INVESTIGATION ON TRANSVORIAL TRANSMISSION OF CHIKUNGUNYA AND DENGUE VIRUSES IN THE WILD POPULATION OF *Aedes* MOSQUITOES AT SELECTED RURAL LOCALITIES IN KUCHING SARAWAK, MALAYSIA

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ABSTRACT

Chikungunya virus (CHIKV) and dengue virus (DENV) are mosquito-borne viruses transmitted by *Aedes aegypti* and *Aedes albopictus.* CHIKV has become endemic in Malaysia since its reemergence in 2006, whereas DENV was first reported in Malaysia in 1902. In 2009, Kampung Pulau Salak in Kuching, Sarawak, Malaysia was one of the areas affected by chikungunya outbreak. Hence, this study was conducted to investigate the potential vector and determine the presence of CHIKV and DENV transovarial transmission among the mosquito population in Kampung Pulau Salak and Kampung Tanjong Bako, Kuching, Sarawak. Mosquito larvae were collected from ovitraps and discarded receptacles found in both villages. The emerged *Aedes* mosquitoes were pooled (223 pools) according to sex, species, and study site before being subjected to RNA extraction and two-step reverse-transcription polymerase chain reaction (RT-PCR). The species of *Aedes* mosquito caught in both villages were *Ae. albopictus* and *Ae. aegypti* which comprises 95.8% and 4.2% of the total number of *Aedes* mosquito collected respectively. CHIKV was detected in one male and one female pool of the emerged adults of *Ae. albopictus* collected from Kampung Pulau Salak, which belongs to the East/Central/South/African (ECSA) genotype, whereas DENV serotype 2 (DENV-2) was detected in one pool of male and two pools of female of emerged *Ae. albopictus* collected from the same village. CHIKV and DENV were not detected in the emerged *Aedes* mosquitoes collected from Kampung Tanjong Bako. The presence of CHIKV and DENV in the emerged adults of mosquitoes revealed evidence of transovarial transmission among the population of *Ae. albopictus* in Kampung Pulau Salak, and because *Ae. albopictus* is the major species collected from the site, this study indicates that the species may be the primary vector of the viruses in the village.

Keywords: Transovarial transmission, *Aedes* mosquitoes, chikungunya virus, dengue virus serotype 2, ECSA genotype

ABSTRAK

Virus chikungunya (CHIKV) dan virus denggi (DENV) merupakan virus bawaan nyamuk *Aedes aegypti* dan *Aedes albopictus*. Virus chikungunya telah menjadi endemik di Malaysia

selepas kemunculan semula virus tersebut pada tahun 2006, manakala kemunculan virus denggi di Malaysia mula dilaporkan pada tahun 1902. Pada tahun 2009, Kampung Pulau Salak di Kuching, Sarawak, Malaysia merupakan salah satu kawasan yang dijangkiti oleh wabak chikungunya. Oleh yang demikian, kajian ini dijalankan untuk mengesan vektor dan untuk menentukan sama ada penyebaran transovari virus chikungunya dan virus denggi berlaku dalam kalangan populasi nyamuk di Kampung Pulau Salak dan Kampung Tanjong Bako, Kuching, Sarawak. Jentik-jentik dikumpulkan dari ovitrap dan bekas terbuang yang dijumpai di kedua-dua buah kampung. Jentik-jentik (223 longgokan) diasingkan mengikut spesies, jantina dan tempat dikumpul setelah menjadi nyamuk dewasa. Setelah menjadi nyamuk dewasa, proses pengekstrakan RNA dan tindak balas rantai polimerase dengan menggunakan kaedah transkripsi berbalik (RT-PCR) secara dua langkah dilaksanakan. Spesies nyamuk yang telah dikumpulkan dari kedua-dua buah kampung tersebut ialah *Ae. albopictus* dan *Ae. aegypti* yang merangkumi 95.8% dan 4.2% masing-masing daripada jumlah keseluruhan nyamuk yang telah dikumpulkan. Virus chikungunya dari genotip *East/ Central/ South/ African* (ECSA) telah dikesan dalam satu longgokan nyamuk jantan dan satu longgokan nyamuk betina *Ae. albopictus* yang dikutip dari Kampung Pulau Salak. Virus denggi serotip 2 (DENV-2) juga dikesan dalam satu longgokan nyamuk jantan dan dua longgokan nyamuk betina *Ae. albopictus* yang dikumpul dari kampung yang sama. Virus chikungunya dan virus denggi tidak dikesan dalam nyamuk yang dikumpul dari Kampung Tanjong Bako. Kehadiran virus chikungunya dan virus denggi dalam nyamuk yang dibesarkan dari peringkat jentik-jentik menunjukkan bukti berlakunya penyebaran transovari dalam kalangan populasi nyamuk *Ae. albopictus* di Kampung Pulau Salak. Hasil kajian ini menunjukkan bahawa nyamuk *Ae. albopictus* berpotensi menjadi vektor utama virus chikungunya dan denggi di Kampung Pulau Salak kerana ianya merupakan spesies nyamuk yang paling banyak dikumpul dari kampung tersebut.

Kata kunci: Penyebaran transovari, nyamuk *Aedes*, virus chikungunya, virus denggi serotip 2, genotip ECSA

INTRODUCTION

Transovarial transmission of arboviruses in mosquito populations is a condition in which infected female mosquitoes pass the viruses to their offspring through their eggs (Heath et al. 2020). The transmission of arboviruses through mosquito progeny may serve to retain the viral pathogen in nature during inter-epidemic periods of the disease (Joshi et al. 2002). Besides, this type of transmission is believed to be the predominant way in which some arboviruses survive in harsh environmental conditions (Chompoosri et al. 2016). The presence of virus in vectors during non-epidemic periods of disease could be the cause of disease re-emergence in the previously exposed areas (Angel & Joshi 2008). Chikungunya virus (CHIKV) and dengue virus (DENV) are two viruses that have been shown to persist in mosquito populations and can co-circulate in areas where *Aedes* mosquito vectors are present (Zim et al. 2013).

Chikungunya is a viral disease that is transmitted to humans by CHIKV-infected mosquitoes. Symptoms of infection include fever, severe joint pain, muscle pain, joint swelling, headache, rash, nausea, and fatigue (WHO 2020). The symptoms may be similar to those of other mosquito-borne diseases such as dengue and Zika, and they are frequently misdiagnosed as it is difficult to distinguish them clinically (Apandi et al. 2010; Paixão et al. 2018; WHO 2020). CHIKV (Family: Togaviridae) was first detected in southern Tanzania in 1952 (Sam et al. 2006). It was suggested that CHIKV originated from Africa and

subsequently spread to Asia (Sam et al. 2006). Since 2004, chikungunya has spread quickly, and it has been identified in over 60 countries throughout Asia, Africa, Europe, and the Americas (WHO 2020).

The first outbreak chikungunya outbreak in Malaysia occurred in Klang, Selangor, between 1998 and 1999 (Lam et al. 2001), and was followed by a second outbreak in Bagan Panchor, Perak, in 2006 (Abu Bakar et al. 2007; Noridah et al. 2007). In the years 2008 to 2009, there was a massive outbreak that began in Tangkak, Johor and spread to almost every parts of Malaysia, including Sarawak (Zainah et al. 2010). Sarawak experienced a large outbreak of CHIKV in 2009 (Diop et al. 2015) and Salak Island in Kuching was one of the areas affected due to the outbreak. The number of reported cases has been increasing, with 2556 cases recorded in 2020 alone (MOH 2020). Currently, there are 409 cases recorded in Malaysia in 2021 (up until 17th March 2021) (MOH 2021).

