DETECTION OF TRANSOVARIAL DENGUE VIRUSES IN *Aedes albopictus* FROM SELECTED LOCALITIES IN KUCHING AND SAMARAHAN DIVISIONS, SARAWAK, MALAYSIA BY REVERSE TRANSCRIPTION POLYMERASE CHAIN REACTION (RT-PCR)

Nor Aliza Abdul Rahim*, Harvie Shukri, Nur Ain Mardiah Rizuan, Lela Suut, Siti Fairouz Ibrahim, Razitasham Safii & Marlini Othman
Faculty of Medicine and Health Sciences,
Universiti Malaysia Sarawak,
94300 Kota Samarahan, Sarawak, Malaysia.
*Corresponding author: arnaliza@unimas.my

ABSTRACT

The current study reports the first detection of transovarial dengue virus in the adult of *Aedes albopictus* raised from immatures including eggs and larvae, collected from December 2014 to February 2016 by using black ovitrap from selected localities in Kuching and Samarahan Divisions, Sarawak. The mosquitoes were screened in the laboratory by using a conventional method of reverse transcription polymerase chain reaction (RT-PCR). Dengue virus serotype 1 (DENV-1) and 2 (DENV-2) were detected in two of the mosquito pools comprising of adult females *Ae. albopictus* which emerged from the immatures. None of the *Ae. albopictus* male mosquitoes were detected with dengue virus. Our findings revealed the first natural evidence of the transovarial transmission in the natural population of *Ae. albopictus* at the selected localities. This discovery may aid in establishing more strategic and effective control of Aedes mosquitoes and may also open more discussion and research on the persistency of dengue viruses in the environment via the vertical or transovarial transmission mainly in Sarawak.

Keywords: Dengue virus, *Aedes albopictus*, transovarial transmission, RT-PCR

ABSTRAK

Kajian semasa melaporkan kajian pertama dalam mengesan virus denggi transovari pada nyamuk *Aedes albopictus* dewasa yang dibesarkan daripada peringkat jentik-jentik, yang dikumpul dari Disember 2014 hingga Februari 2016 dengan menggunakan perangkap jentik-jentik dari kawasan terpilih di Bahagian Kuching dan Samarahan, Sarawak. Nyamuk telah disaringkan di makmal dengan menggunakan kaedah tindak balas rantaian polymerase tranksripsi terbalik *(RT-PCR)* konvensional. Virus denggi serotip 1 (DENV-1) dan 2 (DENV-2) telah dikesan dalam dua kumpulan nyamuk yang terdiri daripada nyamuk *Ae. albopictus* betina yang dibesarkan daripada peringkat jentik-jentik. Walau bagaimanapun, tiada nyamuk *Ae. albopictus* jantan dikesan mengandungi virus denggi. Penemuan ini telah mendedahkan bukti transmisi virus denggi secara transovari yang pertama dalam populasi semulajadi *Ae. albopictus* di kawasan kajian. Hasil kajian ini boleh membantu dalam mewujudkan kawalan

yang lebih strategik dan berkesan bagi nyamuk Aedes dan juga boleh membuka lebih banyak perbincangan dan penyelidikan tentang keupayaan virus denggi untuk hidup berpanjangan di alam sekitar melalui transmisi menegak atau transovari, terutamanya di Sarawak.

Kata kunci: Virus denggi**,** *Aedes albopictus*, transmisi transovari, RT-PCR

INTRODUCTION

Dengue has caused a significant number of mortality and the number of infection has been dramatically increased around the world in recent decades. World Health Organization (WHO) has stated that dengue infection is rampant or prevalent over the world's globe. About 2.5 billion of people around the world are at risk of getting infected (Guzman et al. 2010). Initially, dengue is endemic only to people who live in tropical and sub-tropical countries. However, it has become a continuing threat to countries especially in South-east Asia, Africa and America (WHO 2016).

Dengue infection may result in varying degrees of pathological conditions from mild asymptomatic dengue fever (DF) to severe dengue hemorrhagic fever (DHF) (Murphy & Whitehead 2011). Hemorrhagic manifestation like plasma leakage or bleeding will be observed in the patient suffers from DHF. Dengue can occur in a vast background of people. People of different age, regardless of gender and ethnicity can be infected with dengue (Cheah et al. 2014). Additionally, the distribution or seroprevalance of dengue is also vast as it is not only concentrated in urban areas but rural and sub-rural communities as well.

Dengue is mainly caused by the infection of dengue virus (DENV). DENV is a single-stranded RNA virus and belongs to Flaviviridae and genus *Flavivirus.* There are four serotypes of dengue viruses; DENV-1, DENV-2, DENV-3 and DENV-4. Each of these serotypes is capable of causing dengue infection (Chen & Vasilakis 2011) and from scientific observation the four serotypes may co-circulate in the environment (Cheah et al. 2014). Dengue infection has occurred in human population due to the transmission via mosquitoes. Two species of mosquitoes; *Ae. aegypti* and *Ae. albopictus* are responsible to transmit all the serotypes of dengue viruses. They transmit the virus to human via blood-feeding and indirectly release the virus into the bloodstream (Chan & Johansson 2012). As stated by Rosa et al. (2015) the viral transmission of dengue can occur via two types of transmission; horizontally from Aedes mosquito to human and vertically or transovarially from infected female mosquito to its progeny (Martins et al. 2012).

