ZIBELINE INTERNATIONAL™ PUBLISHING

ISSN: 2521-0858 (Print)
ISSN: 2521-0866 (Online)
CODEN: SHJCAS

Science Heritage Journal (GWS)

DOI: http://doi.org/10.26480/gws.01.2023.09.17

RESEARCH ARTICLE

GENOTYPIC DIVERSITY OF DENGUE VIRUS IN *AEDES* MOSQUITOES COLLECTED FROM DENGUE HOTSPOT AREAS IN KUALA LUMPUR AND SELANGOR, MALAYSIA

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ARTICLE DETAILS

Article History:

Received 23 February 2023
Revised 17 March 2023
Accepted 27 April 2023
Available Online 30 April 2023

ABSTRACT

Dengue fever is endemic in Malaysia and has posed a significant economic and health burden to the country. Malaysia is experiencing the transmission of four dengue serotypes: DENV-1, DENV-2, DENV-3, and DENV-4. To prevent dengue cases, genotypic characterisation is an essential tool to determining the dengue serotypes that are circulating in the dengue cases area. Thus, the objective of this study was to determine the genotype of DENV isolates obtained from DENV-infected *Aedes* collected. A total of 4,438 *Aedes albopictus* and 2,454 *Aedes aegypti* larvae were collected from 132 dengue hotspot localities. They were then reared to adult mosquitoes and pooled. Later, it was tested for determine dengue serotypes using RT-PCR targeting Capsid-preMembrane regions. All DENV serotypes were carefully isolated from DENV positive pools and sequenced. DENV-2, DENV-3, and DENV-4 serotypes were proven positive in 16.83 % of the samples, and were found co-circulating at the study areas. A total 84 isolates were successfully identified, with 17 of them being DENV-2, 36 being DENV-3, and 31 being DENV-4. DENV-2 isolates were of genotype IV, DENV-3 isolates were of genotype III and V, and DENV-4 isolates were of genotype II. This study highlights the importance of active DENV serotype at dengue outbreak areas. This information assists the stakeholders by strengthening dengue genotyping surveillance and providing a solid evidence base for decision making regarding dengue management initiatives. The findings from this study can be utilized to the search for novel genotypes, to the observation of potential genetic alterations in dengue viruses, and to the development of vaccines.

KEYWORDS

dengue genotype, DENV-2 genotype IV, DENV-3 genotype III, DENV-3 genotype V, DENV-4 genotype II.

1. INTRODUCTION

History of dengue in Malaysia began when in March 1954 an outbreak of febrile illness was reported among teachers and students at the Methodist Girls School, Kuala Lumpur. This was the first dengue viruses (DENV) isolated in Malaysia and was identified as DENV-1(Smith,1956). Between November 1962 until July 1963, Malaysia was hit by DHF where cases were reported from Penang Island and was then identified as DENV-2 (Paramaesvaran, 1966; Rudnick et al., 1965). Since 1974, dengue incidents reported in Malaysia have been tabulated and an increasing trend was observed in the last decade (Shepard et al.,2013). It occurs nationally and not limited to urban areas only.

In 2019, as many as 124,777 dengue cases were reported, which was 60% higher than the number of cases recorded in 2018 (WHO, 2019). The states of Selangor and Kuala Lumpur are the areas that have been largely affected by the disease. Both states are reporting high numbers of dengue cases. Two factors that favour dengue transmission such as high population density and rapid development are found very common in both states. One way of preventing dengue is by performing dengue vector surveillance (WHO, 2012; WHO, 2016). Larval and ovitrap surveys are the most common assessment for vector surveillance (WHO, 2016). Larval survey is a systematically searched for water holding containers or any newly discovered standing water for the presence of mosquito larvae from the house or premise. It is an essential part of any effective Integrated Mosquito Management programme because, when mosquitoes are eliminated before becoming adults, they can no longer be a nuisance or a health risk (Beyond Pesticides/NCAMP, 2002).

The dengue virus (DENV) is a member of the flaviviridae family of enveloped, positive-strand RNA viruses. Other viruses that are in the same family are West Nile virus, yellow fever virus, Japanese encephalitis virus, hepatitis C virus and tick-borne encephalitis virus. Flaviviruses are transmitted to humans by arthropod vectors such as mosquitoes or ticks. Dengue viruses are transmitted to humans by the bites of infectious female *Aedes* mosquitoes. Dengue infections are caused by four closely related viruses namely DENV-1, DENV-2, DENV-3 and DENV-4. All four serotypes are known to cause varying degrees of clinical presentation, such as asymptomatic infections, undifferentiated fever, the classic DF (an acute febrile illness accompanied by headache, body aches, and rash), DHF (high fever, hemorrhage phenomena, and frequently hepatomegaly and circulatory failure), and DSS (rapid, weak pulse with narrowing of the pulse pressure (⇕20mmHg (2.7kPa)), regardless of pressure (WHO, 1943; WHO, 2009; Vijay et al., 2022). The clinical and epidemiological association profiles of each dengue serotype have been thoroughly investigated in previous studies (Yung et al., 2015; Pooja Rao et al., 2020; Gupta et al., 2021). Sequencing of dengue viral RNA has further confirmed strain diversity within a serotype, enabling DENVses to be classified into genetically distinct groupings within serotypes known as genotypes (Fatima et al., 2011). Thus, this study aims to characterize the genotypic

Quick Response Code	Access this article online	
	Website: www.jscienceheritage.com	DOI: 10.26480/gws.01.2023.09.17

Cite The Article: Wan Najdah Wan Mohamad Ali, Zurainee Mohamed Nor, Rafidah Ali, Rohani Ahmad, Yvonne Ai-Lian Lim (2023). Genotypic Diversity of Dengue Virus in Aedes Mosquitoes Collected from Dengue Hotspot Areas in Kuala Lumpur and Selangor, Malaysia. *Science Heritage Journal, 7(1): 09-17.*

diversity of DENV isolated from DENV-infected *Aedes* collected from dengue hotspot outbreak areas in Kuala Lumpur and Selangor.

2. Materials and Methods

2.1 Mosquito sampling

Larvae surveys were conducted between January 2017 until December 2018 in 132 dengue hotspot localities across Kuala Lumpur and Selangor. The selection of the localities is based on constant occurrence of dengue cases obtained from idengue web (http://idengue.arsm.gov.my/) published by Vector Borne Disease Control Programme, Ministry of Health Malaysia. Figure 1 shows the map of the selected localities. All larvae collected were transported to the insectarium of Institute for Medical Research (IMR), Kuala Lumpur. The larvae were colonized at 28°C and 80% relative humidity. Partially cook liver was served to the larvae. The pupated larvae were collected using a pipette and transferred into a small plastic container before placed in the cage for emerging. Ten percent glucose supplemented with 1% vitamin B complex was supplied to the newly emerged adult mosquitoes. The adults were then identified using standard taxonomic keys (Jeffery et al., 2012). *Ae. aegypti* and *Ae. albopictus* were pooled separately according to habitat type and locality with maximum 20 adults per pool and then stored at -80ºC fridge until further use.