Dengue fever is another major vector-borne disease that is a major public health concern worldwide. Dengue fever is a disease caused by DENV (Family: Flaviviridae) infection, and the symptoms ranges from asymptomatic to mild fever, as well as more serious manifestations that can be fatal, such as Dengue Hemorrhagic Fever and Dengue Shock Syndrome (Chew et al. 2012; WHO 2020). The first epidemic of dengue was reported in 1653 in the French West Indies (WHO 2011), whereas the first Dengue Hemorrhagic Fever was reported in the Philippines in 1953 (WHO 1997). Dengue fever is known to be endemic in countries throughout South Asia, South-East Asia, Africa, the Americas, the Western Pacific, and the Eastern Mediterranean regions (Salim et al. 2021).

In Malaysia, dengue fever was first reported in 1902 following an outbreak of the disease in 1901 in Penang (Abu Bakar & Shafee 2002). The first Dengue Haemorrhagic Fever outbreak in Malaysia was reported in 1962 in Penang and dengue fever had become endemic in the country by 1960s (Abu Bakar & Shafee 2002; Ali et al. 2020). The four dengue serotypes, which are serotype DENV-1, DENV-2, DENV-3 and DENV-4 (Bhatt et al. 2021), were reported to be co-circulating in Malaysia, and dengue is classified as a highly contagious threat due to increasing trend of virus infection (Pang & Loh 2016). In 2013, a new serotype known as DENV-5 was reported in Sarawak through a genetic sequence analysis in Sarawak (Mustafa et al. 2015). The number of dengue cases has been showing an increasing trend with some fluctuations (iDengue, n.d.), and 8,313 cases have been reported in Malaysia during the first four months of 2021 (MOH 2021).

Aedes mosquitoes play an important role in transmitting these diseases. Malaysia's tropical climate encourages the rapid development and population of *Aedes* mosquitoes, the vector mosquitoes of both CHIKV and DENV (Hii et al. 2016; Ng et al. 2017; WHO 2020). Various strategies include chemical, physical, biological or an integral approach such as Integrated Management Strategy for the Prevention and Control of Dengue by WHO (Sarimin et al. 2020), and several studies on vector control and surveillance have been conducted, especially in Malaysia, to control the mosquito population (Lee et al. 2015; Liew et al. 2019; Ridha et al. 2020). Even though numerous studies have been published on vector control and surveillance in Peninsular Malaysia, data on mosquito or vector surveillance in Sarawak remains scarce. There are no published data on the mosquito vector of dengue and chikungunya viruses on both of the selected rural villages. Therefore, this study aims to provide information regarding vector distribution and the possibility of transovarial transmission of chikungunya and dengue viruses among *Aedes* mosquitoes in Sarawak, Malaysia especially in Kampung Pulau Salak and Kampung Tanjong Bako. *Aedes* mosquito

survey data obtained in this study may further assist in the planning and implementation of mosquito vector control measures. In addition, data on transovarial transmission among *Aedes* mosquito populations can be used to evaluate the importance of transovarial transmission during inter-epidemic periods among wild mosquitoes in nature.

MATERIALS AND METHODS

Study Site

This study was conducted at Kampung Pulau Salak and Kampung Tanjong Bako (negative control site), both of which are in rural areas near the riverbank in Kuching, Sarawak (Figure 1). As the name implies, Kampung Pulau Salak (1º40'0.01'' N, 110º16'60'' E) is located on the Salak island which is a small island located on the Batang Salak river (Figure 2), where majority of the houses are stilt houses on water. The second study site was Kampung Tanjong Bako (1°34'26.9'' N, 110°25'45.8" E), which is located along the Sarawak River (Figure 3) and most of the houses also consist of stilt houses on water. Both villages are home for approximately 200 families respectively and fishing has been their main economic activity in the area. Kampung Pulau Salak has a history of chikungunya outbreaks dating back to 2009, with no re-emergence of the disease in the area since, whereas Kampung Tanjong Bako has no records of chikungunya or dengue outbreaks. However, based on data from Malaysia's Ministry of Health, dengue infections are still active in Sarawak, and chikungunya cases were last reported in 2019, with no cases reported since.

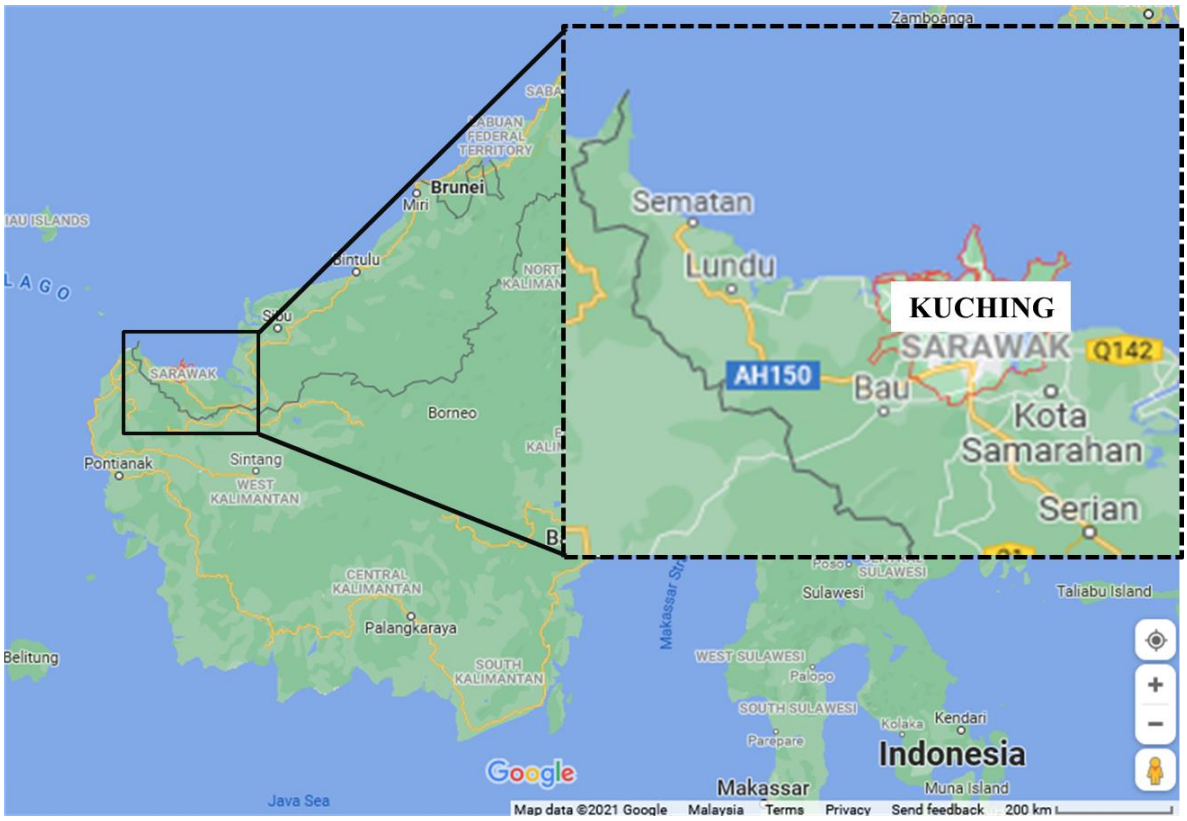

Figure 1. Map showing the location of Kuching in Sarawak

(Source: Adapted from Google maps 2021)

