed and dissolved in 6.7 ml of sterile UHQ water in a sterile glass bottle.
2. 25 mg of xylene cyanol FF was added into the glass bottle and mixed.
3. The mixture was then added with 3.3 ml of glycerol and mixed thoroughly.
4. The ready-to-use 6X loading dye was aliquoted and kept at -20 °C.

Appendix B

Preparation of culture media

I) Leibovitz's L-15 stock medium – 1 liter

1. One pack of the Leibovitz's L-15 powder (Gibco, Life Technologies, USA) was added with 500 ml of sterile UHQ water in a 1 liter conical flask and dissolved by stirring on magnetic stirrer up to 30 minutes.
2. The solution was transferred into a volumetric flask and top up to 1 liter with sterile UHQ water.
3. The medium was subjected to first filtration by using filter paper and then second filtration by using vacuum filter.
4. Resulted medium in a sterile flask was labeled with details of date, medium type and batch number. Following that, 1 ml of the stock medium was transferred into a vial containing nutrient broth and incubated overnight at 37 °C for first quality control (QC 1). Stock medium was kept at 4 °C. The vial was checked for turbidity on the next day.
5. Second QC (QC 2) was performed by same procedure. Medium was only used after the solution in vial of QC 2 was assured to be clear of contamination.

II) Heat inactivation of fetal bovine serum (FBS)

1. FBS (Gibco, Life Technologies, US) was thawed fully and then incubated at 56 °C waterbath for 30 minutes for heat inactivation.
2. The surface of the bottle containing FBS was dried with clean tissue.

III) Penicillin G stock (100X=10,000 U/ml) – 100 ml

1. 0.3 g of penicillin G powder (Sigma, USA) was dissolved in 100 ml of sterile UHQ water and then filter-sterilized through 0.22 µm cellulose acetate membrane (Millipore Corp, Bedford, MA, USA) with positive pressure.
2. QC was done for a week with the same procedure of the QC of L-15 stock medium. Penicillin G stock solution was kept at 4 °C for use after the QC was ensured to be free of contamination.

IV) Streptomycin sulphate stock (100X=10,000 µg/ml) – 100 ml

1. 0.5g of streptomycin sulphate powder (Sigma, USA) was dissolved in 100 ml of UHQ water and then filter-sterilized through 0.22 µm cellulose acetate membrane (Millipore Corp, Bedford, MA, USA) with positive pressure.
2. QC was conducted for a week with the same procedure of the QC of L-15 stock medium. Streptomycin sulphate stock solution was stored at 4 °C for use after the QC was determined to be free of contamination.

V) Tryptose phosphate broth stock – 1 liter

1. 29.5 g of TPB powder was added with 500 ml of UHQ water in a 1 liter conical flask and dissolved by stirring on magnetic stirrer.
2. Fully dissolved TPB solution was transferred into a volumetric flask and top up to 1 liter with UHQ water. The solution was mixed by inverting the flask gently.
3. TPB stock solution was then transferred into Schott bottles and sterilized by autoclave before use for medium preparation.

VI) L-15 medium supplemented with 1% FBS for virus inoculation

1. The following solutions were added into a sterile 250 ml Schott glass bottle aseptically in a laminar flow hood.

1% (v/v) or 2.5 ml Streptomycin antibiotic

1% (v/v) or 2.5 ml Penicillin G antibiotic

1% (v/v) or 2.5 ml FBS (Gibco, Life Technologies, USA)

10% (v/v) or 25 ml TPB (Sigma, USA)

2. The mixtures in Schott bottle was then added with sterile L-15 medium (Gibco, Life Technologies, USA) up to 250 ml.
3. The resulted medium was subjected to two rounds of QC with same procedure of the QC of L-15 stock medium to ensure the medium was free of contamination.

