Malays. Appl. Biol. (2019) 48(1): 51–59

DETECTION OF DENGUE VIRUS FROM FIELD-CAPTURED *Aedes albopictus* IN SEBERANG TAKIR, KUALA NERUS, TERENGGANU, MALAYSIA

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Accepted 24 January 2019, Published online 20 March 2019

ABSTRACT

Dengue is an arthropod-borne disease caused by dengue virus (DENV) infection. The virus is transmitted to humans through the bite of *Aedes* mosquito. Seberang Takir of Kuala Nerus, Terengganu, had been identified as one of dengue hotspots representing a large number of dengue cases in 2016. This study aimed to screen for the presence of DENV in *Aedes* species of Seberang Takir and to estimate the viral infection in mosquitoes through minimum infection rate (MIR) and maximum likelihood estimation (MLE). Mosquitoes were caught using carbon dioxide-baited trap, attractive sugar bait trap and human landing catches (HLC) from October 2016 to January 2017. HLC was found to be the most efficient mosquito collecting method yielding 1151 individuals. Out of these, 337 mosquitoes belonged to *Aedes albopictus*, representing 334 females and 13 males. DENV was screened from these mosquitoes using reverse transcriptase-polymerase chain reaction. Interestingly, two out of 15 mosquito pools (7 females per pool) were found positive for DENV, whereas the remaining pools were shown to be negative for the virus detection. This reveals a MIR 19.05 and MLE 20.24 per 1,000 mosquitoes, which indicates a potential risk of dengue transmission in Seberang Takir. Detection of DENV in *A. albopictus* females is alarming, which requires rigorous vector control in order to prevent severe dengue outbreaks in the future.

Key words: Dengue virus, *Aedes albopictus,* mosquito detection, mosquito traps, reverse-transcriptase PCR

INTRODUCTION

Dengue is endemic in more than 100 countries, particularly in the South East Asia, Western Pacific, and the United States of America (WHO 2011). The disease is estimated to cause 390 million infections per year, which contributes to one of the most important viral diseases worldwide (Bhatt *et al*., 2013). Dengue is caused by dengue virus (DENV), which belongs to the genus *Flavivirus* of the family *Flaviviridae*. The virus is an enveloped positive-sense single stranded RNA virus of approximately 11kb genome size. Infection with DENV may lead to a spectrum of clinical manifestations ranging from mild dengue fever to the more severe and life-threatening forms; dengue haemorrhagic fever and dengue shock syndrome (Gubler, 2006). The complex pathology of dengue illness in humans and the lack of effective dengue vaccine hampers efficacious treatment for humans against dengue.

DENV is transmitted to susceptible vertebrate hosts through the bite of infected mosquito vectors, *Aedes aegypti* and *Aedes albopictus*. Transmission of DENV occurs through human-to-mosquito-to-human cycle or horizontal transmission, which is considered as the major route of virus persistence in an endemic area (Carrington & Simmons, 2014). On the other hand, transovarial or vertical transmission occurs within the mosquito population by which DENV-infected female mosquito transfers the virus to the offspring. This has been suggested as a mechanism that ensures the survival of DENV during adverse conditions without apparent need of human infection (Angel & Joshi, 2008). In the wild, several studies have demonstrated the isolation of DENV from field-collected adult *Aedes* species

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using molecular techniques (Chen *et al*., 2010; Liotta *et al*., 2005). Along with detecting of viral infection, this also allows discrimination of the viral subtypes infecting mosquitoes (Pérez-Castro *et al*., 2016). Other studies on immature stages of mosquitoes similarly detected the co-circulating DENV serotypes during an outbreak and during inter-epidemic periods (Le Goff *et al*., 2011; Martins *et al*., 2012).

Cases of dengue has increased significantly in recent years particularly in urban and semi-urban areas resulting in local spread within a community or to a different geographical region (Messina *et al*., 2014). Virological surveillance through molecular methods has been suggested as an informative and sensitive tool in assessing vector infection thus, the infection risk to human populations (Medeiros *et al*., 2018). Vector control program via virological surveillance remains the only effective method to prevent DENV transmission by monitoring DENV prevalence in *Aedes* population (Macedo *et al*., 2013). Detection of DENV infection from adult *Aedes* sp. can be used as an early warning system to monitor the prevalence of virus-infected mosquitoes and predicting possible dengue outbreaks.

