Entomological Surveillance Associated with Human Zika Cases in Miri Sarawak, Malaysia

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Abstract. Two confirmed human cases of Zika virus (ZIKV) were reported in the district of Miri, Sarawak, in 2016. Following that, a mosquito-based ZIKV surveillance study was conducted within 200-m radius from the case houses. Mosquito surveillance was conducted using five different methods, that is, biogents sentinel mosquito (BG) sentinel trap, modified sticky ovitrap, resting catch, larval surveillance, and conventional ovitrap. A total of 527 and 390 mosquito samples were obtained from the case houses in two localities, namely, Kampung Lopeng and Taman Shang Ri La, Miri, Sarawak, respectively. All mosquitoes collected were identified, which consisted of 11 species. *Aedes albopictus*, both the adult and larval stages, was the dominant species. Resting catch method obtained the highest number of adult mosquitoes (67%), whereas ovitrap showed the highest catch for larval mosquitoes (84%). Zika virus was detected in both adults and larvae of *Ae. albopictus* together with adults of *Culex gelidus*, and *Culex quinquefasciatus* using the real-time reverse transcriptase polymerase chain reaction (PCR) technique. It was noteworthy that *Ae. albopictus* positive with ZIKV were caught and obtained from four types of collection method. By contrast, *Cx. gelidus* and *Culex quinquefasciatus* adults collected from sticky ovitraps were also found positive with ZIKV. This study reveals vital information regarding the potential vectors of ZIKV and the possibility of transovarian transmission of the virus in Malaysia. These findings will be essentials for vector control program managers to devise preparedness and contingency plans of prevention and control of the arboviral disease.

INTRODUCTION

Zika virus (ZIKV) is a mosquito-borne pathogen that belongs to the genus *Flavivirus* of the family *Flaviviridae*. It was isolated from *Aedes africanus* mosquito that were collected from the same locality of the first isolation from monkey in 1947 in Uganda.¹ Individuals with mild infection of ZIKV commonly show symptoms of fever, rash, joint pain, and conjunctivitis (red eyes), which are similar to characteristics and symptoms of dengue infections.² However, during Zika outbreak in Brazil, studies revealed that ZIKV infections have been associated with microcephaly and neurological disorder cases among newborns.³

In 1969, Marchette et al.⁴ reported the first isolation of ZIKV from Malaysian *Aedes aegypti*. They collected mosquitoes in a wide variety of habitats that included rural, urban areas and different forest types. In addition, there were several cases of serological evidence of ZIKV collected from the aborigine population during the 1960s.⁵ There was one confirmed case with clinical evidence of Zika fever reportedly found in a German after visiting Sabah, Malaysia, in 2014.⁶ Following that, a massive outbreak of ZIKV infection was reported in Singapore in 2016. Subsequently, ZIKV infection of individuals with a travel history to Singapore was reported in Malaysia, including two cases in Miri, Sarawak.

Because ZIKV has been isolated from Malaysian *Ae. aegypti* in 1969,⁴ there is a high possibility of the circulation of this virus in the environment. There is also a higher chance of ZIKV to be isolated from *Aedes* mosquitoes in Malaysia. However, it is also important to screen the virus in other vector mosquitoes. A study by Brazilian researchers suggested that the common house mosquito *Cx. quinquefasciatus* could be involved in the

transmission of the virus as it was found infected with ZIKV genome fragments.⁷ Furthermore, a laboratory study in China demonstrated that *Culex quinquefasciatus* has a potential role as a vector in transmitting ZIKV.⁸ Nevertheless, there were laboratory studies that also demonstrated the *Culex* are not competent vectors for ZIKV transmission.^{9–11}

Thus, this study was conducted to sample mosquito adults and larvae from the Miri district in Sarawak, Malaysia, using different types of traps and techniques. The traps were placed at the surrounding area of the patient houses prior within a 200- to 300-m radius before mosquito collection. The real-time reverse transcriptase PCR technique was then conducted to screen ZIKV from these field-caught mosquitoes to determine their potential as vector for ZIKV. Data acquired will be essential for early preventive and control measures for the outbreak of Zika.