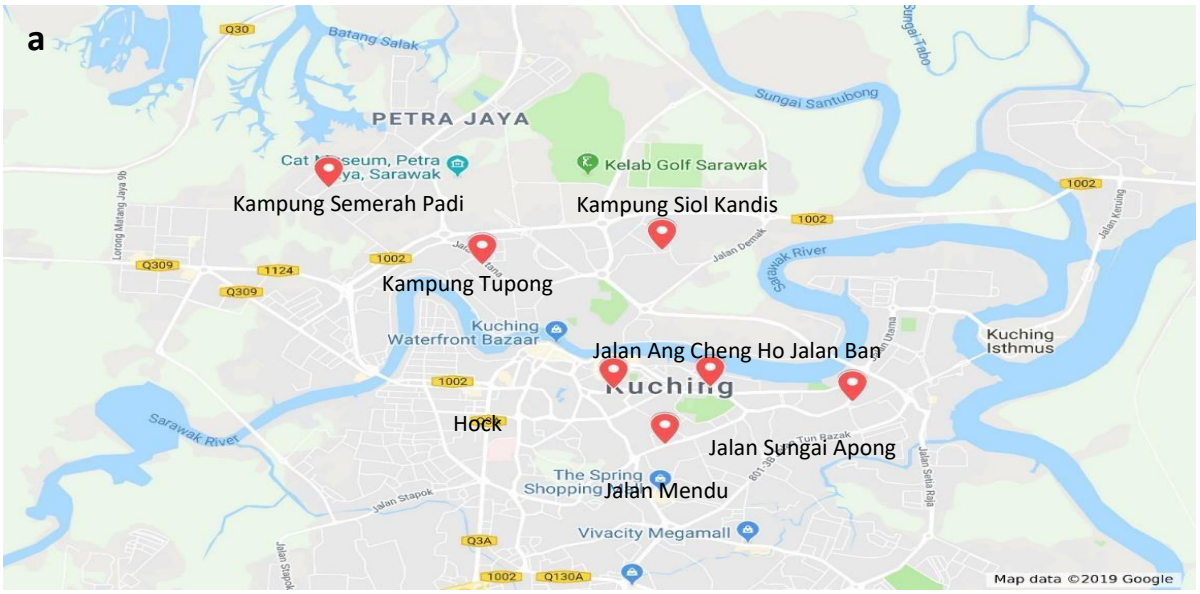
In Malaysia, the occurrence of dengue cases is reported in all states every year (Wan-Norafikah et al. 2012) and all of the four DENV serotypes are present in Malaysia (Cheah et al. 2014). Malaysia experienced its worst dengue epidemic in 2008 with 49,335 cases, and in 2010, Malaysia recorded the highest death toll with 134 of fatalities (Fong et al. 2014). Sarawak, which is the largest state in Malaysia, also experience dengue outbreaks occasionally, and large outbreak is not frequently observed if compared to Peninsular Malaysia which always has large dengue outbreaks (Holmes et al. 2009). However, Sarawak still recorded a notable number of dengue cases every year as reported in the Sarawak Weekly Epid News by Sarawak Health Department. Holmes et al. (2009) have done an experimental study about genetic diversity of DENV in Sarawak and during their studies, three out of the four DENV serotypes; DENV-2, DENV-3 and DENV-4 were successfully detected and isolated from patients with dengue infection in Sarawak. So far, there is no study on the circulating DENV serotypes in the natural population of *Ae. albopictus* in Sarawak. Although

Sarawak is a "sink" population for DENV (Holmes et al. 2009), it would be interesting to study the transmission of DENV serotypes in the natural Aedes mosquito population. High infestation of *Ae. albopictus* in Kuching and Samarahan Divisions, Sarawak might represent a risk to cause a dengue epidemic. Besides that, the potential of *Ae. albopictus* as a dengue vector is far to be known and studied. This study assumed that *Ae. albopictus* may help in the persistency of DENV by adopting silent transmission of virus mainly via vertical or transovarial transmission. Several studies on transovarial transmission were conducted (Cecílio et al. 2009; Rosa et al. 2015; Zeidler et al. 2008) but none is from Sarawak. Our study aimed to detect transovarial DENV in *Ae. albopictus* collected from selected localities in Kuching and Samarahan Divisions and subsequently determine the current circulating DENV serotype at study sites.

MATERIALS AND METHODS

Location

Selected localities in Kuching and Samarahan Divisions, Sarawak were chosen based on history of reported dengue cases retrieved from Weekly Epid News by Sarawak Health Department from 2014 to 2015. The study sites involved residential areas, commercial buildings and a public university. In Kuching Division, our study focused within the Kuching District. Six locations in Kuching District with history of reported dengue cases selected for this study were Kampung Tupong (1°34'22.4"N 110°20'13.2"E), Kampung Siol Kandis (1°34'30.2'N, 110°21'30.0"E), Jalan Ang Cheng Ho (1°33'11.2"N 110°21'50.8"E), Jalan Mendu (1°32'37.1"N 110°21'30.1"E), Jalan Ban Hock (1°33'09.8"N 110°21'09.2"E), and Jalan Sungai Apong (1°33'02.6"N 110°22'51.4"E) (Figure 1a). Meanwhile, one location with no history of dengue cases, Kampung Semerah Padi (1°35'07.5"N 110°19'07.8"E) was randomly chosen as negative control for the study area. While in Samarahan Division, our study concentrated within the Samarahan District and our main study site in this division was Kota Samarahan. One location with history of reported dengue cases in Kota Samarahan was Taman Desa Ilmu Phase 1 (1°27'10.8"N 110°27'23.7"E) (Figure 1b). Besides that, a public university, Universiti Malaysia Sarawak (1°27'53.0"N 110°25'35.3"E) was chosen as a negative control for the study area.