In this study, the presence of DENV in *Aedes* species was evaluated from field-collected mosquitoes of Seberang Takir, Kuala Nerus. Kuala Nerus has recorded 197 dengue cases, the second highest number of cases after Kuala Terengganu, during dengue outbreak in 2016 (Malay Mail, 2016). Seberang Takir is a small town in Kuala Nerus, which consists of 14 small villages and a population of approximately 30,000. The town has been identified as one of the local hotspots for dengue (Moe *et al*., 2016). DENV were screened from the mosquito RNA samples using reverse transcriptase-polymerase chain reaction (RT-PCR), a rapid and highly sensitive molecular technique, which can specifically detect the presence of DENV RNA. Findings from this study allow an estimation of the rate of mosquito population carrying DENV during monsoon season by determining the virus minimum infection rate and maximum-likelihood estimation.

MATERIALS AND METHODS

Sampling locations

Mosquitoes were captured in three locations of Seberang Takir covering approximately one kilometre radius within an area of human premises and small vegetation (Table 1 & Figure 1). The mosquito sampling was conducted between 18:00 h to 19:30 h for at least six times a month, within four months of the study period. In total, 24 times of sampling activities were carried out from October 2016 to January 2017.

Collection and characterisation of *Aedes* mosquitoes

Mosquitoes were collected by human landing catches (HLC), a method that uses humans as bait where mosquitoes are collected from the exposed limb of the collector as they land on the skin. At least two individuals served as human baits for collection of mosquitoes in this study. To increase

Table 1. GPS coordinates of the mosquito sampling locations

Location	GPS coordinate
A	N 05º 21.085', E 103º 07.381'
B	N 05º 21.180', E 103º 07.439'
C	N 05º 21.290', E 103º 07.503'

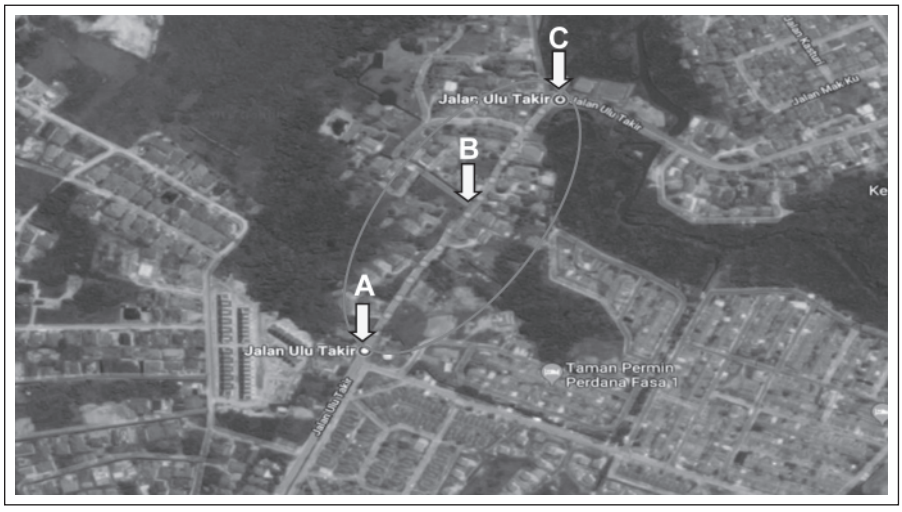

Fig. 1. A map representing three mosquito sampling locations (A, B and C) in Seberang Takir, Kuala Nerus. Image derived from Google Maps (https://maps.google.com/)

the number of mosquitoes collected, two types of mosquito traps; carbon dioxide-baited trap and attractive sugar bait trap, were used at each sampling location. In total, there were six traps set up at three different sampling locations. The carbon dioxide-baited trap uses black container containing water solution of brown sugar and yeast as previously described by Saitoh *et al*. (2004). Attractive sugar bait (ASB) trap was prepared using plastic container baited with cotton and soaked in sucrose attractants from local fruits such as honeydew, banana and guava (Qualls *et al*., 2015). Both mosquito traps were placed under shades at each sampling site overnight and the total number of adult mosquitoes was recorded the following day. Field-captured mosquitoes were taken to the laboratory of School of Fundamental Science, Universiti Malaysia Terengganu (UMT) and characterised under stereomicroscope based on the key identification established for *Aedes* species (CDC, 2012). The mosquitoes were further sorted out as males and females, and subsequently stored at -20°C. Physical parameters such as temperature and humidity were recorded at each sampling site using 3-in-1 hygrometer (Extech 45160).

