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Detection of dengue virus serotype 2 (DENV-2) in population of *Aedes* mosquitoes from Sibu and Miri divisions of Sarawak using reverse transcription polymerase chain reaction (RT-PCR) and semi-nested PCR

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

INTRODUCTION

Dengue has been one of the most important public health concerns worldwide and has caused a significant number of deaths every year. According to the World Health Organization (WHO) (2017), approximately half of the global population is at risk of dengue infection in over 125 endemic countries. Dengue is an acute systemic form of a viral disease which has established itself both epidemically and endemically which can lead to a range of clinical manifestations from mild fevers to severely fatal cases (Bhatt *et al*., 2013). According to the WHO Dengue Guideline (2009), dengue can be classified into two categories: dengue with or without warning signs and severe dengue. A person can have dengue without warning signs if the following criteria are met, such as living in or travelling to dengue endemic areas and having fever followed by nausea, vomiting, rash, and body aches and pain. Patients suffering from dengue with warning signs should have the following criteria: abdominal pain, persistent vomiting, fluid accumulation in the body, mucosal bleeding, lethargy, and blood cell abnormalities such as low platelet counts. Patients having severe plasma leakage, severe bleeding, and severe abnormalities in multiple organ functions are classified as having severe dengue (WHO,

2009). Dengue infection is caused by the dengue virus (DENV), a single-stranded RNA virus belonging to family and genus of *Flaviviridae* and *Flavivirus* respectively, similar to yellow fever virus, West Nile virus, and Zika virus. There are four serotypes of DENV; DENV-1, -2, -3, and -4. However, not all of the dengue virus serotypes can be found in all dengue endemic and epidemic areas. However, some countries like Malaysia are hyperendemic, where all the four DENV serotypes can be found (Cheah *et al.*, 2014; Mohd-Zaki *et al.*, 2014).

DENV is a vector borne virus. The main vector for DENV is the Aedes mosquito, primarily *Aedes aegypti* and *Aedes albopictus*. The Aedes mosquito is classified under the order of Diptera from the family of Culicidae, which mainly consists of mosquitoes (Harbach, 2007). *Ae. aegypti* is one of the main vectors for DENV. *Ae. aegypti* mosquitoes are day feeders and can be found mostly in human dwelling areas, either indoors or outdoors. However, *Ae. aegypti* prefers to blood feed on human indoors (CDC, 2016). Furthermore, it prefers breeding in artificial water containers and in water containing decaying organic materials; preferably dark-coloured containers in shady places (CDC, 2016). *Ae. aegypti* is distributed globally and is believed to have originated from Africa (Powell & Tabachnick, 2013). This species has spread throughout the globe covering most of the tropical and sub-tropical regions, including Malaysia. According to Kraemer *et al.* (2015), it is believed that the increase in transportations, global trades, human travels, and rapid urbanisation have caused the distribution of *Ae. aegypti* to be accelerated.

Ae. albopictus is also a well-known vector for DENV in Asia. It is an indigenous species of Asia, hence nicknamed the Asian Tiger mosquito (Bonizzoni *et al.*, 2013). They feed during the day in outdoor and indoor locations, but are mostly found outdoors. *Ae. albopictus* are strongly attracted to humans, but will also feed on other mammals as well as birds (CDC, 2016). *Ae. albopictus* prefers to breed outdoors mainly in natural water containers such as leaf axils and treeholes. These mosquitoes transmit dengue from humans to humans through blood-feeding, therefore is the vector for the virus. This mode of transmission is called horizontal transmission. Transmission of dengue can also occur in the mosquito population itself, which is known as vertical or transovarial transmission, where the virus is inherited by the progeny from the DENV-infected female Aedes mosquito (Martins *et al.*, 2012). Furthermore, vertical transmission of DENV does not only occur in mosquitoes but also in human beings. There were evidences of DENV being transmitted to infants from their mother during the late stages of pregnancy (Chye *et al.*, 1997; Chotigeat *et al.*, 2000). The state of Sarawak has experienced many outbreaks of dengue over the years, with the highest during 2014 and 2015. However, there are no published data on the circulating dengue virus serotypes in the Aedes mosquito population in Sarawak. Previous studies on the circulating DENV serotype in Sarawak were from clinical samples (Holmes *et al.*, 2009). Therefore, this study aims to provide the latest information on the circulating DENV serotype in the Aedes mosquito population of Sarawak, especially in Sibu and Miri divisions.