VII) L-15 medium supplemented with 10% FBS for maintenance of C6/36 cells

1. The following solutions were added into a sterile 250 ml Schott bottle aseptically in a laminar flow hood.

1% (v/v) or 2.5 ml Streptomycin antibiotic

1% (v/v) or 2.5 ml Penicillin G antibiotic

10% (v/v) or 25 ml FBS (Gibco, Life Technologies, USA)

10% (v/v) or 25 ml TPB (Sigma, USA)

2. The mixtures in Schott bottle was then added with sterile L-15 medium (Gibco, Life Technologies, USA) up to 250 ml.
3. The Schott bottle was labeled with relevant details of date, name of medium and batch number.
4. Two rounds of QC were performed with the same procedure of the QC of L-15 stock medium in order to check for contamination.

Appendix C

I) BLAST search results of C-prM gene junction sequences of DENV isolates

Isolate ID	Serotype	Sequence identity (Accession number of the best match result from GenBank)
864111-Sarawak-Nov-2014	DENV-1	99 % (KX452065)
864208-Sarawak-Nov-2014	DENV-1	99 % (KR919821)
864712-Sarawak-Jan-2015	DENV-1	99 % (KX452065)
865153-Sarawak-Jan-2015	DENV-1	99 % (KX452065)
865973-Sarawak-Apr-2015	DENV-1	100 % (KT827373)
955357-Sarawak-Jun-2015	DENV-1	99 % (KT827373)
955376-Sarawak-Jun-2015	DENV-1	99 % (KT827373)
955496-Sarawak-Jul-2015	DENV-1	99 % (KX452065)
955913-Sarawak-Aug-2015	DENV-1	100 % (KX452065)
992545-Sarawak-Aug-2015	DENV-1	99 % (KT827373)
993335-Sarawak-Oct-2015	DENV-1	99 % (KX452065)
993456-Sarawak-Nov-2015	DENV-1	99 % (KT827373)
993533-Sarawak-Nov-2015	DENV-1	100 % (KT827373)
993546-Sarawak-Nov-2015	DENV-1	100 % (KX452065)
994197-Sarawak-Jan-2016	DENV-1	99 % (KX452065)
994198-Sarawak-Jan-2016	DENV-1	99 % (KT827373)
994312-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
994313-Sarawak-Jan-2016	DENV-1	100 % (KT827373)
994314-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
994316-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
994330-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
994482-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
994506-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
994722-Sarawak-Jan-2016	DENV-1	100 % (KX452065)
01032528-Sarawak-Jan-2016	DENV-1	100 % (KX452065)

Table continued

994773-Sarawak-Feb-2016	DENV-1	100 % (KX452065)
994774-Sarawak-Feb-2016	DENV-1	100 % (KT827373)
994783-Sarawak-Feb-2016	DENV-1	100 % (KX452065)
994793-Sarawak-Feb-2016	DENV-1	100 % (KX452065)
994802-Sarawak-Feb-2016	DENV-1	100 % (KX452065)
01087742-Sarawak-Mar-2016	DENV-1	100 % (KX452065)
01087973-Sarawak-Apr-2016	DENV-1	99 % (KT827373)
864395-Sarawak-Dec-2014	DENV-2	99 % (KY921904)
864528-Sarawak-Dec-2014	DENV-2	99 % (KY921904)
864759-Sarawak-Jan-2015	DENV-2	99 % (EU359009)
865530-Sarawak-Mar-2015	DENV-2	99 % (KX452046)
865795-Sarawak-Mar-2015	DENV-2	99 % (KY921904)
865797-Sarawak-Mar-2015	DENV-2	99 % (KY921904)
865799-Sarawak-Mar-2015	DENV-2	99 % (EU359009)
865974-Sarawa-Apr-2015	DENV-2	99 % (KY921904)
866011-Sarawak-Apr-2015	DENV-2	99 % (KY921904)
866015-Sarawak-Apr-2015	DENV-2	99 % (KY921904)
866078-Sarawak-Apr-2015	DENV-2	99 % (KY921904)
866080-Sarawak-Apr-2015	DENV-2	99 % (KY921904)
866112-Sarawak-May-2015	DENV-2	99 % (KY921904)
993151-Sarawak-Oct-2015	DENV-2	99 % (KX452046)
993152-Sarawak-Oc-2015	DENV-2	99 % (KX452046)
993251-Sarawak-Oct-2015	DENV-2	100 % (KX452046)
993252-Sarawak-Oct-2015	DENV-2	100 % (KX452046)
993346-Sarawak-Oct-2015	DENV-2	99 % (KY921904)
993531-Sarawak-Nov-2015	DENV-2	100 % (KX452046)
01087566-Sarawak-Mar-2016	DENV-2	99 % (KX452046)
01087974-Sarawak-Apr-2016	DENV-2	99 % (KX452046)
761240-Sarawak-Sep-2014	DENV-3	99 % (KJ830751)