Extraction of Dengue virus RNA

Mosquitoes were pooled into 1.5 mL eppendorf tube (seven mosquitoes per pool) and homogenised in phosphate buffered saline using a micro pestle. DENV RNA was then extracted from the mosquitoes using TRIzol Reagent (Invitrogen) according to the manufacturer's protocol. In brief, TRIzol Reagent was added for homogenisation, followed by addition of chloroform and centrifugation of the mixture at 12,000 x g for 15 min at 4°C. RNA was precipitated from the upper aqueous phase with 400 µL of isopropanol by centrifugation at 12,000 x g for 10 min at 4°C. The resulting RNA pellet was washed with 1 mL of 75% of ethanol and centrifuged at 8,000 x g for 5 min at 4ºC. RNA pellet was dried for 5 min, and then resuspended in 30 µL of nuclease free water. The RNA was quantified using NanoDrop and stored at -20°C.

Amplification of Dengue virus gene

DENV gene was amplified by RT-PCR using one-step Access RT-PCR kit (Promega) according to manufacturer's protocol with a minor modification. The modification involved pre-heating of PCR Master Mix containing 1 µg RNA template at 94°C for 2 min to resolve RNA secondary structures. This was followed by addition of 0.1 U/µL of each AMV reverse transcriptase and T*fl* DNA polymerase into the mixture. First-strand cDNA synthesis was performed at 45°C for 45 min followed by denaturation at 94°C for 2 min. Next, the cDNA was amplified for 40 cycles at 94°C denaturation for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. DENV RNA was targeted using dengue primers (D1 and D2) as previously reported by Lanciotti *et al*., (1992). Specific *Actin* primers (Act-F and Act-R) were used to detect the mosquito *Actin* gene. PCR products were resolved on 1.2% Agarose and stained with ethidium bromide.

Infection rate

Minimum infection rate (MIR) was calculated to determine the rate of mosquito population carrying DENV. MIR is estimated from the number of DENV-positive mosquito pools divided by the total number of mosquitoes tested, multiplied by 1000 (Gu *et al*., 2003). A maximum-likelihood estimate (MLE) of the actual number of mosquitoes infected was also calculated based on the following equation as previously described (Gu *et al*., 2003; Condotta *et al*., 2004).

$$MLE = [1- (n-X/n)^{1-m}] \times 1000$$

Where n is the number of pool tested, X is the number of positive pools, and m is the pool size.

Statistical analysis

A binomial test was performed using Minitab® 16.2.4 (Minitab Inc.) to determine the difference in the number of male and female mosquitoes captured in this study.

Ethics statement

Human subjects were trained on how to collect mosquitoes prior to the sampling activity. All practical steps to minimize risk to the collectors have been implemented. Subjects were required to put on protective clothing and exposed only certain part of their arms and legs. Mosquitoes were immediately captured when they land on the skin, before the biting process begins. All subjects were protected with insurance policy should there be any case of disease or injury throughout the study.

RESULTS AND DISCUSSION

Collection of mosquitoes

A total of 1151 adult mosquitoes were collected from Seberang Takir within four months our study, during October 2016 to January 2017 (Table 2). All mosquitoes were successfully captured using HLC method and none were captured by the two baited traps method. Of these field-captured mosquitoes, only 347 (30.1%) were *Aedes,* that were identified as *A. albopictus* and 804 (69.9%) were mosquitoes of other species, mostly *Culex* species. However, none of the mosquitoes obtained were *A. aegypti*,

Table 2. Total number of mosquitoes collected in Seberang Takir from October 2016 to January 2017 and the average environmental parameters

Months	Number of mosquitoes	*Ae. albopictus*		Other mosquito species	Average		Rainfall* (mm)
		Male	Female		Humidity (%)	Temperature (°C)	
October 2016	280	–	37	243	78.2±5.81	27.8±1.54	283.3
November 2016	387	–	46	341	82.6±4.90	27.6±1.47	612.5
December 2016	219	–	61	158	77.7±2.26	26.9±0.90	562.5
January 2017	265	13	190	62	73.0±1.86	28.7±0.51	210.0
Total	**1151**	**13**	**334**	**804**	**–**	**–**	**–**