MATERIALS AND METHODS

Study areas

The study areas were located within the Sibu and Miri divisions of Sarawak, Malaysia (Figure 1). Sibu and Miri were selected as the study areas due to their history of increasing number of dengue cases in the state during late 2016 to 2017 (Sarawak State Health Department, 2017). The division of Sibu recorded the highest number of cases during that period with 398 cases. Miri recorded the second highest with 205 cases. Sibu and Miri are both rapidly developing cities with a constant increase in human population, where numerous number of forested area are being used up for urban development. The localities were chosen randomly covering peridomestic urban and sub-urban areas focusing on residential, commercial, and industrial areas. These areas were chosen due to the higher density

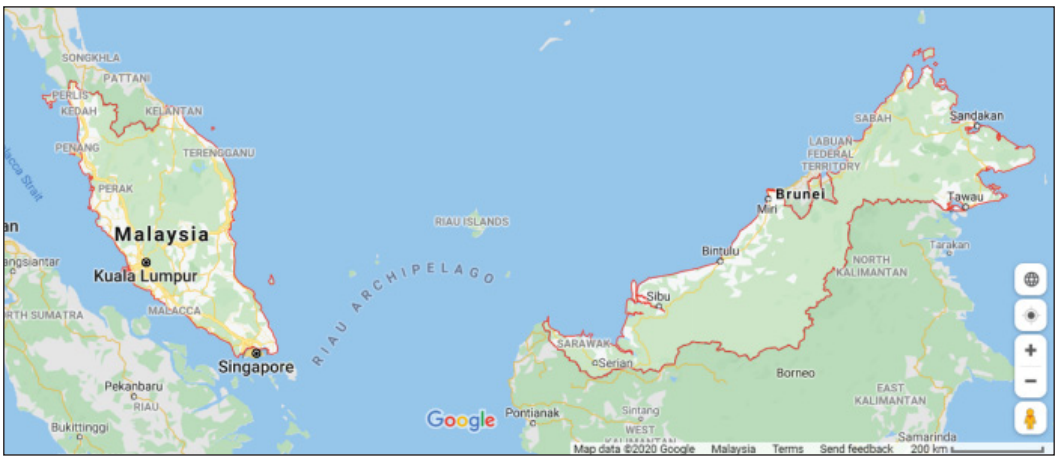

Figure 1. Map of Malaysia. The study sites, Sibu and Miri, are in the state of Sarawak in the island of Borneo, sharing borders with Kalimantan, Indonesia and Brunei Darussalam (Photo courtesy of Google maps).

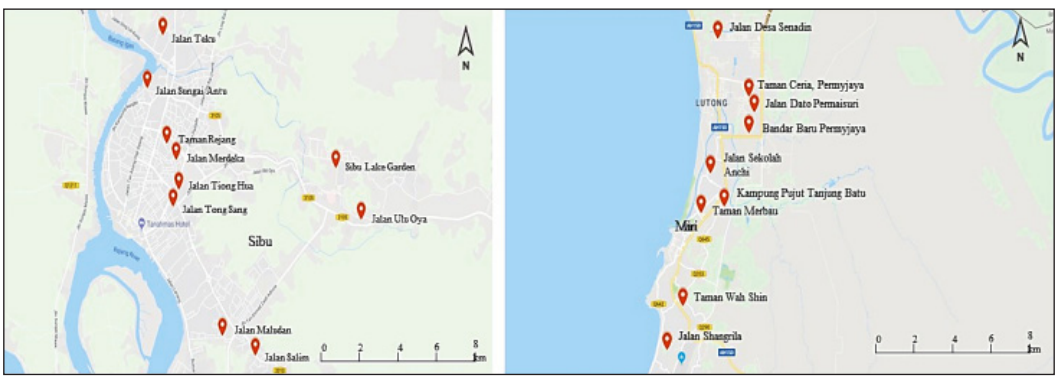

Figure 2. Location map showing the localities selected for sampling in Sibu and Miri Sarawak (Photo courtesy of Google maps).