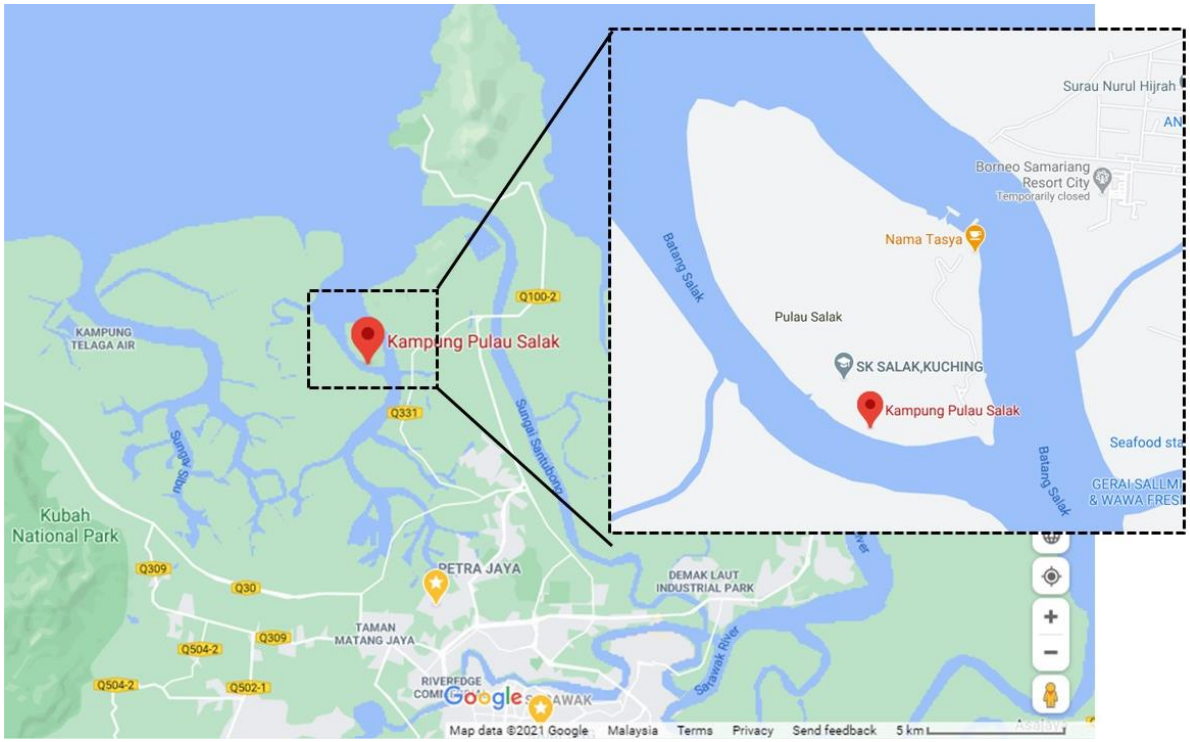

Figure 2. Map showing the location of Pulau Salak in Kuching

(Source: Adapted from Google maps 2021)

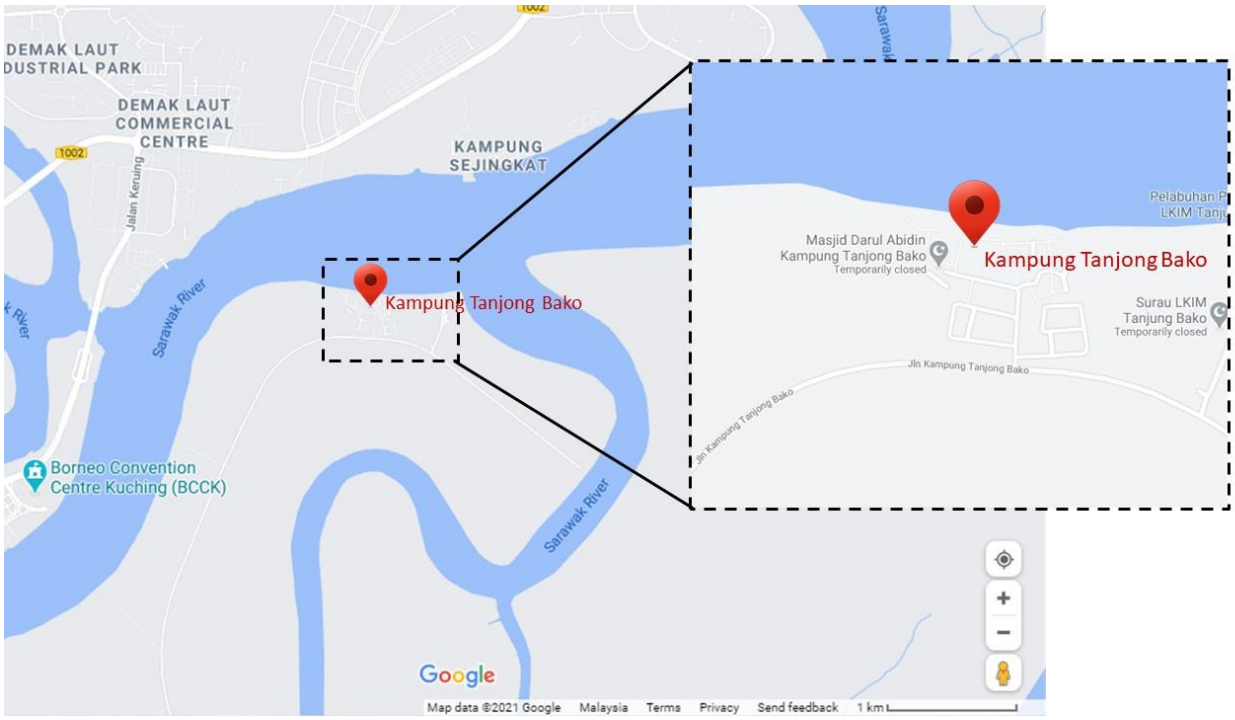

Figure 3. Map showing the location of Kampung Tanjong Bako in Kuching

(Source: Adapted from Google maps 2021)

Mosquito Collection and Identification

The experiment was conducted from September 2018 to November 2019. The mosquito larvae were collected by using ovitraps. The ovitraps used in the sampling were as described by Lee (1992) in Lau et al. (2017) with modifications. Each ovitrap consists of 250 ml black cylindrical plastic containers and the outer wall of the containers was coated with black paint. Oviposition paddle (1.7 cm x 14.9 cm) was placed diagonally in each ovitrap filled with cow-grass infusion solution following methods by Tang et al. (2007). Besides ovitraps, the immatures (larvae and pupae) were also collected from random discarded receptacles that can be found surrounding the houses in both villages. There were 130 ovitraps placed outside of the house of the villagers but in vicinity of their home in both villages (e.g. under the eave or in between flower pots). The houses were chosen randomly and covered about 70% of area in both villages. The ovitraps were left in the village for a week before being collected and brought back to the laboratory. The mosquito collections were conducted three times at each village and there are three to four persons involved in each sampling.