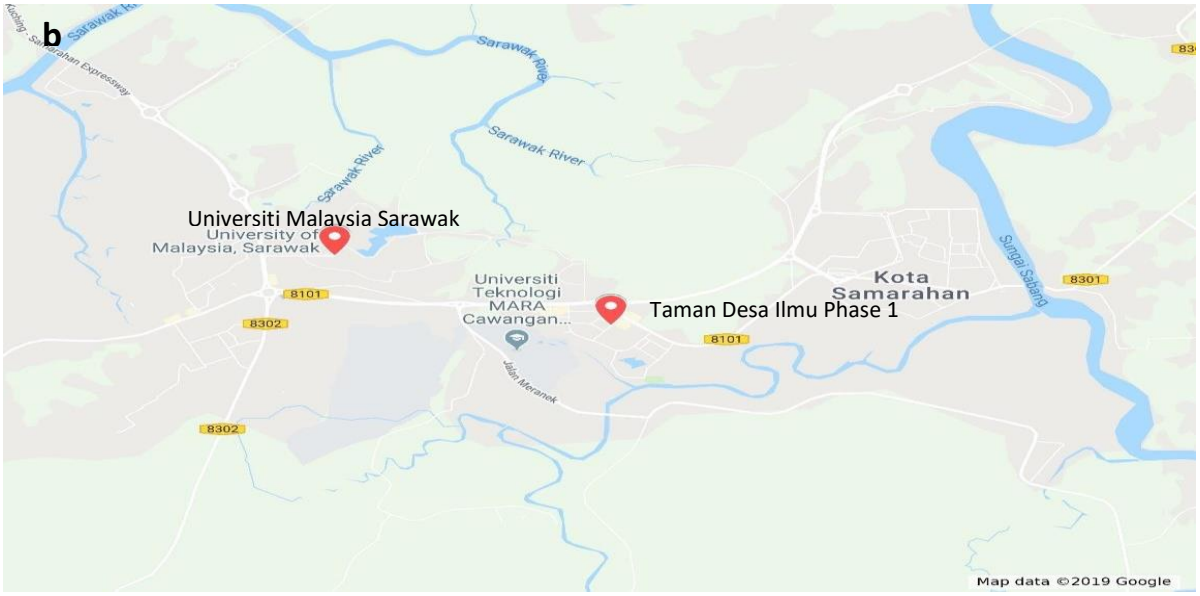


Figure 1. (a) Location map of the selected localities for sampling in Kuching Division. (b) Location map of the selected localities for sampling in Samarahan Division (Souces: Google Maps 2019).

Sample Collection

The mosquito sample collections involving the collection of immatures (eggs and larvae) were conducted by placing 5 black ovitraps at each potential breeding spot at the sampling locations following methods by Roque and Eiras (2008). The collections were conducted three times at each site. The ovitraps were collected and brought back to the laboratory after 7 days. The contents of the ovitraps were poured into medium-sized plastic containers, then each larvae and pupae were placed individually in a small-sized plastic container. The immatures of the mosquito were raised into adults, and subsequently identified using guide by Rueda (2004). Adult male and female of *Ae. albopictus* emerged from the immatures were then pooled according to gender, date of identification and study site. They were then kept in cryogenic vial (1-10 adult per pool/vial) and stored in -80ºC freezer prior to RNA extraction.

RNA Extraction/Virus Isolation

DENV RNA was isolated from the male and female adults of the *Ae. albopictus* emerged from the immatures. DENV RNA was also isolated from DENV infected *Ae. albopictus*. C6/36 cell line culture supernatant was used as a PCR positive control. RNA extractions were done by using Qiagen's Rneasy Mini Kit (Catalogue No: 74104) according to the protocols described by the manufacturer. The final product from the extraction was placed in 1.5 ml microcentrifuge tubes and kept in a -80 ºC freezer pending further use.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR and Semi-Nested PCR)

All the procedures for this conventional RT-PCR were based on protocols described by Reynes et al. (2003) with a slight modification. For the laboratory detection of DENV until the serotype level in mosquito samples, two stages of experimental detection were involved; reverse transcription polymerase chain reaction (RT)-PCR and semi-nested-PCR. Reagents required for the preparation of the master mix in a final total of 15 µl for the RT process were RNase free water, 5X RT buffer (Promega), dNTP mix (Promega) (10mM each), 0.2 µm D2 forward primer (Promega), 20 unit/µl of Rnasin (Promega), AMV transcriptase (Promega) and RNA template. The RT was done in the thermal cycler at 42 °C for 1 hour. After RT, a

PCR process was conducted to amplify the c-DNA produced from the reverse transcription of the RNA. Reagents required for the preparation of the master mix in a final total of 50 µl for the first PCR process were RNase free water, 25 Mm $MgCl_2$ (Promega), dNTP mix (Promega) (10mM each), 0.2 µM D1 and D2 primers (Table 1), 5X GoTaq Buffer (Promega), DNA Taq polymerase (Promega) and DNA template from RT. The thermal cycler was programmed at 95°C for 5 min initial denaturation, followed by 35 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 1 min extension at 72°C and final extension for 10 min at 72°C. If the mosquito sample consist the DENV c-DNA, 511 bp could be expected in 1.5% agarose gel. The experiment proceeded with the serotyping of the DENV by second stage of PCR (Semi-nested). Reagents required for the preparation of the master mix in a final total of 50 µl for second stage of PCR were RNase free water, 25 Mm $MgCl_2$, dNTP mix (10 Mm each), 0.2µ M D1, and 0.2 µM of TS1, TS2, TS3 and TS4 specific primers (Lanciotti et al. 1992) (Table 1), 5X GoTaq buffer, DNA Taq polymerase and DNA template from the first round of PCR. The thermal cycler was programmed at 94°C for 5 min initial denaturation, followed by 25 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C, 1 min extension at 72°C and final extension for 10 min at 72°C. The product was then visualised in 1.5% gel electrophoresis with resulting bands of ~~either~~ 482 bp, 119 bp, 290 bp, and 390 bp for DENV-1, DENV-2, DENV-3 or DENV-4 respectively.

Table 1. Sequences of the universal primers (D1 and D2) and specific primers (TS1, TS2, TS3 and TS4) (Sources: Lanciotti et al. 1992).