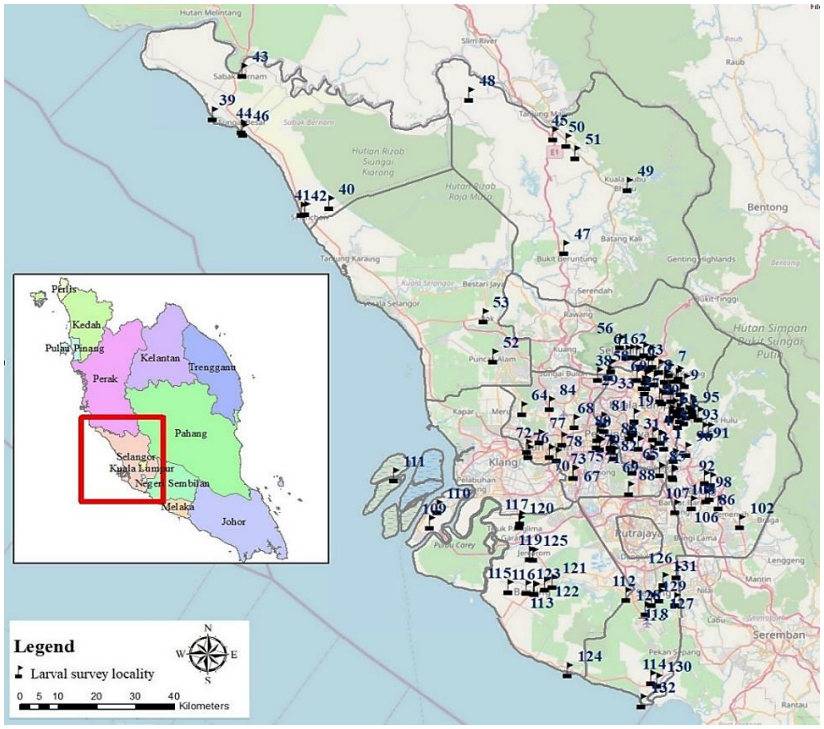

Figure 1: Map of the 132 larval survey study localities in Kuala Lumpur and Selangor

2.2 Detection of DENV

The previously pooled adult mosquitoes were transferred into a nuclease-free 1.5 ml micro centrifuge tube. QIAmp Viral RNA Mini Kit (Qiagen) was used to extract the viral RNA from the mosquito homogenates following the manufacturer's guidelines. Extracted RNA was kept at -80 °C until used to detect transovarial transmission in dengue vectors. Transovarial transmission is basically passing of DENVs to the offspring through the eggs of infected female mosquitoes. Two stages of experimental detection methods were involved. The first method consists of amplification of reverse transcriptase-polymerase chain reaction (RT-PCR) with dengue universal primers (D1 and D2), and the second amplification was semi-nested PCR using 4 separate second amplifications with independent pairs of primers (TS1, TS2, TS3 and TS4) for each DENV serotype.

The protocols and primers were described by with slight modification. Each reaction contained 25.0 µl of 2X MyTaq One-Step Mix (Bioline), 14.5 µl of nuclease-free water, 2 µl of 1mM each universal dengue primer, 0.5 µl of reverse transciptase, 1.0 µl of RNase Inhibitor and 5 µl of RNA template (Lanciotti et al., 1992). The reaction was carried out at 51 °C for 30 minutes to create cDNA, which was then amplified by the following PCR steps: initial denaturation at 92 °C for three minutes, 41 cycles of 92 °C for 30 seconds, 51 °C for 45 seconds and 72 °C for one minute; followed by 72 °C for five minutes. For every RT-PCR run, a positive control (Lab strain *Aedes* mosquito artificially infected with dengue) and negative control (uninfected lab strain *Aedes* mosquito) were included. PCR products were analyzed by performing electrophoresis in 2.0 % agarose gel (FC Bio, USA) at 100 volts and staining with GelStar™ nucleic acid gel stain (Lonza; Rockland, USA). The gel was viewed under ultraviolet illuminator (Ultra Lum Inc.; California, USA) and the resulting bands were photographed with a Polaroid camera. If the pooled sample consist the DENV c-DNA, 511bp product size could be expected.

For universal detection of dengue virus, DENV positive pools were then proceed for dengue type-specific 1, 2, 3 and 4 detection using semi-nested PCR. Reagent required for the preparation of the mastermix in a final total of 50 µl for second stage of PCR were 25 µl of 2X MyTaq mix (Bioline), 11.0 µl of nuclease-free water, 2 µl of 1mM forward primer (D1), 2 µl of 1mM reverse primer (TS1, TS2, TS3 and TS4) specific dengue primer and 10 µl of RNA template. All PCR products were separated by agarose gel electrophoresis, followed by sequencing the positive PCR products. The expected sizes of the amplified products were 482nt for DENV-1, 119nt for DENV-2, 290nt for DENV-3 and 392nt for DENV-4.

2.3 Phylogenetic analysis

MEGA X was used to perform the phylogenetic analysis, which involve the Maximum Likelihood (ML) method based on the Tamura-Nei model with 1,000 bootstrap resampling (Kumar et al., 2018; Tamura et al., 1992). To determine the genotype and genetic relationship of each strain, the nucleotide sequences of the capsid pre-membrane (CprM) gene were analyzed. Reference sequences of DENV genotypes from different years and geographical regions were included in order to characterise the genotypes of each sequence.

2.4 Map development

The coordinates of habitats that contain mosquito larvae were marked using a hand-held Geographic Positioning System (GPS), [Garmin GPSMAP® 60CSx] and processed with MapSource® software (Garmin Ltd: USA). Basic digital administrative boundaries maps of state of Kuala Lumpur and Selangor were downloaded free from https://gadm.org/download_country_v3.html. Coordinates of habitats containing mosquito larvae were later integrated to a GIS database using software ArcGIS 9.3 (ESRI: CA) to quantify spatial heterogeneity in the associated area. Raster images of the site were overlaid with feature layers and entomological data in a GIS database.

3. Results

3.1 Profile of Aedes populations

A total of 6,892 *Aedes* larvae were collected, of which 4,438 (64.39%) were

Ae. albopictus and 2454 (35.61%) were *Ae. aegypti*. Only two *Aedes* species, *Ae. albopictus* and *Ae. aegypti* were captured during this study. T-test demonstrated significant differences (t=-3.685, p<0.05) between mean of *Ae. aegypti* (18.59±2.36) and *Ae. albopictus* (33.62±3.33) larvae collected from the 132 study localities.

3.2 Distribution of DENV positive pools

This study found that from 303 pools gathered only 51 (16.83%) pools were confirmed positive for DENV. Nineteen of those DENV-positive pools were *Ae. aegypti* pool while the other 32 were *Ae. albopictus* pools. This study also confirmed the presence of three DENV serotypes (DENV-2, DENV-3 and DENV-4) from the 51 DENV-positive pools. From the 19 *Ae. aegypti*- positive pools, four pools were detected positive with DENV-3, seven pools were detected positive with DENV-4. There were several *Ae. aegypti*-positive pools that have mix DENV: Five pools were detected positive for DENV-2 and DENV-3, two pools were positive with DENV-3 and DENV-4, and one pool was positive with DENV-2, DENV-3 and DENV-4 (Table 1).