*Data obtained from World Weather and Climate Information (https://weather-and-climate.com/)

which could be due to the natural behavior of this species that prefer to stay indoors compared to *A. albopictus* (Chadee, 2013). There was a significant difference in the number of females and males of *A. albopictus* captured (334 females versus 13 males; Binomial test, two-tailed, $p<0.01$; Table 2). This was not surprising because the HLC method provides a direct human-vector contact and thus attracts female mosquitoes that seek human blood for feeding. Human blood contains the necessary proteins required for the female mosquitoes to develop their eggs and complete their life cycle. Male mosquitoes in contrast, feed on flower nectar and hence, did not easily get captured using HLC. Mosquitoes were collected in Seberang Takir during dusk (18:00 h to 19:30 h) because the *Aedes* species are known to have peak biting activity from 15:00 h until 20:00 h in the evening (Chen *et al*., 2014). Apart from that, it is noteworthy to report that none of the mosquito traps used were successful in collecting mosquitoes. This supports the fact that HLC is the most reliable and efficient method for evaluating viral transmission in mosquito (Ndiath *et al*., 2011; Achee *et al*., 2015).

Environmental parameters such as humidity and temperature were recorded during collection of mosquitoes (Table 2). November was found to be the most humid month (82.6%) compared to the rest of months, probably due to the beginning of rainy season when the north-east monsoon wind blows between November and March in Terengganu. The average temperatures gradually increased by the end of rainy season, shown to be the highest (28.7°C) in January. Although the total number of mosquitoes collected in variable species was the greatest in November, the most *Aedes* species found was three times greater in January 2017. This is expected since previous studies have shown that abiotic factors such as temperature, rainfall and humidity affect the population growth of mosquitoes (Eisen *et al*., 2014; Hashim *et al*., 2008). During rainy season, the weather is relatively warm and humid which provides suitable conditions for breeding of mosquito population (Nurin-Zulkifli *et al*., 2015, Basari *et al*., 2016). We do believe that the number of mosquitoes obtained could be higher if the sampling was conducted after the rainy season, i.e. when the dry season begins. However, it was not feasible to extend the sampling activity after the rainy season because of time constraints and data collection can only be done within the teaching semester (September – January). It is therefore recommended that a more comprehensive study on virological surveillance and correlation with environmental parameters covering a larger area of Kuala Nerus should be conducted in order to support the present findings.

Detection of DENV from mosquitoes

To detect DENV, RNA isolated from the mosquitoes were subjected to RT-PCR using universal dengue primers, D1 and D2 as previously reported (Lanciotti *et al*., 1992). Mosquitoes were pooled into one reaction according to the established method (Condotta *et al*., 2004). Due to the limitation of reagents provided in the Access RT-PCR kit, which only can accommodate 15 reactions, the mosquito pools were selected randomly from each month and subjected to RT-PCR. A total of 105 mosquitoes were tested by dividing them into 15 pools i.e., seven mosquitoes per pool. Three pools of mosquitoes were collected in October, and four pools each in November, December and January. Data obtained from the DENV screening by RT-PCR showed that two out of four pools of female mosquitoes collected in November were positive for DENV, demonstrated by amplification of a specific DENV gene fragment of 511 bp (Figure 2). The remaining samples collected in October, December and January however, did not show any amplification, which indicates no DENV detection. The use of *Actin* gene of RNA mosquito in this study represents good quality of total RNA isolated from mosquitoes.