Primer	**Sequence**	**Genome position**	**Expected size (bp) of dengue DNA product**
D1 (Forward)	5'-TCA ATA TGC TAA AAC GCG CGA GAA ACC G-3'	134-161	511
D2 (Reverse)	5'-TTG CAC CAA CAG TCA ATG TCT TCA GGT TC-3'	616-644	511
TS1 (Reverse)	5'-CGT CTC AGT GAT CCG GGG G-3'	568-586	482 (D1 and TS1)
TS2 (Reverse)	5'-CGC CAC AAG GGC CAT GAA CAG-3'	232-252	119 (D1 and TS2)
TS3 (Reverse)	5'-TAA CAT CAT CAT GAG ACA GAG C-3'	400-421	290 (D1 and TS3)
TS4 (Reverse)	5'-TGT TGT CTT AAA CAA GAG AGG TC-3'	506-527	392 (D1 and TS4)

RESULTS

From December 2014 to February 2016, the immatures of *Ae. albopictus*, which comprised the Aedes larvae and eggs, were collected at the study sites. After three visits per location, a total of 560 mosquitoes from the field-collected immatures from both divisions successfully emerged into female adult *Ae. albopictus*. From 55 pools (560 individuals) of adult female mosquitoes emerged from the immatures, 2 pools (3.6%) of adult female mosquitoes were

positive with DENV. One pool from Jalan Mendu was detected with DENV-2 (Figure 2) while another pool of mosquito from UNIMAS was detected with DENV-1. However, DENV-1 from UNIMAS was unable to be detected using PCR and gel electrophoresis. The DENV-1 was only detected when the sample was sent for gene sequencing at a private laboratory. The infection rate of each locality was also studied and estimated. The minimum infection rate (MIR) for adult female of *Ae. albopictus* emerged from the immatures was defined as the presence of DENV in the pools of adult female *Ae. albopictus* mosquitoes divided by the total number of adult female tested, and multiplied by 1000. From the results obtained, the MIR for the 2 positive pools of adult female mosquitoes with DENV from both divisions was 3.5 (Table 2). For the male mosquito pools, none of the 56 pools (525 individuals) of the male mosquitoes emerged from the immatures showed positive result with dengue virus. The gene sequences for the DENV isolated from the two pools of adult female *Ae. albopictus* mosquito samples emerged from the immatures were obtained. The authenticity of the two sequences obtained was checked with the BLAST program available in the National Center for Biotechnology Information (NCBI) database. The results from the BLAST which was optimized with MEGABLAST (http://www.ncbi.nlm.nih.gov/BLAST/) demonstrated that the samples were highly similar with the reference sequences of DENV-1 and DENV-2 isolates available in the GenBank). Each of the two samples sequences were aligned by using Clustal X 2.1 with references sequences. Multiple alignments of all the sequences using fast fourier transform (MAFFT) (available at http://www.ebi.ac.uk/Tools/msa/mafft/) are shown in Figure 3 & 4. The alignment of the nucleotide sequences showed that the isolated DENV-2 from one of our samples has 95% Identity Value and 89% Query Cover with the GW6 DENV-2 (Asian II) and DENV-2 New Guinea C (Asian II) isolates and corresponded respectively at the position of 110-190 nt and 133-213 nt of their complete genomes. These sequences were available in GenBank with accession no KM587709.1 and AF038403.1 respectively. As for the positive mosquito sample from UNIMAS, it was observed that this particular sample has a 100% identity value with the sequences of dengue virus 1-genotype I (Asia) isolate DENV- 1/VN/BID-V2753/2007 and DENV-1/VN/BID-V2787/2007 isolate available in the GenBank (April, 2016). The alignment of the nucleotide sequences also demonstrated that isolated DENV-1 from our sample corresponded respectively at the position of 115-143 nt and 109-137 nt of their complete genomes. These two reference strains were assigned in the GenBank with their accession numbers JQ287663.1 and GQ199807.1 respectively.

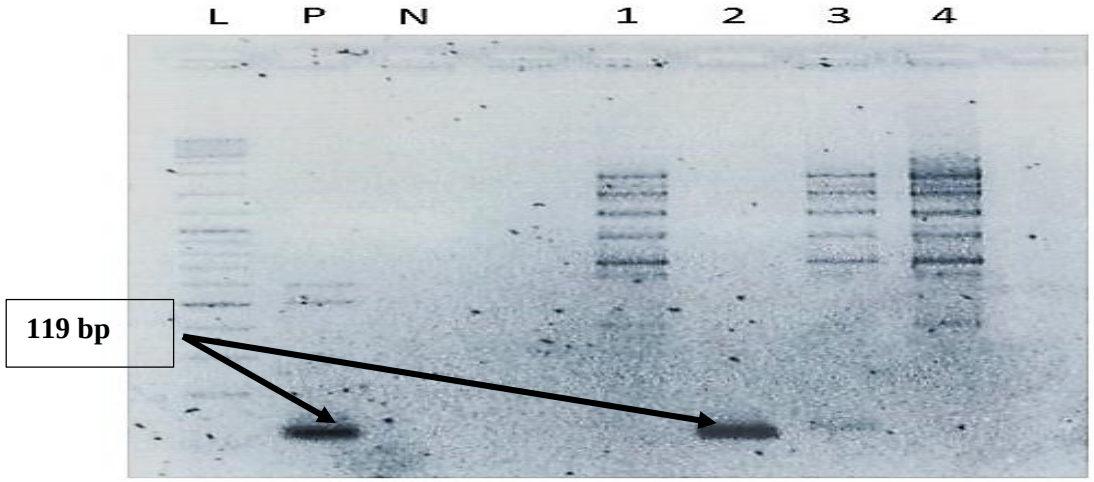


Figure 2. Gel electrophoresis image of female *Aedes albopictus* samples raised from immatures from Jalan Mendu, Kuching. One pool shows positive result indicated by a 119bp c-DNA band of DENV-2. (Lane L: 100bp DNA ladder; Lane P and N: Positive and negative control; Lanes 1, 2, 3, and 4 shows the pooled samples that had undergone amplification with type specific primers TS1, TS2, TS3 and TS4 respectively with positive sample in lane 2 showing 119bp DENV-2 DNA band and negative samples in lane 1,3 and 4. The presence of unwanted bands from lane 1, 3 and 4 might be caused by a technical error/issue while performing the experiment. Further optimization can be done to reduce the unwanted bands but for this study, the band that produced (119bp) was feasible for further analysis.