Table 1: Distribution of 51 DENV-positive serotype pools collected at the 26 localities

Locality number	Locality	tube number & DENV-positive serotype	
		Ae. aegypti	*Ae. albopictus*
1	Angkasa Condominium	ND	LN001/3:DENV-3
6	Blok M Pandan Jaya	ND	LN006/2:DENV-2&3
13	Flat Sect. 1 Wangsa Maju	LN013/4:DENV-4	ND
24	PPR Sri Pantai	ND	LN024/1:DENV-2&3
26	Tmn. Bahagia Kuchai	ND	LN026/1:DENV-3
37	Tmn. Sri Rampai	ND	LN037/2:DENV-3&4
44	Tmn. Berkat Sg. Besar	LN044/3:DENV-3	ND
53	Tmn. Cempaka Sari Ijok	LN053/1:DENV-3	LN053/2, LN053/6:DENV-3
57	Kg. Changkat Greenwood	ND	LN057/5, LN057/6:DENV-3&4
58	Kg. Laksamana Gombak	LN058/4:DENV-3, LN058/12:DENV-2&3	LN058/4:DENV-2&3
60	Selayang Baru	ND	LN060/3:DENV-2&4
68	Apt. Sri Meranti D'mansara	LN068/3:DENV-4	ND
70	Dataran Otomobil	ND	LN070/2:DENV-4
72	Flat Nilam Sari S7 S.Alam	LN072/3:DENV-4	ND
74	Flat Tmn. Dato Harun	LN074/5:DENV-4	LN074/6, LN074/8:DENV-4,
81	SS22 Damansara	ND	LN081/1:DENV-3&4
94	Flat Seri Nilam Ampang	LN094/1:DENV-4	ND
95	Flat Tmn. Dagang Permai	ND	LN095/2:DENV-3&4
98	Tmn. Bkt Mewah Kajang	LN098/2:DENV-2,3&4	ND
104	Tmn. Taming Jaya	LN104/1:DENV-2&3	LN104/3:DENV-2,3&4, LN104/4:DENV-2&4
109	Apt. Samudera Pulau Indah	ND	LN109/11, LN109/17, LN109/18:DENV-2&3, LN109/21, LN109/23:DENV-3&4, LN109/28:DENV-4
113	Tmn. Aman Banting	LN113/3, LN113/5:DENV-3&4,	LN113/9:DENV-4
115	Tmn. Banting Baru	LN115/5:DENV-2&3	LN115/6, LN115/8, LN115/9:DENV-3&4
117	Tmn. Perwira TPG	LN117/1:DENV-3, LN117/6:DENV-2&3	LN117/4:DENV-3&4, LN117/5:DENV-2,3&4
120	Tmn. Seri Medan Jaya	LN120/1:DENV-2&3, LN120/2, LN120/5:DENV-4	LN120/4:DENV-4
124	Tmn. Tanjong Sepat	ND	LN124/5:DENV-2&3

ND: Not detected

As for the 32 *Ae. albopictus*-positive pools, four pools were positive with DENV-3, six pools were positive with DENV-4. Similar to *Ae. aegypti*-positive pools, there were several *Ae. albopictus*-positive pools found with mix DENV: Seven pools were positive with DENV-2 and DENV-3, two pools were with DENV-2 and DENV-4, eleven pools were positive with DENV-3 and DENV-4, and two pools were positive with DENV-2, DENV-3 and DENV-4. Based on DENV serotype sequenced from 51 pools, it was demonstrated that DENV-3 was the highest serotype infecting the *Aedes* mosquito in the dengue outbreak areas studied followed by DENV-4 and DENV-2 being the lowest.

3.3 Distribution map of DENV serotype

Figure 2 shows distribution of DENV serotype detected through the transovarial transmission in Kuala Lumpur and Selangor. The 51 DENV-positive pools were collected from 26 study localities. From the 26-DENV positive localities, four localities (Loc. No: 1, 26, 44, 53) were recorded for having only DENV-3, six localities (Loc. No: 13, 68, 70, 72, 74, 94,) were recorded for having only DENV-4, four localities (Loc. No: 6, 24, 58, 124) were recorded for having DENV-2 and DENV-3, one locality (Loc. No: 60) was recorded for having DENV-2 and DENV-4, five localities (Loc. No: 37, 57, 81, 95, 113) were recorded for having DENV-3 and DENV-4, and six localities (Loc. No: : 98,104, 109, 115, 117, 120) were recorded for having DENV-2, DENV-3 and DENV-4.

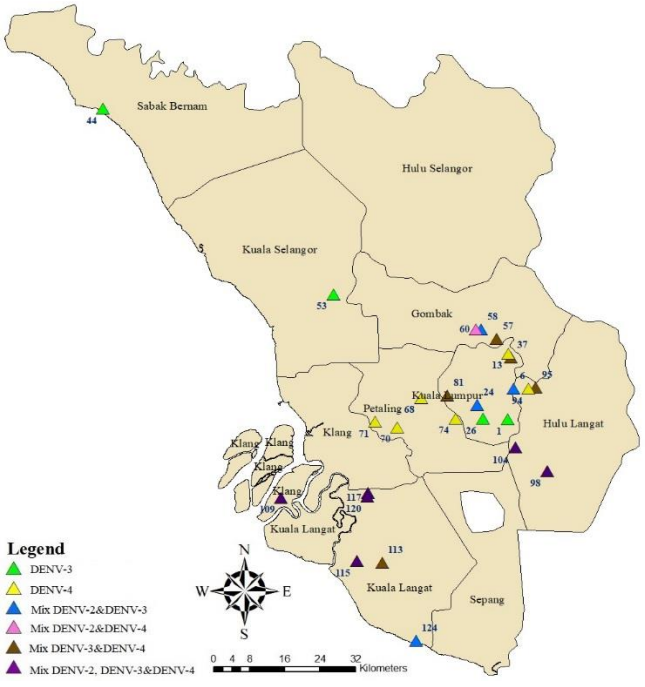

Figure 2: Distribution of DENV-serotype in Kuala Lumpur and Selangor

3.4 Phylogenetic analysis of DENV serotype

From the 19 *Ae. aegypti*-DENV-positive pools, a total of 28 isolates consisting of 6 isolates of DENV-2, 12 isolates of DENV-3 and 10 isolates of DENV-4 were detected. Meanwhile, from the 32 *Ae. albopictus*-DENV-positive pools a total of 56 isolates consisting isolates of 11 of DENV-2, 24 isolates of DENV-3 and 21 isolates of DENV-4 were detected. The nucleotide sequence of the C-prM gene junction of the 84 isolates (17 DENV-2, 36 DENV-3 and 31 DENV-4 virus isolates from both *Ae. aegypti* and *Ae. albopictus* pools) were then submitted to GenBank and accession numbers obtained (Table 2).

Table 2: The Genebank accession numbers of the 84 DENV isolates sequenced from 51 pools