The pH level and temperature of the medium in the ovitraps and discarded receptacles were measured using EcoTester (OAKTON, USA). The mosquito immatures collected were reared in labelled plastic containers according to the medium in which they were collected-in room temperature at the laboratory until they emerged into adulthood. Then, the emerged mosquitoes were frozen in a -80 ºC freezer before subjected to further analysis. The mosquitoes were divided based on species then sex, in each sampling location respectively. Then, in each group, the mosquitoes were pooled together with 20 to 35 individuals per pool for RNA extraction. The collected mosquitoes were identified by using keys provided by the Division of Medical Entomology IMR, Kuala Lumpur (Abdullah 2000) and tabulated accordingly.

Molecular Analysis

RNA extraction and detection

The viral RNA was extracted using the RNeasy Mini Kit by Qiagen. The extraction process was carried out following the manufacturer's protocols (Qiagen, Germany). The RNA samples were analysed using a two-step reverse-transcription polymerase chain reaction (RT-PCR), and the RT-PCR amplified products were then viewed using gel electrophoresis.

Reverse-transcription polymerase chain reaction (RT-PCR) of CHIKV

Two-step RT-PCR was performed based on the protocol described in Thavara et al. (2009) and Nor Aliza et al. (2019), with slight modifications in which the viral RNA was extracted from mosquito's specimen instead of blood specimens as in Thavara et al. (2009). The RNA sample used is different compared with these two studies. RT step was performed to produce the cDNA of CHIKV. The primers used were chikungunya forward primer (5'-ACCGGCGTCTACCCATTCATGT-3') (genome position 10237 to 10258) and chikungunya reverse primer (5'-GGGCGGGTAGTCCATGTTGTAGA-3') (genome position 10544 to 10566) (Dayakar et al. 2015). The RT-PCR assay was carried out by using reagents from Promega (Promega, US).

In the RT step, 4.4 µL of RNase-free water, 3.0 µL of 5X RT reaction buffer, 0.2 µL of deoxyribonucleic acid mix, 0.4 µL of chikungunya reverse primer and 1.0 µL of RNase inhibitor were combined to make a master mix. About 9 µL of the prepared master mix was added into a 0.2 mL PCR tube before 5.0 µL of the sample RNA-template and 1.0 µL of Go-

script reverse transcriptase were added into the mixture. The mixture was placed in an Applied Biosystems®Veriti® thermal cycler for 30 minutes at 48 ºC.

During the PCR step, a master mix of 26.6 µL of RNase-free water, 5.0 µL of magnesium chloride, 1.0 µL of deoxyribonucleic acid mix, 1.0 µL of chikungunya forward primer, 1.0 µL of chikungunya reverse primer and 5X Taq Green Buffer was prepared. About 44.6 µL of the prepared master mix was added to a 0.2 mL PCR tube before 5.0 µL of the RT product and 0.4 µl of Go Taq DNA polymerase were added into the mixture. The PCR was run under the following conditions: Initial denaturation at 94 ºC for 2 minutes, 35 cycles of denaturation at 94 ºC for 1 minute, annealing at 54 ºC for 1 minute, extension at 72 ºC for 1 minute and a final extension at 72 ºC for 10 minutes.

Reverse-transcription polymerase chain reaction (RT-PCR) of DENV

The two-step RT-PCR was performed using the primers suggested by Lanciotti et al. (1992) and the protocol as stated in Reynes et al. (2003), Ayers et al. (2006) and Nor Aliza et al. (2019). Similar to the purpose of the RT step in CHIKV detection, the RT step was carried out to produce DENV cDNA.

The primers used were dengue forward primer (5'-TCAATATGCCTGAAACGCCCGAGAAACCG-3') (genome position 131 to 161) and dengue reverse primer (5'-TTGCACCAACAGTCAATGTCTTCAGGTTC-3') (genome position 616-644). The RT-PCR assay was carried out using reagents from Promega (Promega, US). During the RT step, a master mix of 4.4 µL of RNase-free water, 3.0 µL of 5X RT reaction buffer, 0.2 µL of deoxyribonucleic acid mix, 0.4 µL of dengue reverse primer and 1.0 µL of RNase inhibitor was prepared. About 9 µL of the prepared master mix was added into a 0.2 mL PCR tube before 5.0 µL of the sample RNA-template and 1.0 µL of Go-script reverse transcriptase were added into the mixture. The mixture was placed in an Applied Biosystems®Veriti® thermal cycler for 60 minutes at 42 ºC.

During the PCR step, a master mix of 26.6 µL of RNase-free water, 5.0 µL of magnesium chloride, 1.0 µL of deoxyribonucleic acid mix, 1.0 µL of dengue forward primer, 1.0 µL of dengue reverse primer and 5X Taq Green Buffer was prepared. The prepared master mix of 44.6 µL was added into a 0.2 mL PCR tube before 5.0 µL of the RT product and 0.4 µl of Go Taq DNA polymerase were added into the mixture. The PCR was run under the following conditions: initial denaturation at 95 ºC for 5 minutes, 35 cycles of denaturation at 94 ºC for 1 minute, annealing at 55 ºC for 1 minute, extension at 72 ºC for 1 minute and a final extension at 72 ºC for 10 minutes.

Visualisation of PCR products in agarose gel electrophoresis

Agarose gel (1.6%) was prepared by mixing 1.6 gram of agarose powder (Vivantis, USA) with 100 mL of 0.5X Tris-Borate-EDTA or TBE buffer. SYBR® Safe DNA gel stain was used as a staining dye during the process of DNA visualisation in the gel. About 10 µL of the PCR products and 5.0 µL of 100 base pair (bp) DNA ladder (Promega, US) were loaded into the wells of the gel. The voltage used for the gel electrophoresis process to visualise the PCR products of CHIKV is 100 volts for 75 minutes, whereas the voltage used for the gel electrophoresis to visualize the PCR product of DENV is 100 volts for 60 minutes. The gel was visualised under the Syngene UV transilluminator in a dark room.

Gene sequencing and validation of the CHIKV genotypes and DENV serotypes
The positive PCR products of CHIKV and DENV were sent to First BASE Laboratories, Malaysia, for sequencing in order to verify the results. The gene sequences retrieved from the laboratory were compared with Genbank using the BLAST function in NCBI and analysed for multiple alignments using the Multiple Sequence Comparison by Log-Expectation (MUSCLE) in Molecular Evolutionary Genetic Analysis (MEGA) version 7.0 software. The phylogenetic analysis was also carried out on the positive samples of CHIKV and DENV, which were aligned, respectively, with 22 reference sequences and 27 reference sequences from the GenBank using MEGA version 7.0. The phylogenetic tree was created with the maximum likelihood method and 100 bootstrap replications.

Statistical Analysis
The CHIKV and DENV positive samples were analysed for their minimum infection rates (MIR). The MIR is the ratio of the number of positive pools to the total number of mosquitoes tested. The assumption in MIR is that each positive mosquito pool contains only one infected individual. The MIR was calculated using the following formula:

$$\text{MIR} = \frac{\text{Number of pool with CHIKV/ DENV positive mosquitoes}}{\text{Total number of mosquitoes tested}} \times 1000$$

Chi-square test were performed as well by using IBM Statistical Package for the Social Sciences (SPSS) version 28 to see the association between species of Aedes mosquitoes and the containers in which the immatures were collected from.