of human population, which makes it highly possible for efficient DENV transmission. These areas also have the potential of providing good breeding ground for Aedes mosquitoes. Sibu is the third division in the state of Sarawak with a general population of 300 000 people. It is an inland town located at the central region of the state, with the town centre situated along the banks of the Rajang River. Ten locations (Figure 2) were covered in Sibu division focusing on residential, commercial and a few industrial areas. The names and description of the locations are listed in Table 1. These locations, except for Jalan Sungai Antu and Sibu Lake Garden, are mostly urban residential areas consisting of housing estates. The houses are primarily of single and double storey terrace style brick houses. The houses are arranged close to each other, making the transmission of DENV among the residents highly possible. Some of these housing estates are located near agricultural lands with various types of plantation. The sampling sites in Jalan Sungai Antu are close to light industrial areas such as around food processing factories, automotive workshops, and small furniture factories. These factories are surrounded with strewn discarded materials and items that could provide good breeding places for Aedes mosquitoes. Sibu Lake Garden is a recreational park located approximately 9 km from the town centre and next to the Sibu Division General Hospital. The park is

Table 1. Geographical location and type of localities selected for mosquito sampling in Sibu and Miri, Sarawak

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

also surrounded by residential areas consisting of terrace houses and is densely populated and located next to a forested area. Thus, this area also provides high potential for mosquito to human transmission of DENV.

Miri division, on the other hand, is the fourth division of Sarawak with a general population of 365 000 people. Miri city is the second largest city after the capital city, of Kuching. It is a coastal city, situated near the border of Brunei Darussalam and is located in the north-eastern region of Sarawak. The type of localities selected for mosquito sampling in Miri were similar to the ones in Sibu. Nine locations (Figure 2) were covered focusing on urban and sub-urban residential areas. The names and description of the locations are listed in Table 1. Eight out of the nine locations, except for Kampung Pujut Tanjung Batu, are housing estates and commercial shop lots consisting of brick houses and shop buildings. Kampung Pujut Tanjung Batu is a Malay village located approximately 2 km from the town centre comprising of singly built brick and wooden houses. Sampling activities in these locations were focused in areas surrounding the houses such as the backyard and front lawn.

Mosquito sample collection

Mosquito samples were collected from September 2016 to December 2017. Each division was visited four times during the sampling period. Mosquito sampling were only conducted in outdoor areas due to restricted access to private homes for indoor collections. Two types of Aedes mosquito samples were collected; the circulating adult male and female and the immature forms (larvae and pupae). Both samples were collected in the same area for each locality. Adult Aedes mosquitoes were collected twice daily, in the morning and late afternoon using the human landing catch (HLC) method. Captured adult mosquitoes were kept in liquid nitrogen for transport to the lab to preserve any DENV RNA. The immatures were collected from waste water containers surrounding the locality and placed in plastic containers before being transported to the laboratory for processing. The pH and temperature of the collected water were recorded on-site.

Identification and preservation of mosquitoes

Field-captured adult Aedes mosquitoes were identified using keys by Rueda (2004). They were sorted into pools of 15–20 individual mosquitoes into 1.5 ml tubes according to locality and gender. Immature mosquitoes were bred in breeding trays until they reach adult stage and later identified and sorted similarly. All adult mosquitoes were then stored in -80°C freezers prior to viral RNA extraction.

Viral RNA extraction

Viral RNA extraction was performed using Qiagen's RNeasy Mini RNA extraction kit (Catalogue no: 74104) containing Buffer RLT, Buffer RW1, Buffer RPE, RNase-free water, and RNeasy spin column attached with 2.0 ml collection tube. The extraction procedure was based on the manufacturer's protocol but with slight modification. Extraction protocol was performed on ice to protect RNA from degradation. Each 1.5 ml tube of pooled mosquito sample (15-20 mosquitoes) was added with 350 µL of Buffer RPE (lysis buffer). The sample was then thoroughly homogenised using a sterile plastic homogeniser (OPTIMA Biomasher II). Once uniformly homogenised, the samples were centrifuged for 3 minutes at 12 600 rpm in a refrigerated centrifuge (Eppendorf Centrifuge 5427R) at 0°C. The remaining lysate was transferred into a fresh 1.5 ml tube before adding 700 µL of 70% ethanol, and mixed thoroughly by pipetting repeatedly.