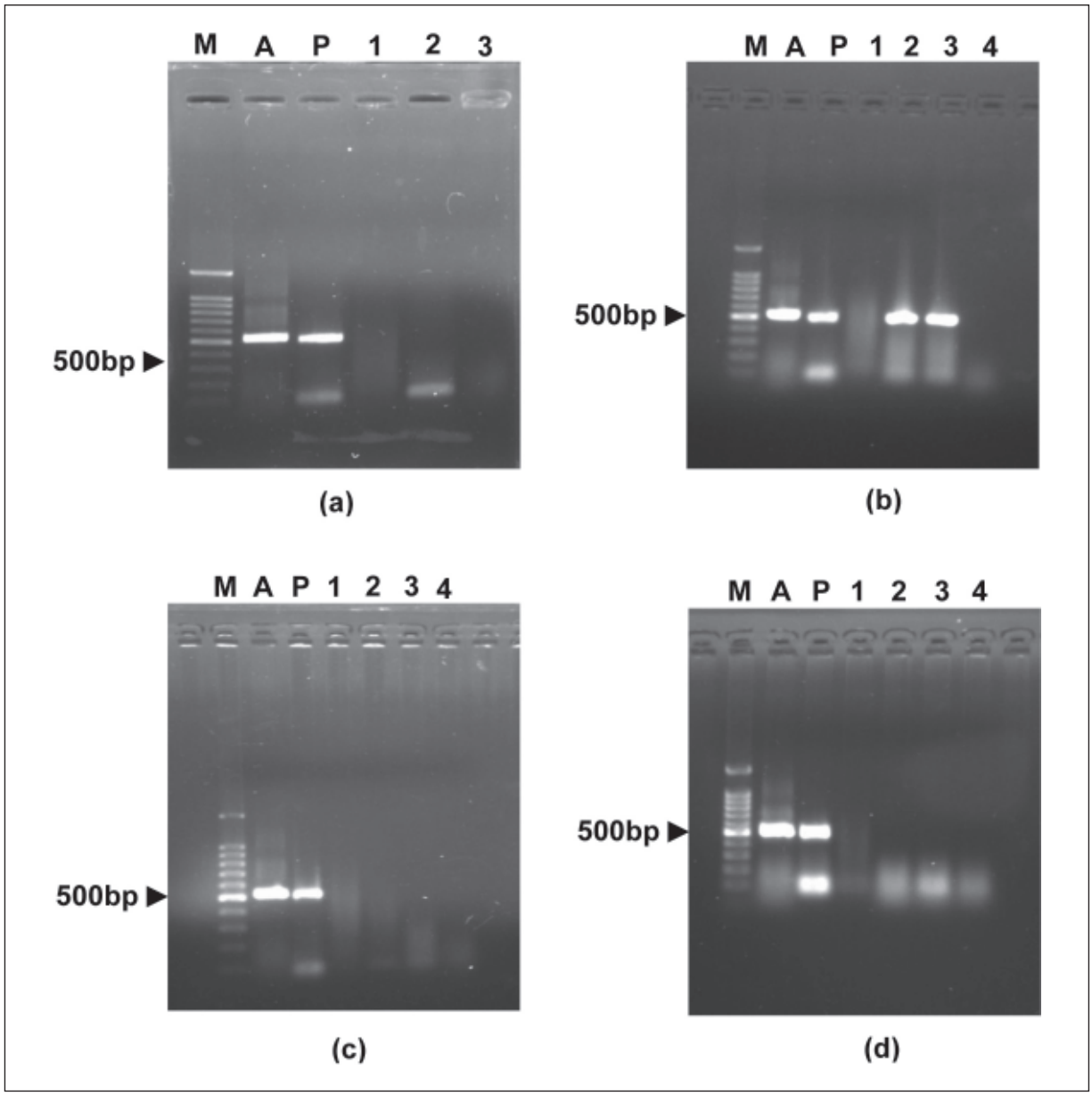

Fig. 2. Amplification of DENV gene from mosquito samples by RT-PCR. Lane M is 100 bp DNA ladder; Lane A is *Actin* gene of mosquito RNA (518 bp); Lane P is DENV-2 positive control (511bp); (a) Lanes 1 to 3 are *Ae. albopictus* females collected in October 2016; (b) Lanes 1 to 4 are *Ae. albopictus* females collected in November 2016; (c) Lanes 1 to 4 are *Ae. albopictus* females collected in December 2016; (d) Lane 1 is male *Ae. albopictus* collected in January 2017, whereas Lanes 2 to 4 are *Ae. albopictus* females.

Data from this study indicated the two pools of female *A. albopictus* collected in November contain DENV, which could be due to the mosquito blood probing from DENV-infected human. It is known that once infected, female mosquito usually will carry the virus for the rest of its life. There is also scientific evidence that female mosquitoes are able to transmit DENV to their offsprings, through transovarial transmission (Thongrungkiat *et al*., 2011; da Costa *et al*., 2017). This allows infected female mosquito to transfer DENV to the eggs, which subsequently hatch into adult mosquito harbouring DENV. There was no male *A. albopictus* in this study, which showed the presence of DENV, although male mosquitoes can be positive for DENV through transovarial transmission (Espinosa *et al*., 2014). Such transmission has been shown to occur prior to dengue incidence in human (Lee & Rohani, 2005).