Table 2. DENV detected by RT-PCR and Semi-nested PCR in adult female and male of *Aedes albopictus* emerged from the immature at selected localities in both divisions from December 2014 to February 2016.

Adult *Aedes albopictus*	**No of pools**	**No of mosquitoes**	**No (%) of positive pools**	**Minimum infection rate (MIR)**	**No (%) of positive pools to serotypes**			
					DENV-1	DENV-2	DENV-3	DENV-4
Female	55	560	2 (3.6%)	3.5	1(0.17)	1(0.17)	0	0
Male	56	525	0	0	0	0	0	0
Total	111	1085	2 (3.6)		1 (0.17)	1(0.17)	0	0

* The MIR was defined as the presence of dengue virus in the pools of adult female/male *Aedes albopictus* mosquitoes emerged from the immature divided by total number of adult female/male emerged from the immature tested multiplied by 1000.

```
Format alignment by MAFFT FFT-NS-i (v7.215)
Positive-DENV2 samples in this study

JMendu_2240193_ AAGGACT--GTCTGCAGCATTCCAGTGAGATCTCTTTGTCAGCTGTTGTACAGTCGA—CACGCGGTTTCTCGCGCGTTTTAGCA---
JAChengHo_22401 AAAGGTCTGTCCTGCAGCATTCCAGTGAGATCTCTTTGTCAGCTGTTGTACAGTCGA—CACGCGGTCTCTCGCGTGTTTTCA-----

Reference sequences in GenBank
gi|770076472|gb ----TTC-AATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTACAACAGCTGA-------CAAAGAGATTCTCACTTGGAATGCTG CAG
gi|2723944|gb|A ----TTC-AATATGCTGAAACGCGAGAGAAACCGCGTGTCGACTGTACAACAGCTGA--A -------------CAAAGAGATTCTCACTTGGAATGCTGCAG
gi|225590496|gb AGTTGTT-AGTCTACGTGGACCGACAAAGAACAGTTTCTTTGAGGAAGCTTGGCTTAACG TAGTTCTAACAGTTTTT-GATTA-------
gi|1021221292|g AGTTGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACG TAGTTCTAACAGTTTTTTAATTA--------
gi|51850374|dbj AGTTGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGAAGCTAAGCTTAACG TAGTTCTAACAGTTTTTTAATTA--------
gi|829642982|gb AGTTGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGAAGCTAAGCTTAACG TAGTTCTAACAGTTTTTTAATTA--------
gi|77024338|gb| AGTAGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACG TAGTTCTAACAGTTTTTTAATTA--------
gi|77024336|gb| AGTAGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACG TAGTTCTAACAGTTTTTTAATTA--------
gi|354990976|gb AGTTGTT-AGGCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACG TAGTTCTAACAGTTTTTTAATTA--------
gi|14585842|gb| AGTTGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTTAACG TAGTTCTAACAGTTTTTGAATTA--------
gi|304360629|gb AGTTGTT-AGTCTACGTGGACCGACAAAGACAGATTCTTTGAGGGAGCTAAGCTCAACG TAGTTCTAACTGTTTTTTGATTA--------
gi|157956543|gb AGTTGTT-AGTCTACGTGCACCGACAAAGACAGATTCTTCGAGGAAGCTAAGCTTAACG TAGTTCTAACAGTTTTTTAATTA--------
          ..     *.*      .  .  ...* *     * *. .   *       .*.. * *   .  * .*.* .   ..
```

* Conserved Region/Conserved nucleotides along the aligned sequences

Figure 3. Nucleotide sequence alignments by MAFFT of positive-DENV-2 mosquito samples from Jalan Mendu and Jalan Ang Cheng Ho.

```
Format alignment by MAFFT FFT-NS-i (v7.215)
Positive-DENV1 sample in this study

Unimas_2240195_ -----GATGCT ----TCGCTGGATCTCGCTTTCTGGAT

Reference sequences in the GenBank
gi|372000986|gb TTCAATATGCTAAAACGCGCGAGAAACCG----------
gi|239840271|gb TTCAATATGCTAAAACGCGCGAGAAACCG----------
                *****      *** .**  .**
```

* Conserved Region/Conserved nucleotides along the aligned sequences

Figure 4. Nucleotide sequence alignments by MAFFT of positive-DENV-1 mosquito sample from Universiti Malaysia Sarawak.

DISCUSSION

The main purpose of this study was to detect the transmission of DENV in the natural population of *Ae. albopictus* via vertical or transovarial (mother-progeny) transmission. This study was conducted in the natural population of *Ae. albopictus* in selected localities within Kuching and Samarahan Divisions, Sarawak based on the history of reported dengue cases in 2014 and 2015.

Our results demonstrated that there was indeed an existence of DENV transmission via transovarial transmission in the natural population of Aedes mosquitoes. In this study, 2 pools of adult female *Ae. albopictus* emerged from the immatures were positive with DENV-1 and DENV-2 respectively. Transmission of DENV from female Aedes mosquito to its progeny has been a topic of research interest in many endemic countries including Malaysia. Although some studies suggested that the transmission via transovarial occurs at a low rate or low frequency (Grunnill & Boots 2015), numerous studies on this area however had been done either by collecting the immatures of dengue vectors in the field or by orally infecting or inoculating DENV into the body of laboratory-grown Aedes mosquitoes (Rohani et al. 2008). Those Aedes mosquitoes were then screened for DENV.