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

Once the column was completely dry, the filtrate was discarded along with the collection tube. A new 1.5 ml collection tube was attached to the column. 80 µL of RNase-free water was added to the column, precisely on the membrane. The column was then centrifuged for 1 minute at 12 600 rpm to elute the RNA from the membrane. The eluted RNA was kept in a labelled tube placed in a -80°C freezer before further analysis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR) for detection of DENV and molecular typing

The RT-PCR analysis method in this study was based on protocols described by Reynes *et al.* (2003), Ayers *et al.* (2006), and Saxena *et al.* (2008). The detection of DENV involved two separate steps, which were the reverse transcription of the viral RNA to produce the first strand cDNA, and two steps of semi-nested PCR for virus detection and serotyping. The PCR primers used in this study were based on primers developed by Lanciotti *et al.* (1992) and Saxena *et al.* (2008), as shown in Table 2. These primers are widely used in DENV related studies, which includes two consensus primers that targets the capsid and pre-membrane (C/prM) genes (D1 and D2) and four serotype specific primers (TS1, TS2, TS3, and TS4).

The RT-PCR process was performed as a two-step method, where the RT step was performed first, followed by the PCR. Five µL of RNA from each sample and one DENV-2 positive control was mixed with 5X RT reaction buffer, 0.2 µL deoxyribonucleic triphosphate (dNTP), 0.4 µL of DENV forward primer (D1), 1 µL of RNase inhibitor, and 1 µL of Moloney Murine Leukemia Virus Reverse Transcriptase enzyme in a 0.2 ml PCR tube. Nuclease free deionised water was added to reach a final volume of 20 µL. The mixture was incubated at 42°C for 60 minutes in the Applied Biosystems Veriti thermal cycler. The RT reaction was proceeded with PCR or stored at -20°C. The cDNA produced from the RT reaction was used to perform the PCR step to detect the presence of DENV. Five µL of RT product was mixed with 1 µL each of both forward and reverse DENV primers (D1 and D2) followed by 5 µL of 25mM $MgCl_2$, 1 µL

of dNTP, 0.4 µL of DNA polymerase enzyme (Promega GoTaq Flexi), and 10 µL of 5X Green PCR buffer (Promega). Nuclease free deionised water was added to reach a final volume of 50 µL. The PCR cycling conditions were 95°C of initial denaturation for 5 minutes, 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute and extension of 72°C for 1 minute, and finally 72°C for 10 minutes. The reaction was then terminated with a downregulation temperature of 4°C at infinity. The end-product of the PCR was then stored at -20°C pending further process. All the primers used in this study were based on primers developed by Lanciotti *et al.* (1992) and Saxena *et al.* (2008) (Table 2).

Samples that were positive for DENV detected through RT-PCR were further identified for their serotypes using a semi-nested PCR employing the use of DENV serotype specific primers TS1 (DENV-1), TS2 (DENV-2), TS3 (DENV-3), and TS4 (DENV-4). Five µL of the DENV positive PCR product (including the positive control) were added with 5 µL of 25mM $MgCl_2$, 1 µL of dNTP, 1 µL of DENV forward primer, 1 µL each of the serotype specific primers, 0.4 µL of DNA polymerase, and 10 µL of 5X Green PCR buffer. Nuclease free deionised water (23.6 µL) was added to make a total final volume of 50 µL. The PCR cycling conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 25 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute and 10 minutes. The reaction was terminated with a downregulating temperature of 4°C at infinity. The end-product of the PCR was visualized using agarose gel electrophoresis.

Agarose gel electrophoresis for detection of PCR product

The amplification of the DENV c-DNA was visualised through agarose gel electrophoresis. A 1.6% agarose gel was prepared containing SYBR® Safe DNA gel stain (Invitrogen Lot 1879547), and 10 µL of PCR product was loaded into the gel along with 6 µL of 100bp DNA ladder. The gel electrophoresis was conducted under conditions of 100 volts for 50 minutes and visualised under UV rays using the Syngene UV transilluminator gel documentation system.

Data Analysis

Positive samples were analysed for its minimum infection rate (MIR). Only positive pools of female or male Aedes mosquitoes, both field-captured and emerges, were calculated for their MIR. The MIR was estimated from the number of female/male mosquitoes in a positive pool divided by the number of female/male mosquitoes tested multiplied by 1000.