RESULTS

***Aedes* Mosquito Larvae Distribution**
This study collected 7,710 mosquito larvae, with 65% (5,014 larvae) of the total mosquito collected from Kampung Pulau Salak and the rest from Kampung Tanjong Bako. *Aedes albopictus* was the most abundant species in both localities, accounting for 93.6% and 99.9% of the total population in Kampung Pulau Salak and Kampung Tanjong Bako, respectively. The other mosquito species collected was *Aedes aegypti*, which made up less than 10% of the total population of mosquito larvae collected, as shown in Table 1.

CHIKV and DENV Detection in Mosquito
From the RT-PCR analysis, the presence of CHIKV and DENV was only detected in *Ae. albopictus* collected from ovitraps. Out of 128 pools of emerged *Ae. albopictus* mosquito collected in ovitraps from Kampung Pulau Salak, only one pool of male and one pool of female mosquitoes were positive for CHIKV. In terms of DENV detection, three pools were found to be positive which consisted of one male pool and two female pools. The presence of viruses was indicated by the presence of DNA bands 325 bp and 511 bp for CHIKV (Figure 4) and DENV (Figure 5), respectively. All positive detections of DENV and CHIKV were detected from *Ae. albopictus* collected from Kampung Pulau Salak. No viruses were detected in mosquito samples from Kampung Tanjong Bako.

In this study, the MIR calculated for CHIKV positive samples is 0.26% for the positive female pool and 0.28% for the positive male pool. On the other hand, the MIR

calculated for positive DENV samples is 0.28% for the positive male pool and 0.52% for the positive female pools.

Table 1. Distribution of *Aedes* mosquito larvae collected from ovitraps and discarded receptacles in Kampung Pulau Salak and Kampung Tanjong Bako

Kampung Pulau Salak				
Species	**Number of individual mosquitoes**		**Total / Percentage (%)**	**Chi-square (χ^2)**
	Ovitraps	**Discarded receptacles**		
Aedes albopictus	4,497	197	4,694 (93.6%)	$\chi^2 = 2.0$ (df = 1, *P* >0.05)
Aedes aegypti	295	25	320 (6.4%)	
Total/ Percentage (%)	4,792 (95.6%)	222 (4.4%)	**5,014 (100%)**	
Kampung Tanjong Bako				
Species	**Number of individual mosquitoes**		**Total / Percentage (%)**	**Chi-square (χ^2)**
	Ovitraps	**Discarded receptacles**		
Aedes albopictus	2558	136	2,694 (99.9%)	$\chi^2 = 2.0$ (df = 1, *P* >0.05)
Aedes aegypti	2	0	2 (0.01%)	
Total/ Percentage (%)	2,560 (95.0%)	136 (5.0%)	**2,696 (100%)**	

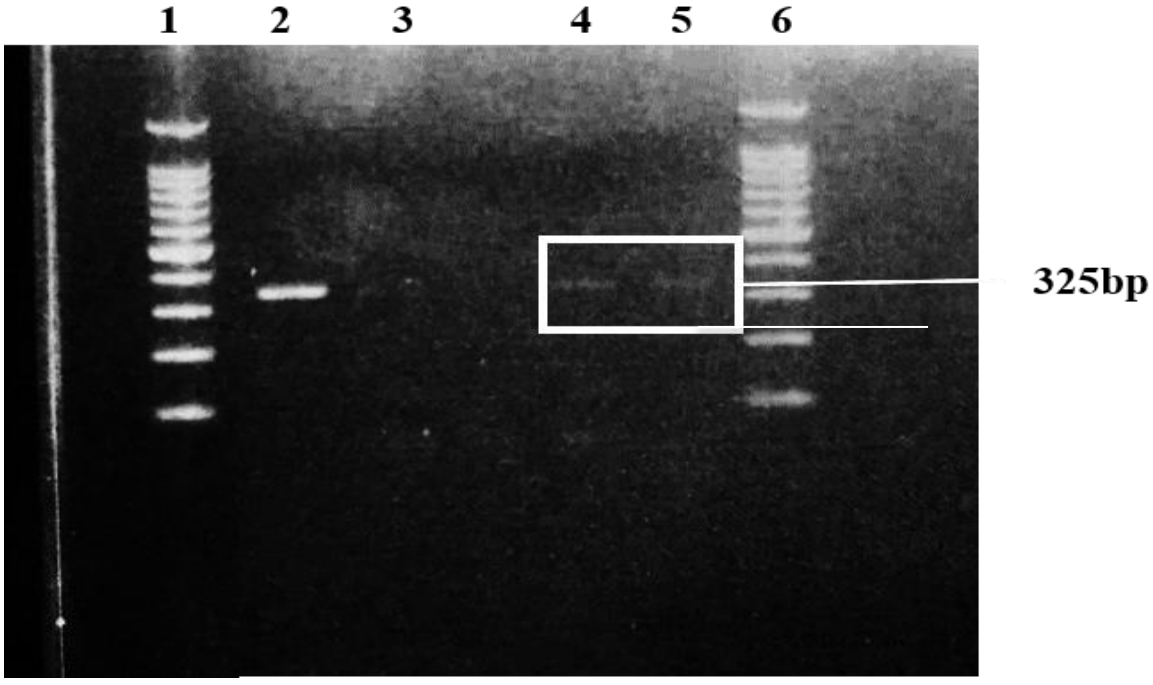

Figure 4. Gel electrophoresis image showing CHIKV positive mosquito pools from Kampung Pulau Salak, as indicated by a 325bp DNA band. (Lane 1,6: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4,5: Positive samples showing 325bp CHIKV c-DNA band). This image is the representative from positive CHIKV detection in samples