Item	Tube number (number of mosquito per pool)	Pool of Aedes species	Gene Bank accession number (C-prM gene)		
			DENV2	DENV3	DENV4
1	LN001/3 (3)	*Ae. albopictus*		MW130091	
2	LN006/2 (9)	*Ae. albopictus*	MW134459	MW130079	
3	LN013/4 (8)	*Ae. aegypti*			MW143269
4	LN024/1 (1)	*Ae. albopictus*	MW134460	MW130092	
5	LN026/1 (3)	*Ae. albopictus*		MW130093	
6	LN037/2 (20)	*Ae. albopictus*		MW130094	MW143270
7	LN044/3 (11)	*Ae. aegypti*		MW130095	
8	LN053/1 (4)	*Ae. aegypti*		MW130096	
9	LN053/2 (2)	*Ae. albopictus*		MW130080	
10	LN053/6 (8)	*Ae. albopictus*		MW130081	
11	LN057/5 (20)	*Ae. albopictus*		MW130097	MW143271
12	LN057/6 (5)	*Ae. albopictus*		MW130098	MW143272
13	LN058/4 (1)	*Ae. aegypti*		MW130099	
14	LN058/7 (1)	*Ae. albopictus*	MW134461	MW130082	
15	LN058/12 (4)	*Ae. aegypti*	MW134462	MW130083	
16	LN060/3 (2)	*Ae. albopictus*	MW134463		MW143273
17	LN068/3 (3)	*Ae. aegypti*			MW143274
18	LN070/2 (6)	*Ae. albopictus*			MW143275
19	LN072/3 (2)	*Ae. aegypti*			MW143276
20	LN074/5 (1)	*Ae. aegypti*			MW143277
21	LN074/6 (20)	*Ae. albopictus*			MW143278
22	LN074/8 (15)	*Ae. albopictus*			MW143279
23	LN081/1 (1)	*Ae. albopictus*		MW130084	MW143280
24	LN094/1 (16)	*Ae. aegypti*			MW143281
25	LN095/2 (20)	*Ae. albopictus*		MW130085	MW143282
26	LN098/2 (20)	*Ae. aegypti*	MW134464	MW130086	MW143283
27	LN104/1 (1)	*Ae. aegypti*	MW134465	MW130100	
28	LN104/3 (20)	*Ae. albopictus*	MW134466	MW130101	MW143284
29	LN104/4 (20)	*Ae. albopictus*	MW134467		MW143285
30	LN109/11 (4)	*Ae. albopictus*	MW134468	MW130102	
31	LN109/17 (2)	*Ae. albopictus*	MW134469	MW130103	
32	LN109/18 (4)	*Ae. albopictus*	MW134470	MW130104	
33	LN109/21 (20)	*Ae. albopictus*		MW130105	MW143286
34	LN109/23 (10)	*Ae. albopictus*		MW130106	MW143287
35	LN109/28 (3)	*Ae. albopictus*			MW143288
36	LN113/3 (6)	*Ae. aegypti*			MW143289
37	LN113/5 (11)	*Ae. aegypti*		MW130107	MW143290
38	LN113/9 (7)	*Ae. albopictus*		MW130108	MW143291
39	LN115/5 (2)	*Ae. aegypti*	MW134471	MW130109	
40	LN115/6 (3)	*Ae. albopictus*		MW130110	MW143292
41	LN115/8 (11)	*Ae. albopictus*		MW130111	MW143293
42	LN115/9 (4)	*Ae. albopictus*		MW130112	MW143294
43	LN117/1 (3)	*Ae. aegypti*		MW130113	
44	LN117/4 (25)	*Ae. albopictus*		MW130114	MW143295
45	LN117/5 (10)	*Ae. albopictus*	MW134472	MW130115	MW143296
46	LN117/6 (1)	*Ae. aegypti*	MW134473	MW130116	
47	LN120/1 (1)	*Ae. aegypti*	MW134474	MW130117	
48	LN120/2 (5)	*Ae. aegypti*			MW143297
49	LN120/4 (8)	*Ae. albopictus*			MW143298
50	LN120/5 (3)	*Ae. aegypti*			MW143299
51	LN124/5 (3)	*Ae. albopictus*	MW134475	MW130118	

The evolutionary history of DENV was inferred using the Maximum Likehood method. All of the sequences were analyzed and aligned with 32 DENV C-prM region sequences previously published globally. One Japanese encephalitis reference sequence was used as the out group. Figure 3 shows the combined phylogenetic tree of all DENV serotypes along with the reference sequences. Phylogenetic tree confirmed that the 84 isolated DENV comprises of DENV-2, DENV-3 and DENV-4.

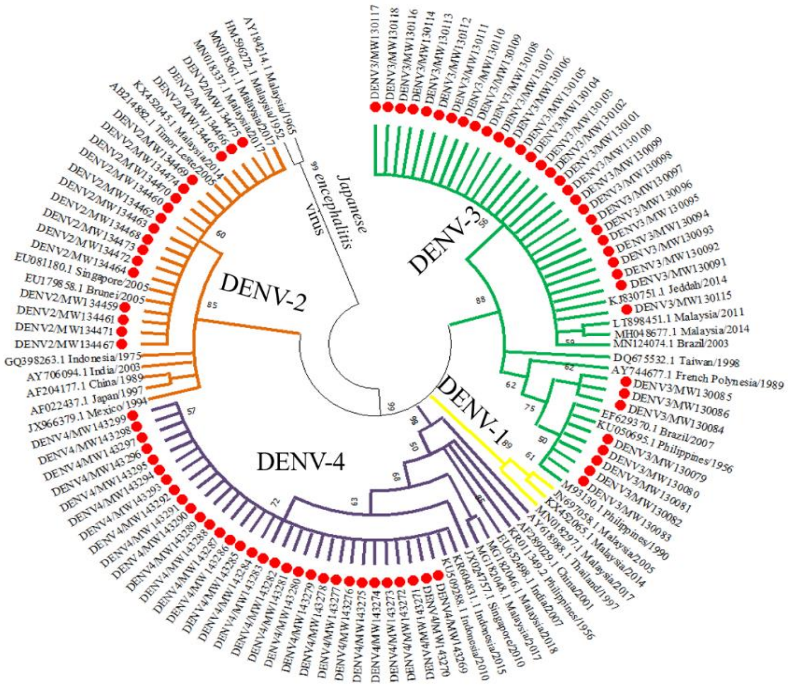

Figure 3: Combined phylogenetic tree of four DENV serotypes constructed by the Maximum-Likelihood (ML) method using Kimura-2-parameter model using 1000 bootstrap replicates, based on consensus 511 bp of C-prM gene junction. Strains in the trees are shown by their GenBank accession number, country of origin and isolated year. All the isolated DENV strains during this study are shown with red circle symbol. The tree was rooted with *Japanese encephalitis* virus from Malaysia as the out-group

3.5 Phylogenetic analysis of DENV-2

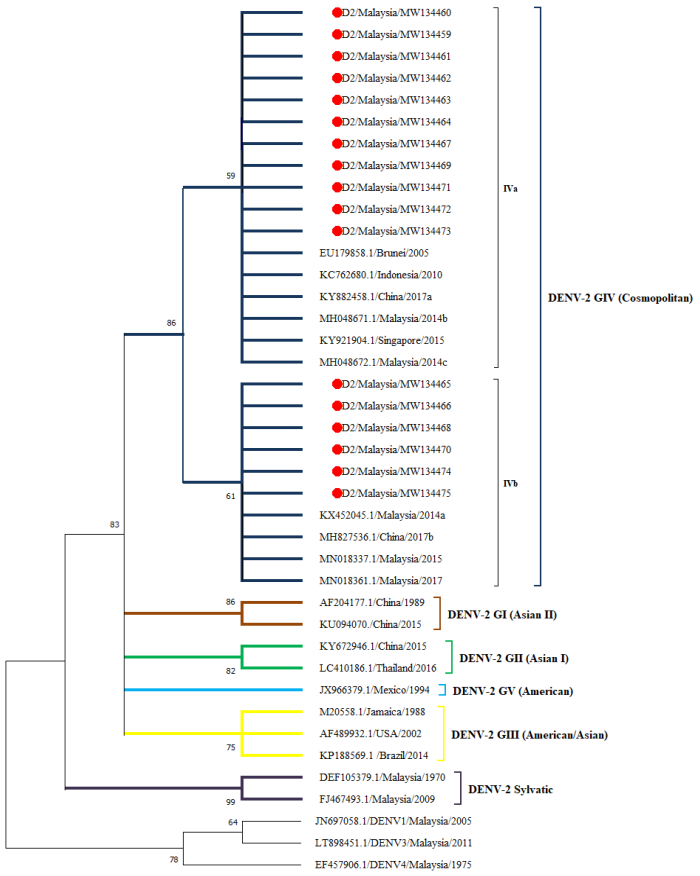

Figure 4: Phylogenetic tree of DENV-2. The tree was generated based on 119 bp region of the CprM gene junction. Strains in the tree are shown by their GenBank accession number, country of origin and isolated year. The DENV strains sequenced in this study are indicated by red circle and labelled with Genbank accession numbers. Bootstrap values (>50) are indicated at the major branch points. The tree was rooted with DENV-1, DENV-3 and DENV-4 from Malaysia as the out-group

All the 17 DENV-2 isolated sequences were compared and aligned with 20 previously DENV-2 genotypes sequences reported globally as references using a 119bp (nt 134-253) region of the C-prM gene. The sequences reference included were five from China (AF204177.1/1989, KU094070.1/2015, KY672946/2015, KY882458.1/2017a and MH827536.1/2017b), seven from Malaysia (EF105379.1/1970,

FJ467493.1/2009, KX452045.1/2014a, MH048671.1/2014b, MH048672.1/2014c, MN018337.1/2015 and MN018361.1/2017), and one sequence each from USA (AF489932.1/2002), Brunei (EU179858.1/2005), Mexico (JX966379.1/1994), Indonesia (KC762680.1/2010), Brazil (KP188569.1/2014), Singapore (KY921904/2015), Thailand (LC410186.1/2016) and Jamaica (M20558.1/1988).