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

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

Table 2. Sequences of the consensus primers (D1 and D2) and the dengue-serotype specific primers (TS1, TS2, TS3, and TS4) (Lanciotti *et al.* (1992), and Saxena *et al.* (1998)

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

RESULTS

Transovarial DENV in field-collected immature Aedes mosquitoes

Immature mosquitoes were collected from either natural or artificial water containers in similar localities to the field-caught adults for Sibu and Miri divisions. The Aedes mosquito larvae and pupae were collected from various type of containers such as car tyres, plastic containers, waste plastic bags, discarded food containers, plastic bottles, flower pots, paint buckets, food cans, and coconut shells (Table 3). The pH and temperature of the water were recorded at the collection sites. The immature mosquitoes preferably thrive in water with pH ranging from 6.6 to 8.6 with temperature range of 25.0°C to 30.0°C. Collectively, a total of 627 and 812 adult female mosquitoes emerged from Sibu and Miri immature samples, respectively. All the female mosquitoes were identified as *Ae. albopictus*. None of the mosquito pools were positive

Table 3. Types of containers, pH and temperature of breeding environment of the immature mosquitoes collected localities in Sibu and Miri

Sibu	**Type of container**	**pH**	**Temperature (°C)**
Jalan Sungai Antu	Car tyre	7.5	25.0
Jalan Maludan	Car tyre	8.0	30.0
	Discarded ceramic bowl	8.2	30.0
Rejang Park	Car tyre	8.4	26.0
Jalan Merdeka	Car tyre	7.5	26.0
	Plastic food container	7.4	25.5
	Paint bucket	8.0	25.0
Jalan Tiong Hua	Plastic food container	7.7	25.5
	Motorcycle tyre	7.4	26.0
	Glass jar	7.9	25.0
Jalan Ulu Oya	Car tyre	7.1	27.0
	Plastic flower pot	7.5	25.0
	Plastic food container	7.2	28.0
Jalan Tong Sang	Car tyre	7.5	25.0
	Paint bucket	8.0	25.0
Jalan Teku	Car tyre	7.5	26.0
	Plastic food container	7.4	26.0
	Polystyrene food container	7.5	25.5
Jalan Salim	Car tyre	8.0	26.0
Sibu Lake Garden	Plastic bottle	7.1	26.7
	Car tyre	6.6	26.7
Miri	**Type of container**	**pH**	**Temperature (°C)**
Taman Merbau	Old paint bucket	7.8	25.0
Bandar Baru Permyjaya	Discarded food can	7.1	25.0
	Car tyre	7.8	25.0
	Coconut shell	6.6	25.0
	Waste plastic bag	8.6	25.0
Kampung Pujut Tg. Batu	Car tyre	7.6	25.0
	Polystyrene food container	7.7	25.0
Jalan Dato Permaisuri	Discarded food can	7.4	25.0
	Car tyre	8.1	25.5
Jalan Sekolah Anchi	Car tyre	8.4	25.5
Taman Wah Shin	Abandoned car	7.8	25.0
	Car tyre	7.8	25.0
	Plastic food container	8.0	25.0
Taman Ceria, Permyjaya	Discarded food can	7.5	26.0
Jalan Desa Senadin	Car tyre	7.2	25.5
Jalan Shangrila	Plastic tray	7.2	26.0
	Plastic bottle	7.5	26.0

for DENV as there was no appearance of a 511 bp DNA band in the gel electrophoresis image.

A total of 675 and 780 adult male mosquitoes emerged from both Sibu and Miri immature samples, respectively. All the male mosquitoes were also identified as *Ae. albopictus*, All the emerged males were screened for transovarial DENV using RT-PCR. A total of 32 pools from Sibu and 39 pools from Miri were screened. All the pools from Sibu were negative and only one pool (n=21) from Taman Ceria, Permyjaya, Miri, was positive for DENV serotype 2 (DENV-2) (Table 4).

DENV in field-caught Aedes mosquitoes

All of the field-caught mosquito samples were identified as *Ae. albopictus*. A total of 1,734 adult *Ae. albopictus* were caught from

Table 4. The percentage, presence, and minimum infection rate (MIR) of DENV-2 in pools of emerged male *Ae. albopictus* from localities in Miri and field-caught female *Ae. albopictus* from localities in Miri and Sibu