The detection of transovarial occurrence in the adult female *Ae. albopictus* emerged from the immatures in this study was in concurrent with other studies. For instance, a study by Martins et al. (2012) reported that DENV-3 was detected in the adult female of *Ae. albopictus* from immatures collected from the natural environment. Likewise, Rohani et al. (1997) also successfully observed DENV from the adult female of *Ae. albopictus* originated from larval stage of the mosquito collected from different states in the Peninsula of Malaysia. Transovarial transmission of DENV have also been reported in laboratory studies performed by Castro et al. (2004) and Shroyer (1990), who were able to observe the positive transmission of DENV to the progeny of experimentally infected female *Ae. albopictus* mosquitoes in the laboratory.

The two positive results of the mosquito samples obtained and which were screened simultaneously with positive and negative controls during this study put an interest why only pools of adult female mosquitoes were detected with DENV but none of the males of *Ae. albopictus.* Furthermore, Kow et al. (2001) who observed the present of all the four dengue serotypes in the field-caught adult male of *Ae. albopictus* suggested that the capability of an adult male Aedes mosquitoes to harbour and transmit DENV should not be underestimated even though an adult male Aedes mosquito naturally does not transmit DENV due to that trait of not requiring to suck blood for egg development unlike female mosquitoes. However, similar to Kow et al. (2001), studies conducted in Brazil (2005-2006) has shown that they were able to confirm the presence of DENV in male *Ae. aegypti* mosquitoes hatched from eggs that were collected at urban residential area in Minas Gerais, Brazil (Vilela et al. 2010). The genotype found was DENV-3 which was the same genotype that was detected in Brazil during its epidemics from year 2002-2004.

As for this study, it is difficult to explain why only pools of adult female *Ae. albopictus* mosquitoes emerged from the immatures were observed with DENV. The filial minimum infection rates for DENV in male and female which emerged from vertically infected eggs are supposed to be exactly similar (Mitchell & Miller 1990). It is only plausible to say that the infection rates of DENV for vertical transmission in the collected immatures in this study were very low and subsequently the presence of the virus were unable to be

detected. Although this study did not evaluate the persistency of the DENV via the vertical transmission for generations, but it is possible to say, the immatures collected in this study might originated from adult mosquitoes that probably already been vertically infected or orally infected with DENV prior to oviposition. Study by Joshi et al. (2002) suggested that DENV can persist in the Aedes mosquitoes for up to several generations through vertical transmission. Wasinpiyamongkol et al. (2003) also observed subsequent increase in the number of infected mosquitoes with DENV following the first generation of infected mosquito during their laboratory studies.

Due to the higher population of *Ae. albopictus* at the selected study sites as compared to *Ae. aegypti*, this research focused to screen for DENV only in *Ae. albopictus*. The negative results reported in this study for other pools of adult female and male *Ae. albpoictus* emerged from the immatures is in agreement with many studies that found no evidence of transovarial transmission specifically in *Ae. albopictus* that originated from immatures (Martins et al. 2012; Hutamai et al. 2007). It has been reported that *Ae. albopictus* is not an efficient vector or less competent to be a vector for the transmission of DENV as compared to *Ae. aegypti* (Rezza 2012). Although it plays a minor role in causing dengue outbreak if compared to its partner *Ae. aegypti* in the transmission of DENV, *Ae. albopictus* still place a health concern to the public due to its dramatic expansion in geographical distribution around the world. However, both Aedes mosquitoes, in term of disseminating DENV via transovarial transmission, there are a wide range of studies and research with contradicting arguments and observations as mentioned by Grunnill and Boots (2015). Some researchers reported that the immatures of *Ae. albopictus* has a higher rate of vertical transmission than *Ae. aegypti* (Das et al. 2013) and even suggested that *Ae. albopictus* could be a reservoir for the dengue virus (Castro et al. 2004), while some other studies showed otherwise (Grunnill & Boots 2015). Nevertheless, there are no statistical studies that can give evidence to which species of Aedes vectors is effective and efficient in transmitting DENV through vertical transmission. Further study must be conducted to produce statistical-based evidence in comparing the infection rates of DENV via transovarial transmission in the immatures of Aedes mosquitoes, of which is still lacking.

According to Rohani et al. (2007), once an Aedes mosquito is infected with DENV it is generally assumed the mosquito is infectious for its lifespan and is indeed capable to infect non-infected human. When the Aedes mosquitoes are virologically monitored for transovarial DENV, many factors can contribute to the success or failure in detecting dengue viruses in the mosquitoes. One major factor is the locality chosen for mosquito collection.
The localities selected for mosquito collection in our study were based on their history for dengue cases in 2014 and 2015, but not based on the current or instant notification of dengue incidence. The locality for mosquito collection was initially placed as the main factor to be considered for entomological and virological studies, and epidemiological surveillance. However, recently, the mosquito collection locality may not be that important because there was one study conducted in Thailand which found no transovarial transmission of DENV in mosquito despite having high incidence of dengue cases during their study (Hutamai et al. 2007).

Secondly, the failure and success in getting DENV positive result might be contributed to factors such as sensitivity of methodologies (Thongrungkiat et al. 2011). The RT-PCR method used in this study has been long proven by researchers that this tool is indeed very highly sensitive especially when it comes to virological studies. It is a rapid, simple, fast and an efficient method to detect DENV serotypes accurately besides being able

to help in predicting the risk of dengue infection (El-badry et al. 2012). This method of detecting dengue viruses however might give false positive results if DNA contamination was not carefully observed. However, in this study, positive control for DENV and negative control with no mosquito samples were used every time the experiment was conducted in order to avoid false-negative result.