Figure 4 shows that all the 17 DENV-2 isolate sequences in this study (MW134459, MW134460, MW134461, MW134462, MW134463, MW134464, MW134465, MW134466, MW134467, MW134468, MW134469, MW134470, MW134471, MW134472, MW134473, MW134474, and MW134475) belonged to genotype IV (GIV: 'cosmopolitan' genotype) and they are branched into two sub-clades (IVa and IVb).

Eleven DENV-2 isolated strains (MW134459, MW134460, MW134461, MW134462, MW134463, MW134464, MW134467, MW134469, MW134471, MW134472, MW134473) formed sub-clade IVa with reference sequences in GIV strains from Brunei/2005, China/2017a, Indonesia/2010, Malaysia/2014b, Malaysia/2014c, and Singapore/2015 with 59 bootstrap value. And the rest of DENV-2 isolate strains (MW134465, MW134466, MW134468, MW134470, MW134474, MW134475) formed sub-clades IVb with reference sequences in GIV strains from China/2017b, Malaysia/2014a, Malaysia/2015 and Malaysia/2017 with 61 bootstrap value.

3.6 Phylogenetic analysis of DENV-3

All the 36 DENV-3 isolated sequences were compared and aligned with 20 previously DENV-3 genotypes sequences reported globally as references using a 290bp (nt 132-421) region of the C-prM gene. The sequence references included were six from Malaysia (AB010990.1/1998, LT898451.1/2011, LT996904.1/2007, MH048677.1/2014a, MH051731/2014b and MH377085.2/2016), three each from Brazil (EF629370.1/2007a, EF629371.1/2007B and MN124074.1/2003) and Thailand (AY676353.1/1987, AY912458.1/1998 and MK506265.1/2007), two each from Singapore (AY662691.1/2004 and EU081192.1/2005), and Philippines (KU050695.1/1956 and M93130.1/1990) and one sequence each from Indonesia (AB189128.1/1998), French Polynesia (AY744677.1/1989), Saudi Arabia (KJ830751.1/2014), and India (MH734371.1/2007).

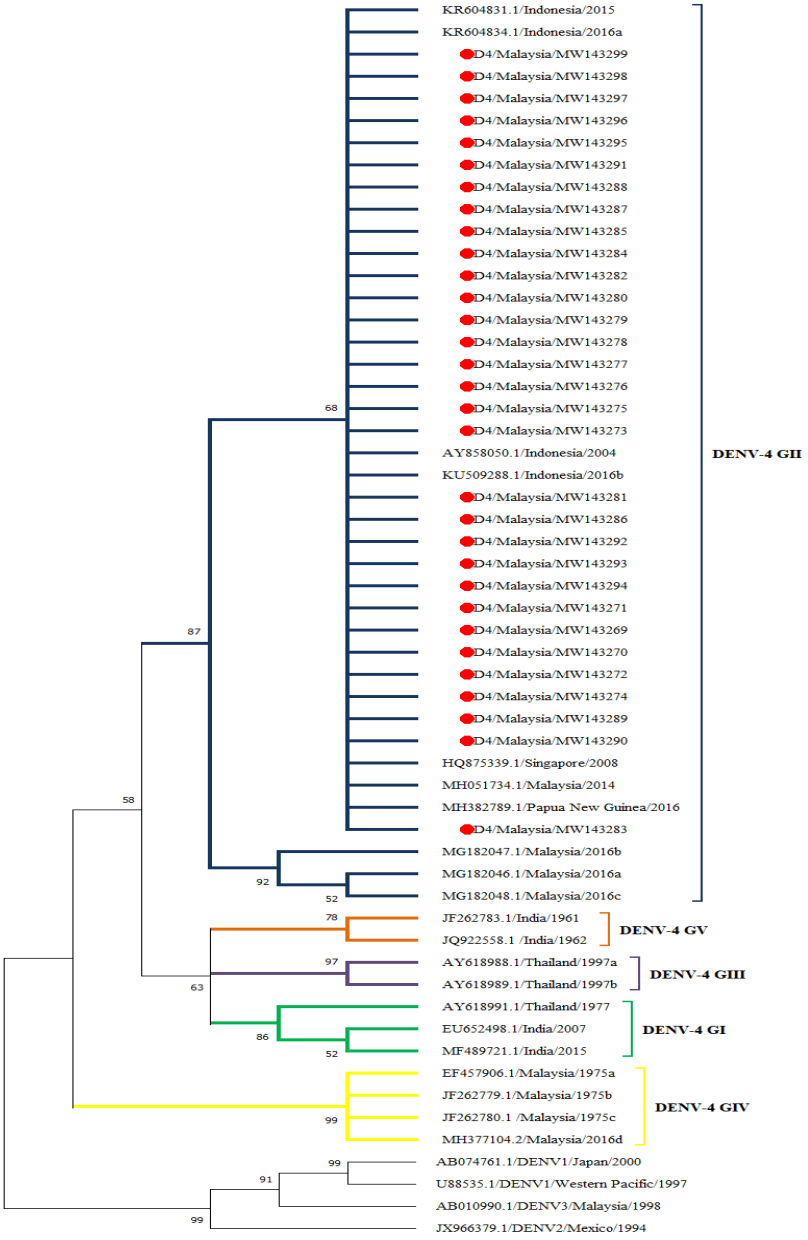

Figure 5: Phylogenetic tree of DENV-4. The tree was generated based on 389bp region of the CprM gene junction. Strains in the trees are shown by their GenBank accession number, country of origin and isolated year. The DENV strains sequenced in this study are indicated by red circle and labelled with Genbank accession number. Bootstrap values (>50) are indicated at the major branch points. The tree was rooted with DENV-1 from Japan and Western Pacific, and DENV-2 and DENV-3 from Malaysia as the out-group

Figure 5 shows that 28 of DENV-3 isolated sequences in this study (MW130091, MW130092, MW130093, MW130094, MW130095, MW130096, MW130097, MW130098, MW130099, MW130100, MW130101, MW130102, MW130103, MW130104, MW130105,

MW130106, MW130107, MW130108, MW130109, MW130110, MW130111, MW130112, MW130113, MW130114, MW130115, MW130116, MW130117 and MW130118) belonged to genotype III (GIII) and the other eight DENV-3 isolated strains (MW130079, MW130080, MW130081, MW130082, MW130083, MW130084, MW130085 and MW130086) belonged to genotype V (GV).

All the 28 DENV-3 GIII isolated during this study formed sub-clade with reference sequences from Malaysia/2007, India/2007 and Saudi Arabia/2014 with 50 bootstrap value. And the rest 8 of DENV-3 GV isolated strains were closed to reference sequences in GV strains from Brazil/2007a, Brazil/2007b, Philippines/1956, Philippines/1990, Thailand/ 2007 and Malaysia/2016 with 81 bootstrap value.