Table continued

761255-Sarawak-Sep-2014	DENV-3	99 % (KJ830751)
863931-Sarawak-Oct-2014	DENV-3	99 % (KJ830751)
865437-Sarawak-Jan-2015	DENV-3	99 % (KJ830751)
993837-Sarawak-Dec-2015	DENV-3	99 % (MF370226)
993841-Sarawak-Dec-2015	DENV-3	99 % (MF370226)
01088844-Sarawak-Jun-2016	DENV-3	99 % (MF370226)
10054734-Sarawak-Jun-2016	DENV-3	99 % (KY921906)

II) BLAST search results of E gene sequences of selected DENV isolates

Isolate ID	Serotype	Sequence identity (Accession number of the best match result from GenBank)
864111-Sarawak-Nov-2014	DENV-1	99 % (KX452055)
864208-Sarawak-Nov-2014	DENV-1	99 % (EU359008)
865153-Sarawak-Jan-2015	DENV-1	99 % (KX452055)
865973-Sarawak-Apr-2015	DENV-1	99 % (KJ806959)
955376-Sarawak-Jun-2015	DENV-1	100 % (KX225492)
993335-Sarawak-Oct-2015	DENV-1	99 % (KX452055)
993456-Sarawak-Nov-2015	DENV-1	99 % (KJ806959)
994198-Sarawak-Jan-2016	DENV-1	99 % (KX225492)
01087742-Sarawak-Mar-2016	DENV-1	99 % (KX452055)
01087973-Sarawak-Apr-2016	DENV-1	99 % (KX225492)
864528-Sarawak-Dec-2014	DENV-2	99 % (KU666946)
864759-Sarawak-Jan-2015	DENV-2	99 % (JF327392)
865530-Sarawak-Mar-2015	DENV-2	99 % (KX452017)
865797-Sarawak-Mar-2015	DENV-2	99 % (KF479405)
866078-Sarawak-Apr-2015	DENV-2	99 % (KF479405)
866112-Sarawak-May-2015	DENV-2	99 % (KF479405)
993152-Sarawak-Oct-2015	DENV-2	99 % (KX452038)
993251-Sarawak-Oct-2015	DENV-2	99 % (KX452045)
993346-Sarawak-Oct-2015	DENV-2	99 % (KU666946)
01087974-Sarawak-Apr-2016	DENV-2	99 % (KX452045)
761240-Sarawak-Sep-2014	DENV-3	99 % (LT898452)
761255-Sarawak-Sep-2014	DENV-3	99 % (LT898452)
863931-Sarawak-Oct-2014	DENV-3	99 % (LT898452)
865437-Sarawak-Jan-2015	DENV-3	99 % (KJ830751)
993841-Sarawak-Dec-2015	DENV-3	99 % (KY921907)
01088844-Sarawak-Jun-2016	DENV-3	99 % (KY921907)
10054734-Sarawak-Jun-2016	DENV-3	99 % (KC762684)

Appendix D

C-prM gene junction sequences of DENV isolates

I) DENV-1 isolates

>864111-Sarawak-Nov-2014.seq

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AGTTATGTGAGGATACAATGAC
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>864208-Sarawak-Nov-2014.seq

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>864712-Sarawak-Jan-2015.seq

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>865153-Sarawak-Jan-2015.seq

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>865973-Sarawak-Apr-2015.seq

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>955357-Sarawak-Jun-2015.seq

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>955913-Sarawak-Aug-2015.seq

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>992545-Sarawak-Aug-2015.seq

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>993335-Sarawak-Oct-2015.seq