MATERIALS AND METHODS

Site selection and mosquito collection. The study was conducted in the Miri district of Sarawak, Malaysia, which has an estimated population of 67,000 households (Department of Statistics Malaysia, 2010). Based on the reported confirmed case of Zika infection, two sampling sites, Kg. Lopeng (N 4°22' 41.455"E 113°59'23.593") and Shang Ri La (N 4°19'47.622"E 113°58'40.602"), were selected (Figure 1).

Five mosquito collection methods were used during the study, namely, BG sentinel trap, modified sticky ovitrap, resting catch, larval surveillance, and conventional ovitrap. A total of 100 modified sticky ovitraps and 30 conventional ovitraps were placed in 50 households indoor and outdoor within a 200- to 300-m radius from each case house, respectively. For modified sticky ovitrap, the standard ovitrap container was improvised before catching the adult mosquitoes. A small pit was made at the side of the container to prevent overflowed water from flushing away the specimens, especially during heavy rains. A sticky sheet (21 cm × 6 cm) was placed at the inner wall of the container beforehand and

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FIGURE 1. Study site locations: Kg. Lopeng and Shang Ri La, Miri, Sarawak. (Source of image: OpenStreetMap).

filled up with water. In this study, “indoor” refers to any parts of the premise that are directly under the roof, whereas “outdoor” refers to the areas outside the roof but within the immediate vicinity of the premise.

BG sentinel traps were placed near to the infected case house for 14 hours, that is, 5:00 AM to 7:00 PM. Adult mosquito collection was also conducted by the resting catch method using aspirators at mosquito resting sites such as bushes, trees, potted plants, and wall. Resting mosquitoes were collected using electrically operated aspirator into clean paper cups, before the samples were transported back to the laboratory for processing. Blood-fed mosquitoes were allowed to digest the blood for 2–3 days before being processed.

Larval sample collection was carried out within a 200-m radius from the case-infected area. All potential indoor and outdoor mosquito-breeding sites were inspected. Larvae and pupae were collected using pipettes and placed in separate labeled containers. Important information such as the type of habitat (natural or artificial), number of samples, and stages of life cycle were recorded on prescribed forms.

Specimen identification and transportation. Adult mosquitoes collected were identified morphologically using standard taxonomy keys. Later, they were sorted out and pooled into groups according to the species, sex, locations, and type of collection method. However, mosquito specimens obtained from modified sticky ovitrap were processed individually. All specimens were kept in a cold box at sites and during transportation before virus detection process. For conventional ovitrap and larval surveillance specimens, all immature stages were brought back to the laboratory and bred to adulthood before species identification.

Virus detection in mosquito pool. Pooled mosquitoes (maximum of 25 per pool) were homogenized with 300 μ L of Minimum Essential Media Eagle solution using sterile pestle

on ice. In total, 200 μ L of homogenate was used for the RNA extraction step using the QIAamp RNA Viral Kit (Qiagen, Heiden, Germany) following the manufacturer’s protocol. The final RNA pellet was dissolved in 60 μ L of the provided buffer AVE and stored at -80° before virus screening. ZIKV detection was performed using real-time reverse transcription–PCR (RT-PCR) assay. RNA was amplified using the iTaq™ Universal Probes One-Step Kit in the BioRad CFX96 Touch Real-Time PCR (Bio-rad Laboratories, Hercules, CA) machine in a 20- μ L reaction volume containing 5 μ L of extracted RNA, 0.5 μ L of iScript advanced reverse transcriptase, 10 μ M of each primers, and 25 μ M of fluorogenic probe, and addition of