3.7 Phylogenetic analysis of DENV-4

All the 31 DENV-4 isolated sequences were compared and aligned with 21 previously DENV-4 genotype sequences reported globally as references using a 389bp (nt 137-536) region of the C-prM gene. The sequence references included were eight from Malaysia (EF457906.1/1975a, JF262779.1/1975b, JF262780.1/1975C, MG182046.1/2016a, MG182047.1/2016b, MG182048.1/2016c, MH051734.1/ 2014 and MH377104.2/2016), four from Indonesia (AY858050.1/2004, KR604831.1/2015, KR604834.1/2016a and KU509288/2016b), three each from Thailand (AY618988.1/1997a, AY618989.1/1997b and AY618991.1/1977) and India (EU652498.1/2007, JF262783.1/1961 and JQ922558.1/1962) and one sequence each from Singapore (HQ875339.1/2008) and Papua New Guinea (MH382789.1/2016).

Figure 6 shows that all 31 of DENV-4 isolated sequences in this study (MW143269, MW143270, MW143271, MW143272, MW143273, MW143274, MW143275, MW143276, MW143277, MW143278, MW143279, MW143280, MW143281, MW143282, MW143283, MW143284, MW143285, MW143286, MW143287, MW143288, MW143289, MW143290, MW143291, MW143292, MW143293, MW143294, MW143295, MW143296, MW143297, MW143298 and MW143299) belonged to genotype II (GII). All the 31 DENV-4 GII isolated during this study were closed to the reference sequences from Malaysia/2014, Indonesia/2004, Indonesia/2015, Indonesia/2016a, Indonesia/2016b, Singapore 2008 and Papua New Guinea/2016 with 68 bootstrap value.

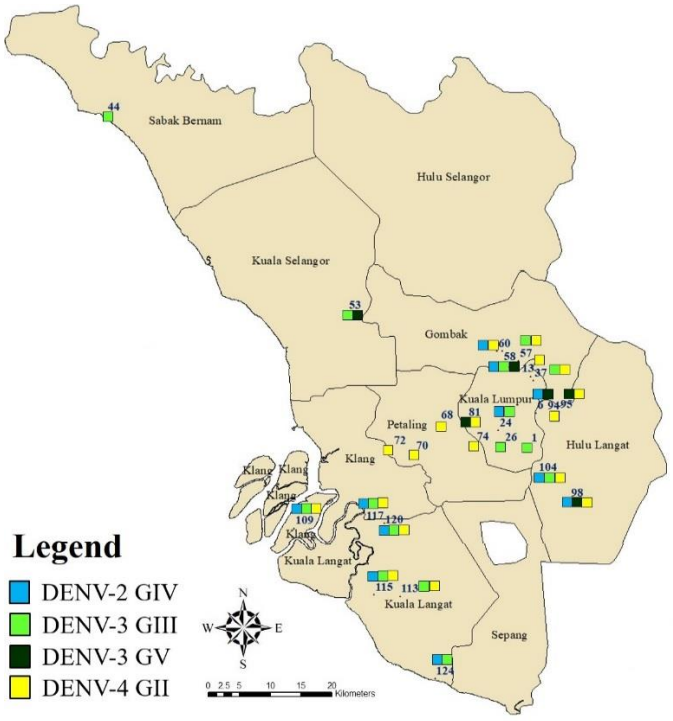

Figure 6: Distribution of the DENV genotype in Kuala Lumpur and Selangor

3.8 Distribution of the DENV genotype

Figure 6 illustrates the distribution of DENV-2, DENV-3 and DENV-4 genotypes isolated in this study. DENV-2 GIV was isolated from 11 study localities (Loc. No: 6, 13, 58, 60, 98, 104, 109, 115, 117, 120 and 124); DENV-3 GIII was isolated from 15 study localities (Loc. No: 1, 24, 26, 37, 44, 53, 57, 58, 104, 109, 113, 115, 117, 120 and 124); DENV-3 GV was isolated from 6 study localities (Loc. No: 6, 53, 58, 81, 95 and 98), and DENV-4 GII was isolated from 18 study localities (Loc. No: 13, 37, 57, 60, 68, 70, 72, 74, 81, 94, 95, 98, 104, 109, 113, 115, 117 and 120). Detection of two genotypes of DENV-3 (GIII and GV) at Loc. No 53 and 58 were also demonstrated in this study.

4. Discussion

This study reports the detection of three DENV serotypes amongst field collected *Ae. aegypti* and *Ae. albopictus* mosquitoes in dengue hotspot areas in Kuala Lumpur and Selangor, Malaysia. Although previous studies demonstrated that co-circulation of all 4 DENV serotypes have been demonstrated in Kuala Lumpur and Selangor, this current study however detected only 3 serotypes (DENV-2, DENV-3, and DENV-4) (Chew et al., 2012; Noraishah et al., 2018; Suppiah et al., 2018; Johari et al., 2019). In addition, DENV-3 was recorded as the highest DENV isolated from the infected *Aedes* mosquito followed by the DENV-4 and DENV-2. This finding is in contrast to study by who found that DENV-2 was the most frequent serotype. Their study however was based on human cases (Kalayanarooj and Nimmannitya, 2000).

According to who also reported the presence of all four serotypes in Malaysia, dominance by any one serotype was never detected for the period between 1971 to 1985 (Abu Bakar and Shafee, 2002). Switching of DENV serotype however was later observed whenever there was an outbreak of dengue cases (Suppiah et al., 2018; Johari et al., 2019; Kalayanarooj and Nimmannitya, 2000; Abu Bakar and Shafee, 2002). In 1986 it was reported that DENV-3 has becoming the dominant serotype in Malaysia (Abu Bakar and Shafee, 2002). Meanwhile reported that DENV-1 was the predominant serotype in Selangor for three years in a row from 2013 to 2015 (Noraishah et al., 2018). She in fact predicted that the dominance of DENV-1 would be replaced by DENV-3 for at least two years based on dengue trends in Selangor at that time (Noraishah et al., 2018; Suppiah et al., 2018). This current study indirectly proves that her prediction is correct.

Apart from co-circulation of serotypes, this study also captured the presence of co-infection of dengue serotypes in mosquito in the area. Co-infection by several dengue serotypes in a mosquito is not uncommon. For example, co-infection of DENV-1 and DENV-2 was observed in 6.7% of the *Ae. aegypti* larvae evaluated in Brazil (Costa et al., 2017). More than one DENV serotype was present in 78.7% of larval collected from 31 disease endemic district towns of Rajasthan state, in India (Angel et al., 2015). Two serotypes of dengue viruses (DENV-2 and DENV-3) were also detected in *Ae. aegypti* and *Ae. albopictus* in Thailand (Thavara et al., 2006). Co-infection between DENV-2 and DENV-4 was also observed within lab strain of *Ae. aegypti* mosquitoes (Muturi et al.,2017).

This current study showed that out of 17% (51/303) pools positive for DENV, 41% (21/51) of them demonstrated single serotype infection and the other 59% (30/51) indicated infection by more than one serotypes. Given that only six out of that 30 pools had mix serotypes detected in the same mosquito, indicate that only 20% (6/30) of pools with more than one serotypes demonstrated co-infection while the other 80% (24/30) of pools showed co-circulation of DENV. Co-infection of the DENV serotypes is said to be driven by the massive multiple co-circulation of four dengue serotypes in hyperendemic areas (Chew et al., 2012; Senaratne et el.,2020). Thus it proves that co-circulation of co-infection DENV serotypes in mosquitos in this study will be linked to human co-infection. As a result, the incidence of concurrent infections is growing, and this might become an alarming system for dengue outbreaks.