Location	No. of pools	No. of emerged male mosquitoes	Percentage of positive pools (%)	Presence of DENV	MIR
Miri					
Bandar Baru Permyjaya	12	231	0	–	–
Taman Ceria, Permyjaya	1	21	100.0	DENV-2	47.6
Taman Merbau	6	115	0	–	–
Jalan Dato Permaisuri	6	120	0	–	–
Taman Wah Shin	4	75	0	–	–
Kampung Pujut Tanjung Batu	2	49	0	–	–
Jalan Sekolah Anchi	1	20	0	–	–
Jalan Desa Senadin	2	43	0	–	–
Jalan Shangrila	5	106	0	–	–
Total	**39**	**780**			
Location	No. of pools	No. of field-caught female mosquitoes	Percentage of positive pools (%)	Presence of DENV	MIR
Miri					
Bandar Baru Permyjaya	3	52	0	–	–
Taman Ceria, Permyjaya	4	80	20.0	DENV-2	12.5
Taman Merbau	1	19	0	–	–
Jalan Dato Permaisuri	7	131	0	–	–
Taman Wah Shin	9	159	0	–	–
Kampung Pujut Tanjung Batu	2	27	0	–	–
Jalan Sekolah Anchi	1	20	0	–	–
Jalan Desa Senadin	2	35	0	–	–
Jalan Shangrila	8	137	0	–	–
Total	**37**	**660**			
Sibu					
Jalan Sungai Antu	2	43	0	–	–
Jalan Maludan	2	38	0	–	–
Rejang Park	1	23	0	–	–
Jalan Merdeka	5	99	0	–	–
Jalan Tiong Hua	3	68	0	–	–
Jalan Ulu Oya	1	21	0	–	–
Jalan Tong Sang	4	85	23.5	DENV-2	11.8
Jalan Teku	2	36	0	–	–
Jalan Salim	1	24	0	–	–
Sibu Lake Garden	4	80	25.0	DENV-2	12.5
Total	**25**	**517**			

Sibu and Miri, comprising of 1,177 females and 557 males. All captured adult females *Ae. albopictus* were screened for DENV using conventional RT-PCR. The mosquitoes were tested in pools of 15-20 mosquitoes per sample tube. A total of 25 (n=517) pools of adult mosquitoes from Sibu were screened for DENV. Out of the 25 pools analysed, only 2 pools of female mosquitoes were positive for DENV-2, which was one pool (23.5%) from Jalan Tong Sang and one pool (25.0%) from Sibu Lake Garden (Table 4), as indicated by a 119 bp DNA band in the gel electrophoresis image. As for Miri, 37 (n= 660) pools of adult mosquitoes were screened for DENV, but only one pool (20.0%) from Taman Ceria, Permyjaya was positive for DENV-2, indicated by a clear 119 bp DNA band in the gel electrophoresis image. The MIR for these positive samples were estimated at 11.8 and 12.5 from the two pools from Sibu and 12.5 from the only positive pool from Miri (Table 4).

Gene Sequence of DENV-2 in samples

All PCR samples positive for DENV-2 were sent for gene sequencing for confirmation. The DNA sequences obtained were compared with reference sequence from GenBank. DENV positive samples from this study were confirmed to be DENV-2 when compared with highly similar DENV-2 sequences from GenBank.

DISCUSSION

***Aedes albopictus* in all selected localities in Sibu and Miri divisions of Sarawak**

In this study only one species was identified in all the samples collected, that is *Ae. albopictus*. A recent ovitrap surveillance study conducted by Lau *et al*. (2017) throughout Sarawak, showed a similar result, where only *Ae. albopictus* were found in high density in both urban and suburban residential areas, and no *Ae. aegypti* or other container breeding mosquitoes were detected. Studies conducted three decades ago also indicated that *Ae. albopictus* were widespread across the state of Sarawak (Chang and Jute, 1982). They found *Ae. aegypti* but in lower density as compared to *Ae. albopictus*. Their subsequent study 12 years later also observed that *Ae. albopictus* was much more abundant than *Ae. aegypti* (Chang and Jute, 1994). The dominance of *Ae. albopictus* may be because it is an indigenous species in Asia, including Malaysia (Bonizzoni *et al*., 2013), which explains the abundance of *Ae. albopictus* in the study areas. A study conducted in Shah Alam, Selangor, Malaysia indicated an abundance of *Ae. albopictus* in the samples collected in outdoor peridomestic areas (Faiz *et al*., 2017), similar to findings in this study. Another study conducted in Penang Island, Malaysia also indicated that *Ae. albopictus* were present in higher numbers than *Ae. aegypti* in both outdoor and indoor samplings (Rahim *et al*., 2018). Inclusion of indoor sampling in this study could have shown a more significant result. In addition, studies in previous years stated that *Ae. aegypti* are present in higher numbers in highly developed urban areas as compared to rural areas (Saifur *et al*., 2013). The findings in this study on *Ae. albopictus* indicated otherwise.