The success in establishment of DENV infection and transmission in mosquitoes is generally determined by the competency of mosquito host. This ability varies upon the biting rate, survival rate and its ability to acquire host (Severson & Behura,

Table 3. Minimum infection rate and maximum likelihood estimation of dengue virus in adult mosquitoes of Seberang Takir from October 2016 to January 2017

Month	Number of mosquitoes tested	Number of pools	Positive pools	MIR	MLE
October 2016	21	3	0		
November 2016	28	4	2	**19.05**	**20.24**
December 2016	28	4	0		
January 2017	28	4	0		
Total	**105**	**15**	**2**		

2016). Mosquito acquires the virus from feeding on blood, and the virus must be able to overcome the innate immune mechanisms and organ-specific antiviral effectors of mosquitoes before being transmitted to a susceptible vertebrate host (McFarlane *et al.*, 2014).

Estimation of viral infection rate

In the past, *Aedes* surveillance has relied heavily on larval stages of the mosquito due to inefficient methods of capturing adult mosquitoes (Mackay *et al*., 2013; CDC, 2016). However, since adult mosquitoes are directly involved in dengue transmission to humans, this prompted us to seek for the mosquitoes with virus detection and establish the infection rate thresholds for further risk assessment of human disease. Accurate estimation of infection rate is an important element in public reporting, which in turn relates to infection control in an area. Basically, there are two common approaches in estimating the proportion of infected individuals from pooled samples, namely MIR and MLE. MIR is calculated, as the ratio of the number of positive pools to the total number of tested mosquitoes, assuming that there is only one infected individual in each positive pool. This is a general practice in studies with large number of mosquito samples which estimates the lower limit of the true infection rate caused by pathogenic microorganisms (Clements, 2012). Although the actual infection rate is ideally determined by testing the presence of DENV in an individual mosquito, this approach has limitations of time and cost. Analysis of pooled mosquitoes of similar species are rather simple, cost-effective where the pool size is kept small and constant (Condotta *et al*., 2004). Another way of estimating the proportion of infected mosquitoes in pooled sample is by MLE, which estimates the true infection rate and loosen the MIR assumption of only one infected individual occuring in a positive pool (Gu *et al*., 2004).

In this study, a total of 15 *A. albopictus* pools (105 individuals) were screened for DENV. Out of these, two pools were found positive for DENV (Figure 2), which resulted in MIR 19.05 and MLE 20.24 per 1,000 mosquitoes (Table 3). The rate of infected mosquitoes however, is below the value obtained in Selangor (MIR 38.02), which has the most dengue cases and dengue related deaths in Malaysia (Lau *et al*., 2015), while MIR 50-57.6 has been reported in *Aedes* collected in Singapore (Chow *et al*., 1998). Estimation of MIR is useful in monitoring dengue transmission, thus this finding correlates well with the incidence of dengue in Kuala Nerus, which suggests a potential risk of DENV transmission in Seberang Takir. Our MIR value was supported by maximum-likelihood estimation, which usually gives values closer to the actual infection rate in mosquitoes (Gu *et al*., 2004). The close agreement of MIR and MLE data may be due to the constant and small number of mosquito pool size (Condotta *et al*., 2004), which suggests both estimates can be used to assess potential transmission risk of disease. This also highlights the likelihood that only one or very few of the positive pools were infected with DENV, which usually occurs in area of low dengue virus transmission.

In assessment of infection rate of arboviruses, MIR higher than 0.1% has been used as an indicator of potential outbreak (Gu *et al*., 2008). Some countries however, reported higher MIR values with infection rates as high as 2.8% in Brazil (Eiras *et al*., 2018) and 11.6% in Columbia (Romero-Vivas *et al*., 1998). Our data indicate that Seberang Takir is an area of significant importance considering its percentage of infection rate above the indicator of potential outbreak (MIR 1.9%). Detection of DENV in female *A. albopictus* is appalling as the mosquito is permitted to re-infect other susceptible human hosts. Effective vector control programs and environmental management are thus important to minimise human contact with the infected mosquito by destroying the habitats of mosquitoes. Such actions should be able to reduce the risk of dengue infection. It is worth mentioning that this study was carried out in order to use the infection rate as a baseline to benchmark Seberang Takir, Kuala Nerus. There was no previous study reporting the risk of viral transmission in Seberang Takir, thus it is essential to obtain this data for assessment of mosquito control program and disease prevention strategies. In conclusion, Seberang Takir has shown

a potential risk of dengue transmission. This finding highlights the importance of virological surveillance and effective vector control strategies to prevent dengue outbreak in the future.

ACKNOWLEDGEMENTS

We would like to acknowledge Assoc. Prof. Dr. Chee Hui Yee, Universiti Putra Malaysia for providing the DENV-2 control strain. We thank the Universiti Malaysia Terengganu for providing research funds and lab facilities to carry out this project.

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