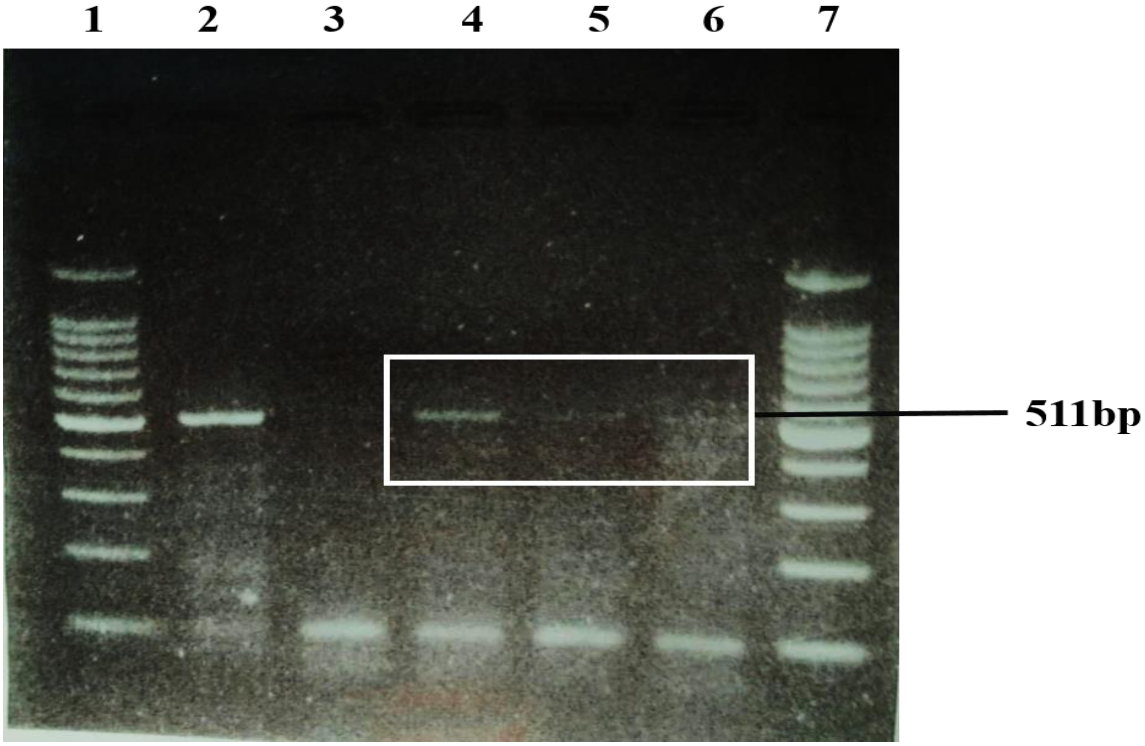

Figure 5. Gel electrophoresis image showing DENV positive mosquito pools from Kampung Pulau Salak, as indicated by a 511bp DNA band. (Lane 1,7: 100bp DNA ladder, Lane 2: Positive control, Lane 3: Negative control, Lane 4,5,6: Positive samples showing 511bp DENV c-DNA band). This image is the representative from positive DENV detection in samples

Phylogenetic Analysis of CHIKV and DENV

The gene sequencing and phylogenetic analysis of positive CHIKV samples revealed that the positive samples belong to the East Central South African (ECSA) genotype (Figure 6-A). The analysis revealed that the CHIKV isolate from the female pool of mosquitoes collected in Kampung Pulau Salak (Pulau Salak/2018/FAe.alb) is closely related to strains isolated from Sarawak (KU196264/Sarawak/2009), as the strains were grouped in the maximum likelihood tree. Meanwhile, the isolate from the male pool (Pulau Salak/2018/Mae.alb) was grouped with strains from Thailand (KX009170/Thailand/2013) (Figure 6-B).

The analysis of the positive DENV samples revealed that the DENV belongs to serotype 2. The maximum likelihood tree also shows that two of the DENV isolates from Kampung Pulau Salak are closely related to the strains isolated in Malaysia (KU666945.1/Malaysia/TM78 and KU666948.1/Malaysia/TM296) (Figure 7-A), while one of the isolates is closely related to strains from India (MG885749.1/India/2017) (Figure 7-B).

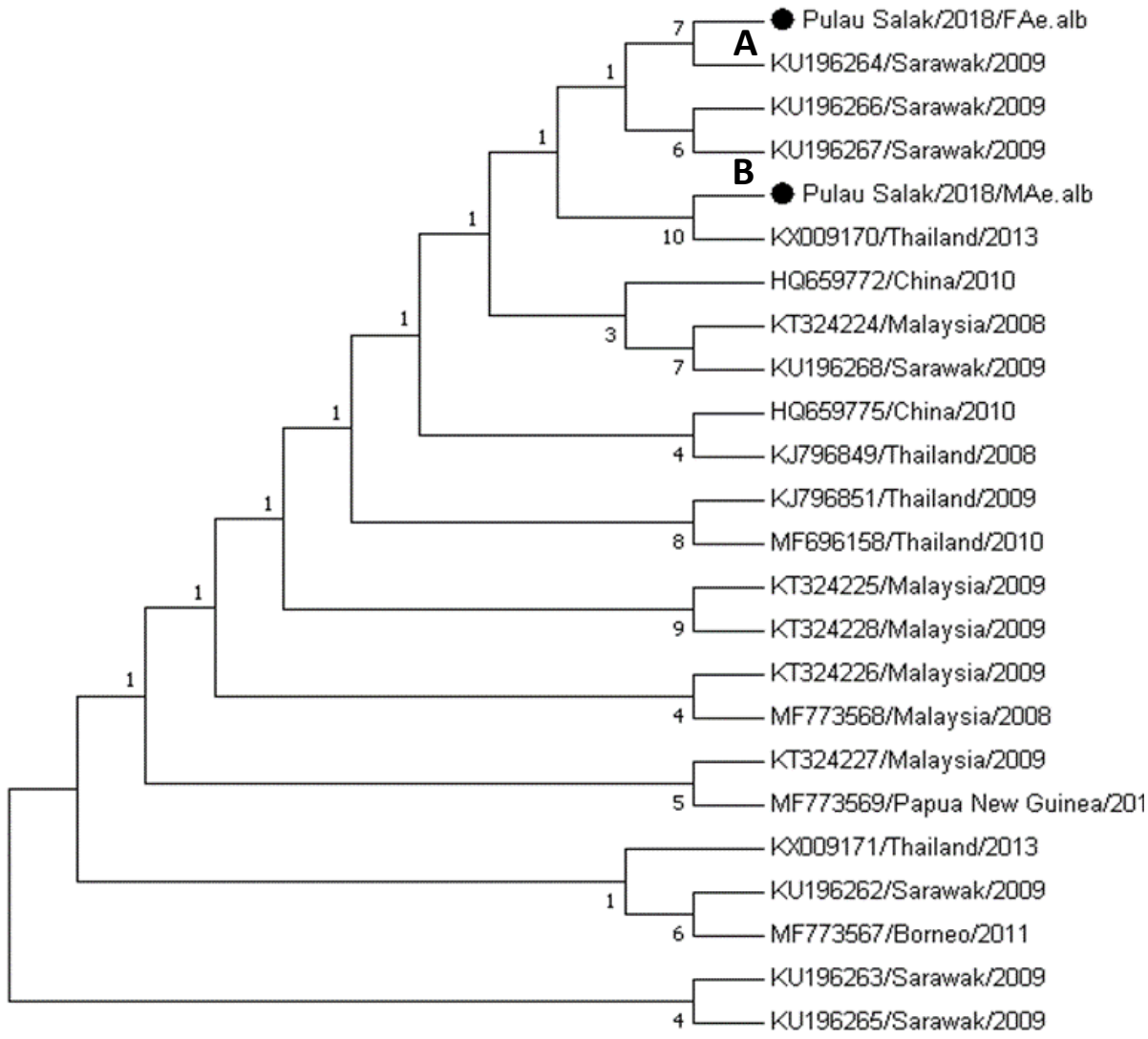

Figure 6. Maximum likelihood tree of 24 gene sequences of ECSA genotype of CHIKV (2 from this study, 22 from GenBank) created by using MEGA 7. The black dots represent the isolates from Kampung Pulau Salak (Isolates A and B). The sequences were labelled according to the accession number/ country/ year of detection or isolate number