Phylogenetic study conducted on 51-DENV positive pools confirmed the presence of 84 DENV isolates that consist of 17 isolates of DENV-2, 36 isolates of DENV-3 and 31 isolates of DENV-4. All 17 DENV-2 isolates from this study were of genotype IV (Cosmopolitan). Cosmopolitan genotype is probably quite common in Klang (ie. Kuala Lumpur, Selangor, and Putrajaya) valley as it was continuously detected in several studies reported from 1989 to 2017 (Chee et al., 2003; Chew et al., 2015). Therefore, not surprising when it was also detected in this current study.

Thirty-six isolates of DENV-3 in this study were of two genotypes: genotype III (GIII) and genotype V (GV). DENV-3 GIII was previously documented in Malaysia from 2007 to 2017(Tan et al.,2017). DENV-3 GIII, on the other hand, has been reported as being spread to Malaysia from nearby regions over the last three decades (Tan et al., 2018). Meanwhile, DENV-3 GV was reported only found in a few older strains (Arauji et al., 2009). Interestingly, all DENV-3 GV in this current study were found similar to the oldest strain reported in Philippines in 1956 and in Brazil in 2007 (Arauji et al., 2009). Nonetheless, the DENV-3 GV determined in this current study were also found close to previous DENV-3 GV from *Ae.*

aegypti in Selangor in 2016 and has been registered in Genbank under the reference number MH377085.2/Malaysia (Johari et al., 2019). Both genotypes of DENV-3 in this study were found co-circulated in Selangor only.

It was interesting to note that both genotypes of DENV-3 (genotype GIII and GV) were found co-circulating at the same two localities (Loc. No: 53 and 58). As genetic variations co-circulate in the same place, dengue epidemics have been reported to increase in number and severity (Ahamed et al.,2019). Clearly, this information is useful because it can be used as an indicator of possible dengue outbreak with increasing magnitude and severity. Thirty one strains of DENV-4 determined were all of genotype II (GII). Despite being identified as a rare serotype in Malaysia, DENV-4 serotype was reported as the dominant DENV serotype in two states namely Kelantan and Sabah and as predominant DENV serotype in the states of Pahang (Suppiah et al., 2008; Ng et al., 2015). The DENV-4 GII strain determined in this current study were found close to previous DENV-4 GII isolated from dengue patients in Selangor in 2014 and has been registered in GenBank with a reference number MH051734.1.

The categorization of dengue genotypes is based on nucleotide divergence of 6% to 8% within a chosen genomic area (Rico-Hesse, 1990; Goncalvez et al., 2002; Weaver and Vasilakis, 2009; Dey et al., 2015). Previous research has revealed that genotypes variation influences illness severity, the likelihood of an epidemic, and the capacity of dengue virus serotypes to function as vectors (Rico-Hesse, 2010; OhAinle et al., 2011; Aguas et al., 2019).

Findings from this study assists the stakeholders by strengthening dengue genotyping surveillance and providing a solid evidence base for decision making regarding dengue management initiatives. Evidences from this study can be utilized to the search for novel genotypes, to the observation of potential genetic alterations in dengue viruses, and to the development of vaccines.

5. Conclusion

This study conducted in 132 dengue hotspot areas in Kuala Lumpur and Selangor, Malaysia between January 2017 until December 2018 demonstrated the presence of only 3 DENV serotypes (DENV-2, DENV-3, and DENV-4). Out of the three serotypes, 84 DENV-positive isolates were DENV-2 serotype, all 17 isolates were of GIV genotype, from DENV-3 serotype, 28 isolates were of GIII genotype and 8 isolates were of GV genotype while from DENV-4 serotype, all 31 isolates were of GII serotypes. As they were discovered in only about 20% of the study region (26/132 hotspot localities) by transovarial transmission by *Ae. aegypti* and *Ae. albopictus*, transmission of the remaining localities is expected to occur vertically. Thus, further studies to identify the main mechanisms for virus transmission either vertical or horizontal for repeated hotspot dengue outbreak areas are suggested.

This study however was not able to carry out a detailed evaluation with regards to relationship between the different isolates since sequences available in this study were obtained only from the CprM region of the genome. Any attempt to determine the origins and/or pathogenicity of the virus requires full sequence of the genotype. Maps developed not only allowed the distribution of DENV serotypes and genotypes co-circulation of hotspot areas to be visualized clearly but help strengthen the dengue information system and facilitate the planning of more accurate and focused control programmes and more effective dengue surveillance by the local health authority.

Acknowledgements

The authors are grateful to the Director-General of Health, Malaysia for permission to publish this paper. We especially thanked the staff of Medical Entomology Unit of IMR without whose diligence and hard work under difficult field conditions, this research would not have been accomplished. This study was conducted under grant NIH-866334-8009(b).

References

Abu, B.S., and Shafee N., 2002. Outlook of dengue in Malaysia: a century later. Malaysian J. Pathol, 24 (1), Pp. 23–27.

Aguas, R., Dorigatti, I., Coudeville, L., Luxemburger, C., Ferguson, N.M., 2019. Cross-serotype interactions and disease outcome prediction of dengue infections in Vietnam. Sci Rep., 9 (1), Pp. 9395. doi:10.1038/s41598-019-45816-6

Ahamed, S.F., Vivek, R., Britto, C., 2019. Emergence of new genotypes and lineages of dengue viruses during the 2012–15 epidemics in southern India. International Journal of Infectious Diseases, 84S, Pp. S34–S43.

Angel, B., Angel, A., Josh, V., 2015. Multiple dengue virus types harbored by individual mosquitoes. Acta Tropica., 150, Pp. 107–110.

Araújo, J.M.G.D., Bello, G., Schatzmyr, H.G., Santos, F.B.D., Nogueira, R.M.R., 2009. Dengue virus type 3 in Brazil: a phylogenetic perspective. Mem Inst Oswaldo Cruz, Rio de Janeiro, 104 (3), Pp. 526-529.

Chew, M.H., Rahman, M.M., Jelip, J., Hassan, M.R., Isahak, I., 2012. All serotypes of dengue viruses circulating in Kuala Lumpur, Malaysia. Current Research Journal of Biological Sciences, 4 (2), Pp. 229-234.

Chew, M.H., Rahman, M.M., Hussin, S., 2015. Molecular epidemiology and phylogenetic analysis of Dengue virus type-1 and 2 isolated in Malaysia. Pakistan Journal of Medical Science, 31 (3), Pp. 615–620.

Chee, H.Y., Abu, B.S., 2003. Phylogenetic investigation of dengue virus type 2 isolated in Malaysia. Dengue Bulletin, 27, Pp. 100-107.