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>993456-Sarawak-Nov-2015.seq

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>993533-Sarawak-Nov-2015.seq

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>993546-Sarawak-Nov-2015.seq

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>994197-Sarawak-Jan-2016.seq

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>994506-Sarawak-Jan-2016.seq

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>994722-Sarawak-Jan-2016.seq

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>994773-Sarawak-Feb-2016.seq

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>994774-Sarawak-Feb-2016.seq

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>994783-Sarawak-Feb-2016.seq

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>994793-Sarawak-Feb-2016.seq

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>994802-Sarawak-Feb-2016.seq

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>01032528-Sarawak-Jan-2016.seq

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>01087742-Sarawak-Mar-2016.seq

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>01087973-Sarawak-Apr-2016.seq

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II) DENV-2 isolates

>864395-Sarawak-Dec-2014.seq

CAACTGTGCAGCAGCTGACAAAGAGATTCTCACTTGGAATGCTACAGGGACGAGGACCATTGAAAC
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>864528-Sarawak-Dec-2014.seq

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>864759-Sarawak-Jan-2015.seq

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>865530-Sarawak-Mar-2015.seq

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>865795-Sarawak-Mar-2015.seq

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>865797-Sarawak-Mar-2015.seq

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>865799-Sarawak-Mar-2015.seq

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>865974-Sarawak-Apr-2015.seq

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>866011-Sarawak-Apr-2015.seq

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>866015-Sarawak-Apr-2015.seq

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>866078-Sarawak-Apr-2015.seq

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>866080-Sarawak-Apr-2015.seq

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>866112-Sarawak-May-2015.seq

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>993151-Sarawak-Oct-2015.seq

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>993152-Sarawak-Oct-2015.seq

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>993251-Sarawak-Oct-2015.seq

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>993252-Sarawak-Oct-2015.seq

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>993346-Sarawak-Oct-2015.seq

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>993531-Sarawak-Nov-2015.seq

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>01087566-Sarawak-Mar-2016.seq

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>01087974-Sarawak-Apr-2016.seq

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III) DENV-3 isolates

>761240-Sarawak-Sep-2014.seq

CTGCTGAACGGCCAGGGACCAATGAAATTGGTTATGGCGTTCATAGCTTTCCTCAGATTTCTAGCC
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CTCTGTCTCTTGATGATATTGCCAGCAGCACTTGCTTTCCACTTGACTTCACGAGATGGAGAGCCG
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>761255-Sarawak-Sep-2014.seq

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>863931-Sarawak-Oct-2014.seq

CTGCTGAACGGCCAGGGACCAATGAAATTGGTTATGGCGTTCATAGCTTTCCTCAGATTTCTAGCC
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>865437-Sarawak-Jan-2015.seq

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>993837-Sarawak-Dec-2015.seq

TTGCTGAACGGCCAGGGACCAATGAAATTGGTCATGGCGTTCATAGCCTTCCTTAGATTTCTGGCC
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>993841-Sarawak-Dec-2015.seq

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>01088844-Sarawak-Jun-2016.seq

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CTCTGTCTCATGATGATATTGCCAGCAGCACTTGCTTTCCACTTGACTTCACGAGATGGAGAGCCG
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>10054734-Sarawak-Jun-2016.seq

CTGCTGAATGGCCAAGGACCAATGAAATTGGTTATGGCGTTCATAGCTTTCCTCAGATTTCTAGCC
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CTGAAAGGCTTCAAGAGGGAGATCTCAAATATGCTGAGCATTATCAACAGACGGAAAAAGACATCG
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CGCATGATTGTGGGGAAGAATGAAAGAGGAAAATCCCTACTTTTTTAAGACAGCCTCTGGAATCAAC
ATGTGCACACTCATAGCCATGGATCTGGGAGAAATGTGTGATGATACGGTCACTT

E gene sequences of selected DENV isolates

I) DENV-1 isolates

>864111-Sarawak-Nov-2014.seq

ATGCGATGCGTGGAATAGGCAGCAGGGACTTCGTGGAAGGACTGTCAGGAGCAACTTGGGTAGAC
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