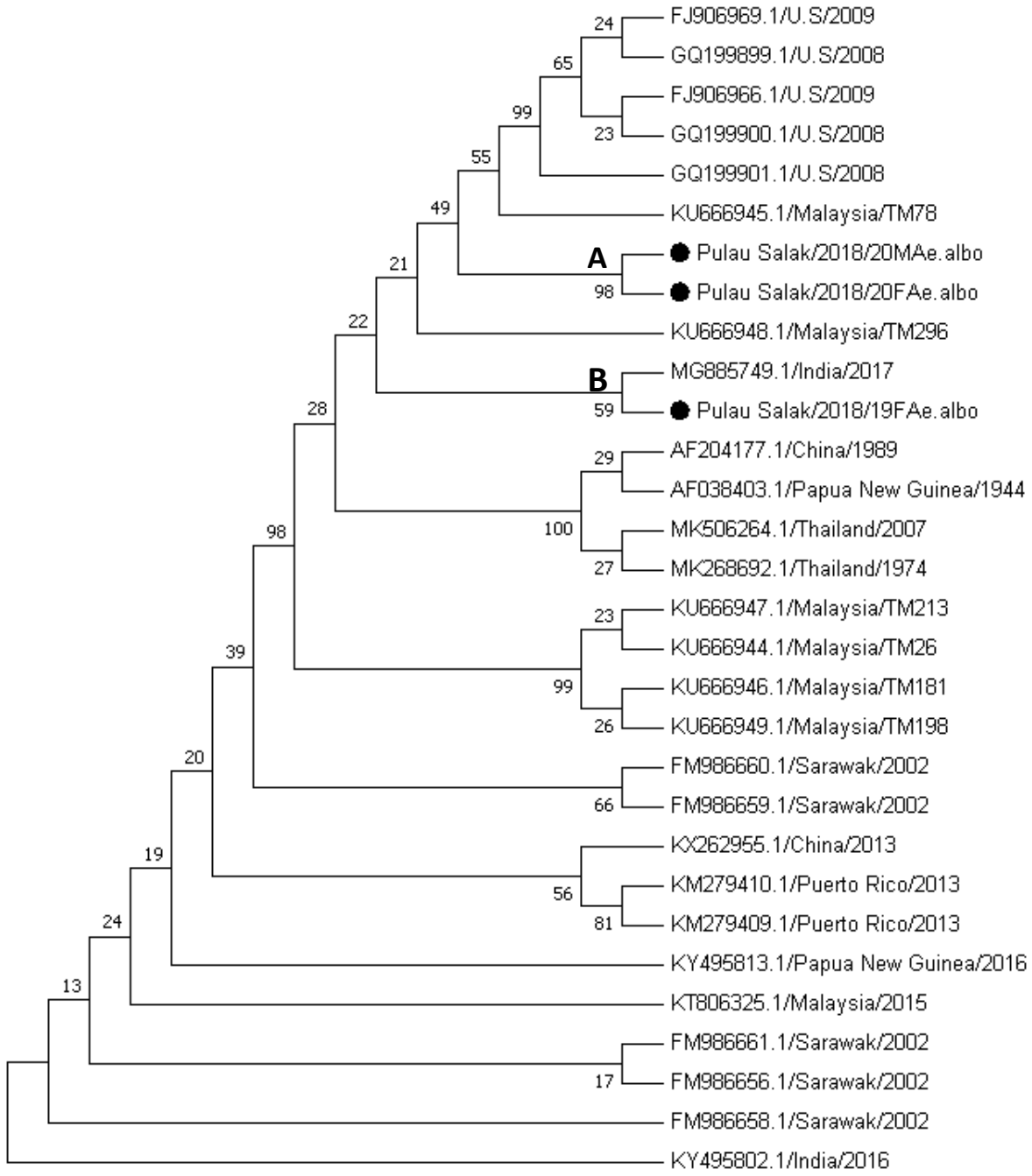

Figure 7. Maximum likelihood tree of 30 gene sequences of DENV-2 (3 from this study, 27 from GenBank) created by using MEGA 7. The black dots represent the isolates from Kampung Pulau Salak (Isolates A and B). The sequences were labelled according to the accession number/ country/ year of detection or isolate number

The *Aedes* Mosquito Survey and Population

Ae. albopictus was the most common species of mosquito collected in this study. This is not unusual, as Chang and Jute (1982) have stated that the population of *Ae. albopictus* is more extensive in Sarawak compared to *Ae. aegypti*. The authors conducted their study at 73 localities across seven divisions in Sarawak and *Ae. albopictus* was found in most of the localities (97.3%), while *Ae. aegypti* was detected only in 37 localities in the study (50.7%) (Chang & Jute 1982). The population of *Ae. aegypti* seems to be decreasing in comparison to 37 years ago, as a recent study by Lau et al. (2017) showed no presence of *Ae. aegypti* in eight divisions of Sarawak. This could be because of the population of *Ae. albopictus* is dominant that it has reduced the abundance and range of *Ae. aegypti* (Noor Afizah et al. 2015). It is not fully understood regarding the reasons of failure of the establishment of *Ae. aegypti* in all the divisions (Lau et al. 2017).

This contrasts with the distribution of *Aedes* mosquitoes in Peninsular Malaysia, where *Ae. aegypti* is still detected in surveillances, with the species being more dominant indoors (Noor Afizah et al. 2018; Rozilawati et al. 2015;). The surveillances were carried out in selected areas of Selangor, Federal Territory of Kuala Lumpur, and Penang Island. Although *Ae. aegypti* is more dominant indoors, it is not always the major *Aedes* species collected in peninsular Malaysia. Similar to our findings, Rozilawati et al. (2017) and Wan-Norafikah et al. (2020) have found that *Ae. albopictus* is the most dominant species collected during their surveillances.

Our findings showed the presence of *Ae. aegypti*, though in smaller numbers than *Ae. albopictus*. It was reported that the population of *Ae. aegypti* is lower in rural areas compared to urban areas, and the species has adapted to breed outdoors in various artificial containers that are available around human dwellings (Ndenga et al. 2017). There was no proper supply of water in Kampung Pulau Salak before 2007, which may have contributed to the presence of *Ae. aegypti*, as villagers stored rainwater in containers placed around their houses. These containers could provide an ideal breeding ground for *Ae. aegypti*.

In addition, the low number of *Ae. aegypti* in this study could be affected by communication barriers such as a lack of adequate transportation between the village and the urban areas. The communication barrier may affect the migration of *Ae. aegypti* and leads to an uneven distribution of the species in the village (Chang & Jute 1982). Also, *Ae. albopictus* has been reported to be a more important vector in village areas (Holmes et al. 2009). *Ae. albopictus* prefers to breed in containers surrounded by vegetation, whereas *Ae. aegypti* prefers to breed in artificial containers located in densely populated areas (Tedjou et al. 2020). Since Kampung Pulau Salak is a rural area, *Ae. albopictus* is more likely to thrive there.

Evidence of Transovarial Transmission of CHIKV and DENV in Mosquitoes

The presence of CHIKV and DENV in emerged adults of *Ae. albopictus* collected from the ovitraps indicates the occurrence of transovarial transmission among the mosquito population in this study. It was known that the eggs of *Aedes* mosquitoes can survive in dryness and remain viable for a longer period in harsh environmental condition, which allows for the retention of CHIKV and DENV in the mosquito eggs (Chompoosri et al. 2016; WHO 2020). Besides, it was also reported that the ribonucleic acid (RNA) of CHIKV can be retained for an extended period in mosquito eggs, which can be detected in the infected emerged adults (Wong et al. 2016).