DENV in *Aedes albopictus* population of Sibu and Miri divisions of Sarawak

This study showed the presence of circulating DENV in the natural population of *Ae. albopictus* in Sibu and Miri divisions of Sarawak. Three pools of mosquito were positive for DENV-2, out of 62 pools of female *Ae. albopictus* tested. These positive pools comprised of one pool (23.5%) from Jalan Tong Sang, Sibu, one pool (25.0%) from Sibu Lake Garden, and one pool (25.0%) from Taman Ceria, Permyjaya, Miri. All the positive pools contained similar numbers of individual mosquitoes, so the percentage of positive pools are quite similar. The percentage can be higher or lower depending on the number of positive DENV pools. A study conducted by Urdaneta *et al.* (2015) only showed 5.2% of positive mosquito pools out of 154 pools of mosquito collected. Study conducted by Pinheiro *et al.* (2005) also obtained a low percentage of positive pools, where only 17.1% of the mosquito pools were positive for DENV-3.

There are no other studies to date in the state of Sarawak in terms of entomological aspects which can be compared directly to this study. However, the detection of DENV in adult mosquitoes is possible and has been done as far as two decades ago. A study conducted by Chow *et al.* (1998) in Singapore showed that all the DENV serotypes could be isolated from both mosquito vectors, *Ae. albopictus* and *Ae. aegypti*, tested in pools. Studies conducted by Urdaneta *et al.* (2015) also detected multiple dengue virus serotypes such as DENV-1, DENV-2, and DENV-3 in field-caught adult mosquitoes. Besides that, DENV can also be detected in a single individual mosquito, as reported by Chung and Pang (2002), from mosquito population in Singapore. Male adult *Ae. albopictus* caught in this study were all negative for DENV. However, other studies have reported that all DENV serotypes can be found in male mosquitoes as well, an indication that the virus is being transmitted vertically or transovarially from gravid female mosquito to its progeny (Kow *et al.*, 2000).

The MIR value for each positive pool were 11.8, 12.5, and 12.5 for Jalan Tong Sang, Sibu, Sibu Lake Garden, and Taman Ceria, Permyjaya respectively. It can be suggested that Sibu Lake Garden, Sibu and Taman Ceria, Permyjaya, Miri has high potential of dengue transmission among the population compared to the other areas due to its higher MIR value. According to Vikram *et al.* (2015) the higher the MIR, the higher the risk of dengue transmission. Areas of high MIR values in the Aedes mosquito population are also prone to dengue outbreaks. For example, a study conducted by Peña-Garcia *et al.* (2016) in Colombia reported MIR values of up to 73.53, which is much higher when compared to the MIR values in this study.

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

Transovarial transmission of DENV was detected to occur among immature mosquitoes sampled in this study. Among all the pools of immatures collected from natural and artificial containers, only one pool of male *Ae. albopictus* from Taman Ceria Permyjaya, Miri was positive from DENV-2. This confirms that transovarial transmission of DENV does occur at least in this area. Transovarial transmission of DENV have been reported since three decades ago (Jousset, 1981; Khin and Than, 1983; Hull *et al.*, 1984). There were similar studies that reported evidence of transovarial transmission of DENV-2 (Khin and Than, 1983) and DENV-4 (Hull *et al.*, 1984) from immatures of *Ae. aegypti* collected from natural environment. The immatures were raised from eggs (Hull *et al.*, 1984) and larvae (Khin and Than, 1983) to adult before subjected to RT-PCR. Another study demonstrated that transovarial transmission of DENV-2 in *Ae. aegypti* can be achieved *in vivo* using adult gravid females infected through intrathoracic inoculation (Jousset, 1981).

Studies regarding transovarial transmission of DENV in Aedes mosquito population were mainly conducted in South America and Asia, where these areas are endemic for dengue (Ferreira-de-Lima & Lima-Camara, 2018). This is the first study to report transovarial transmission of DENV in *Ae. albopictus* population in Miri division, Sarawak. Similar study conducted in several states across Peninsular Malaysia reported dengue virus in adults of *Ae. albopictus* emerged from immatures using PAP staining and RT-PCR methods (Rohani *et al.*, 1997). However, the serotypes of dengue virus were not reported.

The transovarial transmission of DENV could be maintained in successive generations of *Ae. aegypti* mosquitoes for up to five generations in laboratory studies using infected female *Ae. aegypti* (Joshi *et al.*, 2002; Rohani *et al.*, 2008). A study conducted in the late 1980's observed that in laboratory conditions, DENV could also be transmitted transovarially at a higher rate in other species of orally infected Aedes mosquito such as *Aedes mediovittatus*, a forest and peridomestic mosquito found in the Caribbean (Freier and Rosen, 1988).