The detection of DENV-1 and DENV-2 in our mosquito samples were unable to be compared with any other entomological or virological study in Sarawak, especially studies that mainly probes the natural population of Aedes mosquitoes to detect DENV. Out of the four dengue virus serotypes, DENV-1 and DENV-2 were the only serotypes detected in our samples during the study period. However, an earlier study by Holmes et al. (2009) was able to isolate not only DENV-2, but DENV-3 and DENV-4 as well which were isolated during their studies from patient's serum of suspected dengue cases. However, the study by Holmes et al. (2009) was unable to detect DENV-1 in the serum retrieved from patients with dengue infection. DENV-1 and DENV-2 detected in the adult of *Ae. albopictus* during this study period would probably indicate that the DENV serotype circulating in the natural population of the vector at selected study sites within the year 2014 and 2016 could be DENV-1 and DENV-2.

Collecting the immature mosquitoes by using ovitrap is the most practical and non-laborious method unlike collecting adult Aedes mosquito in the field which is more laborious, time consuming and unsafe as it is prone to viral infection. Zeidler et al. (2008) in contrast suggested that screening for DENV in larvae is not the best method to predict dengue epidemic if compared to the screening of the field-collected adult Aedes mosquitoes which more likely have been infected by DENV prior to virus screening in the laboratory. In contrary to this, Guedes et al. (2010) postulated that screening DENV in the immatures may provide good and beneficial information about dengue serotypes currently circulating in the natural population of Aedes mosquitoes.

CONCLUSION

This study suggests that transovarial transmission of DENV in the population of *Ae. albopictus* does occur in nature. This mode of transmission might be widely occurring in Peninsula Malaysia, but this study shows that it might be a natural phenomenon in Sarawak, and Malaysia as well. However, further discussion regarding the role played by Aedes mosquito in transmitting the DENV transovarially and the mechanism of transmission itself need to be debated and evaluated further. Additionally, further work need to be done collectively by taking into consideration of other factors such as period of mosquito collection, extrinsic incubation period of DENV in Aedes mosquitoes and the sensitivity of method used. Active surveillance on the vector population and monitoring of the circulating dengue serotypes are important for reference to dengue control as vaccine for dengue virus is still on its way.

ACKNOWLEDGEMENTS

We would like to thank the Faculty of Medicine and Health Sciences, University Malaysia Sarawak (UNIMAS) for providing the facilities to conduct this study and the Institute of Health and Community Medicine UNIMAS for providing DENV isolates as positive control. This study was funded by the Fundamental Research Grant Scheme by the Ministry of Higher Education Malaysia, Grant no: FRGS/01(24)/835/2012(75)

REFERENCES

Castro, M.G.de, Nogueira, R.M.R., Schatzmayr, H.G., Miagostovich, M.P. & Lourenço-de-oliveira, R. 2004. Dengue virus detection by using reverse transcription- polymerase chain reaction in saliva and progeny of experimentally infected *Aedes albopictus* from Brazil. *Memórias Do Instituto Oswaldo Cruz* 99(8): 809–814.

Cecílio, A.B., Campanelli, E.S., Souza, K.P.R., Figueiredo, L.B. & Resende, M.C. 2009. Natural vertical transmission by *Stegomyia albopictus* as dengue vector in Brazil. *Brazilian Journal of Biology Revista Brasleira de Biologia* 69(1): 123–127.

Chan, M. & Johansson, M.A. 2012. The incubation periods of dengue viruses. *PLoS ONE* 7(11): 1–7.

Cheah, W., Ng, K.S., Marzilawati, A.-R. & Lucy, C.S.L. 2014. A review of dengue research in Malaysia. *Medical Journal of Malaysia* 69: 59–67.

Chen, R. & Vasilakis, N. 2011. Dengue—quo tu et quo vadis? *Viruses* 3(9) :1562-1608.

Das, B., Das, M., Dwibedi, B., Kar, S.K. & Hazra, R.K. 2013. Molecular investigations of dengue virus during outbreaks in Orissa state, Eastern India from 2010 to 2011. *Infection, Genetics and Evolution* 16: 401–410.

El-badry, A.A., El-beshbishy, H.A. & Al-ali, K.H. 2012. Molecular study on the dengue virus in *Aedes aegypti* from Al- Madinah, Saudi Arabia using one step real time RT-PCR. *Science Parasitology* 13(4): 133–138.

Fong, L.F., Edwards, A., Wani Muthiah, Sivanandam, H. & Ruban, A. 2014. Change in dengue virus variation behind outbreak. http://www.thestar.com.my/news/nation/2014/02/15/ new-virus-variation-behind-outbreak-den2-dengue-serotype-leaves-behind-more-harmful-effects-says-d/ [30 September 2019].

Grunnill, M. & Boots, M. 2015. How important is vertical transmission of dengue viruses by mosquitoes (Diptera: Culicidae)? *Journal of Medical Entomology* 53(1): 1–19.

Guedes, D.R.D., Cordeiro, M.T., Melo-Santos, M.A.V, Magalhaes, T., Marques, E., Regis, L. & Ayres, C.F.J. 2010. Patient-based dengue virus surveillance in *Aedes aegypti* from Recife, Brazil. *Journal of Vector Borne Diseases* 47: 67–75.

Guzman, M.G., Halstead, S.B., Artsob, H., Buchy, P., Farrar, J., Gubler, D.J. & Pelegrino, J.L. 2010. Dengue: a continuing global threat. *Nature Publishing Group* 8(12): S7–S16.

Holmes, E.C., Tio, P.H., Perera, D., Muhi, J. & Cardosa, J. 2009. Importation and co-circulation of multiple serotypes of dengue virus in Sarawak, Malaysia. *Virus Research* 143(143): 1–5.

Hutamai, S., Suwonkerd, W., Suwannchote, N., Somboon, P., Prapanthadara, L., Section, D.

& Mai, C. 2007. A survey of dengue viral infection in *Aedes aegypti* and *Aedes albopictus* from re-epidemic areas in the north of Thailand using nucleic acid sequence based amplification assay. *Southeast Asian Journal of Tropical Medicine and Public Health* 38(3): 448–454.