Costa, C.F.D., Passos, R.A.D., Lima, J.B.P., 2017. Transovarial transmission of DENV in Aedes aegypti in the Amazon basin: a local model of xenomonitoring. Parasites & Vectors, 10, Pp. 249. doi:10.1186/s13071-017-2194-5

Dey, S., Nandy, A., Nandy, P., Das, S., 2015. Diversity and evolution of the envelope gene of dengue virus type 1 circulating in India in recent times. Int J. Bioinform Res Appl., 11 (6), Pp. 469-482. doi:10.1504/ijbra.2015.073235

Fatima, Z., Idrees, M., Bajwa, M.A., 2011. Serotype and genotype analysis of dengue virus by sequencing followed by phylogenetic analysis using samples from three mini outbreaks-2007-2009 in Pakistan. BMC Microbiol., 11, Pp. 200. doi:10.1186/1471-2180-11-200

Goncalvez, A.P., Escalante, A.A., Pujol, F.H., 2002. Diversity and evolution of the envelope gene of dengue virus type 1. Virology, 303 (1), Pp. 110-119. doi:10.1006/viro.2002.1686

Gupta, A., Rijhwani, P., Pahadia, M.R., 2021. Prevalence of Dengue Serotypes and its Correlation with the Laboratory Profile at a Tertiary Care Hospital in Northwestern India. Cureus, 13 (5), Pp. e15029. doi:10.7759/cureus.15029

Johari, N.A., Voon, K., Toh, S.Y., Sulaiman, L.H., Yap, I.K.S., Lim, P.K.C., 2019. Sylvatic dengue virus type 4 in Aedes aegypti and Aedes albopictus mosquitoes in an urban setting in Peninsular Malaysia. PLoS Negl Trop Dis., 13 (11), Pp. e0007889.

Jeffery, J., Rohela, M., Muslimin, M., Abdul, Aziz, S.M.N., Jamaiah, I., Kumar, S., Tan, T.C., Lim, Y.A.L., Nissapatorn, V., Abdul-Aziz, N.M., 2012. Illustrated keys: some mosquitoes of peninsular Malaysia. Malaysia: University of Malaya Press, Pp. 87.

Kalayanarooj, S., and Nimmanitya, S., 2000. Clinical and laboratory presentations of dengue patients with different serotypes. Dengue Bulletin, 24, Pp. 53-59.

Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: Molecular Evolutionary Genetics Analysis across computing platforms. Molecular Biology and Evolution, 35, Pp. 1547-1549.

Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J., Vordam, A.V., 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. J. Clin. Microbial., 30, Pp. 545-551.

Muturi, E.J., Buckner, E., Bara, J., 2017. Superinfection interference between dengue-2 and dengue-4 viruses in Aedes aegypti mosquitoes. Tropical Medicine and International Health, 22 (4), Pp. 399-406.

Noraishah, M.S., Syed Sharizman, S.A.R., Faridah, A., 2018. Trend of dengue virus serotype in Selangor, Malaysia: a descriptive study. Journal of Biostatistics, 15 (2), Pp. 127-138.

Ng, L.C., Yu-kie, C., Koo, C., 2013. Dengue Outbreaks in Singapore and Malaysia caused by different viral strains. Am. J. Trop. Med. Hyg., 92 (6), Pp. 1150–1155.

OhAinle, M., Balmaseda, A., Macalalad, A.R., 2011. Dynamics of dengue disease severity determined by the interplay between viral genetics and serotype-specific immunity. Sci Transl Med., 3 (114), Pp. 114ra128. doi:10.1126/scitranslmed.3003084

Paramaesvaran, N., 1966. Haemorrhagic fever in children in Penang: A report on 41 cases. Bulletin of the World Health Organization, 35 (1), Pp. 40.

Pooja, R., Basavaprabhu, A., Shenoy, S., Dsouza, N.V., Sridevi, Hanaganahalli, B., Kulkarni, V., 2020. Correlation of Clinical Severity and Laboratory Parameters with Various Serotypes in Dengue Virus: A Hospital-Based Study. Int J Microbiol., Pp. 6658445. doi:10.1155/2020/6658445

Rico-Hesse, R., 2010. Dengue virus virulence and transmission determinants. Curr Top Microbiol Immunol., 338, Pp. 45-55. doi:10.1007/978-3-642-02215-9_4

Rudnick, A., Tan, E.E., Lucas, J.K., Path, M.C., Omar, M.B., 1965. Mosquito-borne Haemorrhagic Fever in Malaya. British Medical Journal, 1, Pp. 1269-1272.

Smith, E.G., 1956. Isolation of three strain of type 1 dengue virus from a local outbreak of the disease in Malaya. Journal of Hygiene, 54 (4), Pp. 569-580.

Shepard, D.S., Lees, R., Ng, C.W., Undurraga, E., Halasa, Y., Lum, L., 2013. Burden of Dengue in Malaysia. In Report from a Collaboration between Universities and the Ministry of Health of Malaysia. Putrajaya: Malaysia.

Senaratne, U.T.N., Murugananthan, K., Sirisena, P.D.N.N., Carr, J.M., Noordeen, F., 2020. Dengue virus co-infections with multiple serotypes do not result in a different clinical outcome compared to mono-infections. Epidemiology and Infection, 148, Pp. e119, 1–9.

Suppiah, J., Ching, S.M., Amin-Nordin, S., 2018. Clinical manifestations of dengue in relation to dengue serotype and genotype in Malaysia: A retrospective observational study. PLoS Negl Trop Dis., 12 (9), Pp. e0006817.

Tamura, K., 1992. Estimation of the number of nucleotide substitutions when there are strong transition-transversion and G+C content biases. Mol. Biol. Evol., 9, Pp. 678–687.

Tan, K.K., Nurul-Izzani, Z., Juraina, A.J., 2017. Disruption of predicted dengue virus type 3 major outbreak cycle coincided with switching of the dominant circulating virus genotype. Infection, Genetics and Evolution, 54, Pp. 271–275.

Tan, K.K., Nurul-Izzani, Z., Syuhaida, S., 2018. Emergence of the Asian lineage dengue virus type 3 genotype III in Malaysia. BMC Evolutionary Biology, 18 (58).

Thavara, U., Siriyasatien, P., Tawatsin, A., 2006. Double infection of heteroserotypes of dengue viruses in field populations of Aedes aegypti and Aedes albopictus (diptera: culicidae) and serological features of dengue viruses found in patients in Southern Thailand. Southeast Asian J Trop Med Public Health, 37 (3), Pp. 1-9.

Vijay, J., Anuradha, N., Anbalaganm, V.P., 2022. Clinical Presentation and Platelet Profile of Dengue Fever: A Retrospective Study. Cureus., 14 (8), Pp. e28626. Published 2022 Aug 31. doi:10.7759/cureus.28626

Weaver, S.C., Vasilakis, N., 2009. Molecular evolution of dengue viruses: contributions of phylogenetics to understanding the history and epidemiology of the preeminent arboviral disease. Infect Genet Evol., 9 (4), Pp. 523-540. doi:10.1016/j.meegid.2009.02.003

World Health Organization (WHO). 1943. The clinical diagnosis. Acta Radiologica [Old Series], 24, Pp. 19-22. Retrieved from https://www.who.int/csr/resources/publications/dengue/012-23.pdf

WHO. 2009. Dengue: guidelines for diagnosis, treatment, prevention and control (New edt.). Geneva, Switzerland. 2009. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/44188/9789241547871_eng.pdf?sequence=1&isAllowed=y

WHO. 2012. Global Strategy for Dengue Prevention and Control 2012–2020. Geneva, Switzerland. pp. 43. 2012. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/75303/9789241504034_eng.pdf

WHO. 2016. Vector Surveillance and Control at Ports, Airports, and Ground Crossings. Genava, Switzerland: World Health Organization. 2016. Retrieved from https://apps.who.int/iris/bitstream/handle/10665/204660/9789241549592_eng.pdf;jsessionid=B8A3EC8266A42E751EBC3BE2B6A3A91C?sequence=1

WHO. 2019. Dengue situation updates 2019.

Yung, C.F., Lee, K.S., Thein, T.L., 2015. Dengue serotype-specific differences in clinical manifestation, laboratory parameters and risk of severe disease in adults, singapore. Am J. Trop Med Hyg., 92 (5), Pp. 999-1005. doi:10.4269/ajtmh.14-0628.