Influence of water temperature and pH on mosquito breeding

Majority of the immature mosquitoes collected in the study areas were thriving well in water with temperature ranging

from 25.0°C to 30.0°C and pH range of 6.6 to 8.6. Temperatures ranging from 15.0°C to 30.0°C are suitable for the survival of Aedes larvae in the natural environment (Williams and Rau, 2011). Other studies have also demonstrated that temperatures of water ranging from 26.5°C to 29.3°C in containers supports the increase in mosquito abundance (Joseph *et al*., 2013). On the contrary, temperatures exceeding 30.0°C have been shown to affect the survival rate of mosquito larvae and pupae (Bayoh and Lindsey, 2004). Certain pH levels could also influence the livelihood of mosquito larvae. Studies have shown that varying pH levels of water could seriously affect mosquito larvae (Okogun *et al*., 2005). Acidic conditions may not be suitable for mosquito breeding. According to Joseph *et al*. (2015), a pH range of 7.1 to 7.3 supports mosquito breeding in all habitats.

Circulation of DENV-2 in population of *Aedes albopcitus* of Sibu and Miri divisions of Sarawak

During the entire period of this study, DENV-2 could be the current circulating DENV serotype within the *Ae. albopictus* populations in the selected localities. DENV-2 was detected in the field-caught adult females and male adults emerged from immatures in the laboratory. However, there are no published data in Sarawak regarding the current circulating DENV serotype in the study area, so findings in this study could not be compared with current settings. Studies on human serum samples have been performed in Sarawak, where DENV-2 were reported in clinical isolates from Sibu (Holmes *et al*., 2009). The finding was reported in 2002, therefore the circulating DENV serotype at present may differ. Thus, the correlation between the positive DENV-2 mosquito samples and the current circulating DENV serotypes cannot be determined. The state of Sarawak neighbours with the country of Brunei Darussalam and Kalimantan, Indonesia. Frequent entry of travellers and migrant workers from these countries could facilitate for the entry and spread of multiple DENV serotypes. Serological studies in Brunei showed that DENV-2 was predominant from 2005 to 2006 (Osmali *et al*., 2007). DENV-1 was also detected in low amount. Recent studies conducted in Kalimantan, Indonesia indicated that all four DENV serotypes were present in the region and DENV-3 was the predominant serotype at the moment (Sasmono *et al*., 2019). Furthermore, studies conducted in Peninsular Malaysia specifically in the state of Selangor and the east coast region reported multiple predominant serotypes of DENV ranging from DENV-1 to DENV-3 isolated from human serum samples of dengue patients (Vinomarlini *et al*., 2011; Ab-Fatah *et al*., 2015). A similar study conducted in Kuala Lumpur, Malaysia and its adjacent areas with the state of Selangor indicated that DENV-4 was the predominant serotype during 2011 (Chew *et al*., 2012).

Prior to this study, the positive female mosquitoes might be horizontally infected with DENV or could obtained the virus through transovarial infection. Horizontal infection of DENV is a primary mechanism for virus maintenance and happens when a mosquito is orally infected with DENV when it feeds on viremic blood of an infected human being (Mulyatno *et al*., 2012). Likewise, the DENV-2 positive from male mosquitoes discovered in this study were infected with DENV through transovarial transmission of the virus.

Furthermore, there is also a possibility that the field-caught adult mosquitoes originated from a different locality. According to the WHO (2017), an average adult Aedes mosquito can fly up to 400 meters from where they initially emerged. In addition, with increasing connectivity in transports, infected mosquitoes can also travel from different states or country. The only study performed regarding the circulation of DENV serotypes was reported in 2009. Holmes *et al*. (2009), managed to identify co-circulation of multiple DENV serotypes in Sarawak which includes DENV-2, DENV-3, and DENV-4. However, these data were based on clinical isolates of infected patients and not from the entomological survey. In conclusion, *Ae. albopictus* is found to be the major Aedes mosquito population in Sibu and Miri divisions of Sarawak, and DENV-2 was the current circulating dengue virus serotype. Further investigations on spatial and

temporal occurrence of circulating and transovarial dengue serotypes in vector mosquitoes may serve as valuable reference for vector control planning of the areas.

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