Joshi, V., Mourya, D.T., & Sharma, R.C. 2002. Persistence of dengue-3 virus through transovarial transmission passage in successive generations of *Aedes aegypti* mosquitoes. *American Journal of Tropical Medicine and Hygiene* 67(2): 158–161.

Kow, C.Y., Koon, L.L., & Yin, P.F. 2001. Detection of dengue viruses in field caught male *Aedes aegypti* and *Aedes albopictus* (Diptera:Culicidae) in Singapore by type-specific PCR. *Journal of Medical Entomology* 38(4): 476–479.

Lanciotti, R.S., Calisher, C.H., Gubler, D.J., Chang, G.J., & Vorndam, A.V. 1992. Rapid detection and typing of dengue viruses from clinical samples by using reverse transcriptase-polymerase chain reaction. *Journal of Clinical Microbiology* 30(3): 545– 551.

Martins, V.E.P., Alencar, C.H., Kamimura, M.T., Araujo, F.M.de C., Simone, S.G.de, Dutra, R.F. & Guedes, M.I.F. 2012. Occurrence of natural vertical transmission of dengue-2 and dengue-3 viruses in *Aedes aegypti* and *Aedes albopictus* in Fortaleza, Ceara. *PLoS ONE* 7(7): 1–9.

Mitchell, C. & Miller, B. 1990. Vertical transmission of dengue viruses by strains of *Aedes albopictus* recently introduced into Brazil. *Journal of the American Mosquito Control Association* 6: 251–253.

Murphy, B.R., & Whitehead, S.S. 2011. Immune response to dengue virus and prospects for a vaccine. *Annual Review of Immunology* 29: 587-619.

Reynes, J.M., Ong, S., Mey, C., Ngan, C., Hoyer, S. & Sall, A. A. 2003. Improved molecular detection of dengue virus serotype 1 variants. *Journal of Clinical Microbiology* 41(8): 3864–3867.

Rezza, G. 2012. *Aedes albopictus* and the re-emergence of dengue. *BioMed Central Public Health* 12: 1–3.

Rohani, A., Asmaliza, I., Zainah, S., & Lee, H. 1997. Detection of dengue virus from field-caught *Aedes aegypti* and *Aedes albopictus* adults and larvae. *Southeast Asian Journal of Tropical Medicine and Public Health* 28(1): 138–142.

Rohani, A., Zamree, I., Joseph, R.T., & Lee, H.L. 2008. Persistency of transovarial dengue virus in *Aedes aegypti* (Linn.). *Southeast Asian Journal of Tropical Medicine and Public Health* 39(5): 813–816.

Rohani, A., Zamree, I., Lee, H.L., Mustafakamal, I., Norjaiza, M.J. & Kamilan, D. 2007. Detection of transovarial dengue virus from field-caught *Aedes aegypti* and *Aedes. albopictus* larvae using C6/36 cell culture and reverse transcriptase-polymerase chain reaction (RT-PCR) techniques. *Dengue Bulletin* 31: 47–57.

Roque, R.A. & Eiras, A.E. 2008. Calibration and evaluation of field cage for oviposition study with *Aedes* (*Stegomyia*) *aegypti* female (L.) (Diptera: Culicidae). *Neotropical Entomology* 37: 478–485.

Rosa, E., Dahelmi, S, S. & Syamsuardi. 2015. Detection of transovarial dengue virus with RT-PCR in *Aedes albopictus* (Skuse) larvae inhabiting phytotelmata in endemic DHF areas in West Sumatra, Indonesia. *Journal of Infectious Diseases and Microbiology* 3(1): 14-17.

Rueda, L.M. 2004. Pictorial keys for the identification of mosquitoes (Diptera: Culicidae) associated with dengue virus transmission. *Zootaxa* 589: 1–60.

Shroyer, D.A. 1990. Vertical maintenance of dengue virus in sequential generations of *Aedes albopictus*. *Journal of the American Mosquito Control Association* 6(2): 312–314.

Thongrungkiat, S., Maneekan, P., Wasinpiyamongkol, L. & Prummongkol, S. 2011. Prospective field study of transovarial dengue-virus transmission by two different forms of *Aedes aegypti* in an urban area of Bangkok, Thailand. *Journal of Vector Ecology* 36(1): 147–152.

Vilela, A. P., Figueiredo, L. B., dos Santos, J. R., Eiras, A. E., Bonjardim, C. A., Ferreira, P. C., & Kroon, E. G. 2010. Dengue virus 3 genotype I in *Aedes aegypti* mosquitoes and eggs, Brazil, 2005–2006. *Emerging Infectious Diseases* 16(6): 989.

Wan-Norafikah, O., Nazni, W.A., Noramiza, S., Shafa'ar-Ko'ohar, S., Heah, S.K., Nor-Azlina, A.H. & Lee, H.L. 2012. Distribution of Aedes mosquitoes in three selected localities in Malaysia. *Sains Malaysiana* 41(10): 1309–1313.

Wasinpiyamongkol, L., Thongrungkiat, S., Jirakanjanakit, N. & Apiwathnasorn, C. 2003. Susceptibility and transovarial transmission of dengue virus in *Aedes aegypti*: A preliminary study of morphological variations. *Southeast Asian Journal of Tropical Medicine and Public Health* 34(l 2): 131–135.

WHO (World Health Organization). 2016. *Dengue and severe dengue.* http://www.who.int/mediacentre/factsheets/fs117/en/ [11 March 2016].

Zeidler, J.D., Acosta, P.O.A., Barrêto, P.P. & Cordeiro, J.D.S. 2008. Dengue virus in *Aedes aegypti* larvae and infestation dynamics in Roraima, Brazil. *Revista de Saude Publica* 42(6): 2–6.