Similar to our study, the occurrence of transovarial transmission of CHIKV was observed in a study conducted by Niyas et al. (2010) in which the authors have reported the presence of CHIKV in emerged adults from larvae collected in the households of chikungunya patients in Kerala, India. The larvae were reared in the laboratory before being subjected to RNA extraction and RT-PCR for CHIKV detection. In Malaysia, however, the presence of CHIKV was detected in field-caught adult female *Ae. albopictus* mosquitoes rather than the emerged *Ae. albopictus* mosquitoes (Rozilawati et al. 2017). Heath et al. (2020) also collected mosquito larvae from the field through ovitraps and water-holding containers; CHIKV was detected in their emerged *Aedes* mosquito samples.

DENV transovarial transmission was also observed in our study, which is similar to previous studies conducted in Kenya (Heath et al. 2020) and in urban localities of Kuching and Samarahan (Nor Aliza et al. 2019). The first detection of DENV transovarial transmission in selected localities in Kuching and Samarahan areas was described by Nor Aliza et al. (2019), where they found both DENV-1 and DENV-2 in emerged *Ae. albopictus* samples. Previous studies in Peninsular Malaysia found DENV in their mosquito samples, with most of the samples isolated from *Ae. albopictus* (Lee et al. 2005; Rohani et al. 1997; 2014). These results were similar to our result that DENV was only detected in emerged adults of *Ae. albopictus* in Kampung Pulau Salak.

CHIKV and DENV were not detected among collected *Ae. aegypti* in either village. As the population of this mosquito species is low in the study site, the presence of both viruses in the mosquitoes cannot be determined. There is a possibility that the viruses could be detected in *Ae. aegypti* if the presence of the mosquito species is higher in the villages as according to WHO (2020), this species is also a vector of both of the viruses. *Aedes aegypti* was the primary vector involved in human transmission before the outbreak in Reunion Island in 2005 to 2006 (Tsetsarkin et al., 2007). This large-scale epidemic happened in a region lacking this typical vector, and Tsetsarkin et al. (2007) found that a mutation in the E1 gene of the CHIKV (A226V) was responsible for CHIKV adaptation to *Ae. albopictus* and caused a significant increase in CHIKV infectivity. There is a possibility that the CHIKV detected in this study has this mutation, which would explain the presence of the virus in *Ae. albopictus* mosquitoes.

Furthermore, there is a difference in the ability of *Ae. albopictus* and *Ae. aegypti* to transmit one of the CHIKV genotype, which was the Indian Ocean lineage (Chompoosri et al. 2016). Although *Ae. albopictus* is known to be a less competent vector than *Ae. aegypti* (Joanne et al. 2017), they are more competitive at CHIKV infection and transmission (Chompoosri et al. 2016) and maintaining dengue infection due to their possible high rate of transovarial transmission (Joanne et al. 2017).

The Minimum Infection Rate of CHIKV and DENV and Association Between Species of Aedes Mosquitoes Collected and Its Breeding Containers

MIR is one of the alternatives commonly used in arbovirus surveillance, and one of the benefits of arbovirus surveillance is to monitor the presence of pathogen (Gu & Novak 2003). In this study, the MIR for the CHIKV positive female pool and male pool of the emerged mosquitoes are 0.26% and 0.28%, respectively, whereas the MIR calculated for the positive samples of DENV is 0.28% for the positive male pool and 0.52% for the female pools in this study. The MIR value of positive samples of both CHIKV and DENV obtained are extremely low when compared to the MIR values recorded in studies by Rohani et al. (2005). This

might be because the colonies of mosquitoes in their study were artificially infected with CHIKV in the laboratory, resulting in a high MIR value (71%).

Meanwhile, the MIR value obtained in a study by Lee et al. (2005) for DENV positive pools of *Ae. albopictus* ranged from 2.35% to 14.30% with a larger number of mosquito samples collected, which included 19,434 *Ae. albopictus* larvae and 3,759 *Ae. aegypti* larvae. The MIR values recorded are much higher compared to the MIR value recorded in this study. In addition, the MIR value for the positive pools of DENV in Kampung Pulau Salak is also lower compared to those in Sibu and Miri divisions in which a value of 11.8% is considered high in the study (Harvie et al. 2020). The low MIR value calculated from the positive pools of mosquitoes in this study indicates that the chance of CHIKV and DENV outbreaks in Kampung Pulau Salak is low, as higher mosquito infection rates are assumed to increase the rate of arbovirus transmission (Bustamante et al. 2010). Thus, the maintenance of CHIKV and DENV among the vector mosquito population in the village will be low as the MIR is low. However, since there are infected mosquitoes in this study, greater vector surveillance are required as the viruses can persist in a community through transovarial or transvertical transmission among the mosquitoes population (Vikram et al. 2015). On the other hand, statistical analysis by chi-square test (χ^2=2.0, df=1, P >0.05) shown there was no significant difference on the species of mosquitoes collected and the breeding containers as the p-value is greater than 0.05. Value of p-value would be significant if it is less than 0.05 (Oladapo et al. 2020).

Phylogenetic Analysis of CHIKV and DENV

The CHIKV genotype detected in this study is the ECSA genotype. In Malaysia, two CHIKV genotypes are known to be circulating: Asian and ECSA genotypes. Asian genotype was detected during the first and second outbreaks of CHIKV in Klang and Bagan Panchor, respectively (Abu Bakar et al. 2007; Lam et al. 2001). The ECSA genotype in Malaysia was first reported in Kinta District, Perak, in 2006 (Noridah et al. 2007; Sam et al. 2009). Similar to the situation in Malaysia, a shift of the Asian genotype to the ECSA genotype of CHIKV caused outbreak in India (Yergolkar et al. 2006). The ECSA genotype may have arrived in Malaysia because of human travel to CHIKV-affected regions such as India (Noridah et al. 2007). Apandi et al. (2011) also reported that the ECSA genotype was responsible for the massive outbreak affecting all states in Malaysia. The CHIKV detected in this study may be directly related to the genotypes reported previously as the strains were grouped in the phylogenetic trees.

In terms of DENV, dengue of serotype 2 (DENV-2) was detected in the positive samples from Kampung Pulau Salak. This is not surprising given that DENV-1 and DENV-2 were detected in emerged adults of *Ae. albopictus* in certain areas of Kuching and Kota Samarahan in Sarawak (Nor Aliza et al. 2019). In addition, Holmes et al. (2009) reported the presence of DENV-2 in the isolates obtained from Serian, Lundu, and Sibu from 1997 to 2002, indicating that the virus has circulated this serotype in Sarawak since 1997.

CONCLUSION

This is the first study to demonstrate the presence of circulating of CHIKV and DENV in Kampung Pulau Salak. The findings of this study have shown that there is a presence of transovarial transmission of CHIKV of ECSA genotype and DENV serotype 2 among the population of *Aedes albopictus*, as the virus was only detected in this mosquito species. This study also indicates that *Ae. albopictus* has the potential to be the main vector of CHIKV and DENV in Kampung Pulau Salak, Kuching, due to its dominance. More samples should be

collected in the future to conduct a thorough investigation into the presence of transovarial transmission of CHIKV and DENV, which can serve as a reference in case of a future outbreak. This study may also help in estimating the risk of infection of both viruses in Kampung Pulau Salak.

ACKNOWLEDGEMENTS

We would like to thank the Faculty of Medicine and Health Sciences, Universiti Malaysia Sarawak, for providing the necessary facilities this study, as well as the Institute of Health and Community Medicine, Universiti Malaysia Sarawak, for providing positive control of CHIKV and DENV. This study was funded by Special MyRA Assessment Funding Grant (F05/SpMYRA/1714/2018).

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