TABLE 1
Collection of mosquitoes in Kg

Species	Collection site			
	Kg Lopeng		Shang Ri La	
	No. collected	%	No. collected	%
<i>Aedes aegypti</i>	10	1.9	13	3.3
<i>Aedes albopictus</i>	423	80.3	344	88.2
<i>Aedes butleri</i>	3	0.6	2	0.5
<i>Aedes niveus</i>	3	0.6	0	0.0
<i>Aedes sp.</i>	1	0.2	5	1.3
<i>Anopheles sundaicus</i>	1	0.2	0	0.0
<i>Culex gelidus</i>	19	3.6	0	0.0
<i>Culex pseudovishnui</i>	13	2.5	0	0.0
<i>Culex quinquefasciatus</i>	42	8.0	25	6.4
<i>Culex sitiens</i>	5	0.9	0	0.0
<i>Culex sp.</i>	1	0.2	0	0.0
<i>Culex tritaeniorhynchus</i>	2	0.4	0	0.0
<i>Culex whitmorei</i>	2	0.4	0	0.0
Unknown (damaged specimens)	2	0.4	1	0.3
Total	527		390	

Lopeng and Shang Ri La, Miri, Sarawak.

FIGURE 2. Percentage of adult mosquitoes captured in Kg. Lopeng and Shang Ri La, Miri, Sarawak. This figure appears in color at www.ajtmh.org.

FIGURE 3. Percentage of adult mosquitoes captured based on methods. This figure appears in color at www.ajtmh.org.

FIGURE 4. Total percentage of larvae collected in Kg. Lopeng and Shang Ri La, Miri, Sarawak, by larval surveillance and ovitrapping. This figure appears in color at www.ajtmh.org.

nuclease-free water. The primers, probe, and PCR cycles were used as described by Lanciotti et al.²

Data analysis. The data were analyzed using descriptive statistics for comparison between numbers of mosquitoes based on collection methods and locations. Poisson regression analysis was used to calculate the significance of sampling methods from both locations. The virus infection rate was calculated with PooledInfRate, version 4.0 by Biggerstaff. The add-in application in Microsoft Excel (Microsoft Excel for Windows, Redmond, WA) computes the IR using data from pooled samples even though the pool sizes were different from each other. The IR values were calculated by both minimum infection rates (MIRs) and maximum likelihood estimation. Locations of the mosquito samples with ZIKV detection were mapped using the QGIS: QGIS Geographical Information System (2019).

RESULTS

Mosquito species composition. A total of 527 and 390 mosquitoes were collected from Kg. Lopeng and Shang Ri La, respectively, with the total captured comprising 11 species, that is, *Aedes aegypti*, *Aedes albopictus*, *Aedes butleri*, *Aedes niveus*, *Anopheles sundaicus*, *Culex gelidus*, *Culex pseudovishnui*, *Culex quinquefasciatus*, *Culex sitiens*, *Culex tritaeniorhynchus*, and *Culex whitmorei* (Table 1). Our results showed a high number of adult *Ae. albopictus* captured in Kg. Lopeng (161, 67.9%), whereas *Ae. albopictus* and *Cx. quinquefasciatus* shared the abundance (25, 44.6%) from Shang Ri La. Furthermore, *Cx. quinquefasciatus* was recorded as the second highest number of predominant mosquito collected from the sites (Figure 2). This study also demonstrated that the resting catch method captured the most numbers of adult mosquito species compared with the modified sticky ovitrap and BG Sentinel trap, with nine species, five species, and one species, respectively. In addition, the resting catch method recorded the highest number of *Ae. albopictus* adult in both locations (Figure 3).

For larval collection, a total of 624 mosquito larvae comprising six species from two genera were obtained from the study sites. *Ae. albopictus* represented the highest number of collected larvae from both Kg. Lopeng and Shang Ri La with 262 (90.34%) and 317 (95.5%), respectively. This study also reveals that ovitrapping method is more effective than the larval surveillance method. Moreover, this study also revealed that *Ae. albopictus* larvae were the predominant species collected through larval surveillance and conventional ovitrap (Figure 4).

Statistical analysis using Poisson regression showed that there is a significant difference between sampling methods used in this study, with a *P*-value < 0.001, whereas the differences between locations were not significant, with a *P*-value = 0.914.

Zika virus detection. Zika virus was detected from mosquitoes caught in both study locations. The MIRs of ZIKV were calculated based on mosquito species and locations (Table 2). It was noteworthy that mosquitoes positive with ZIKV were obtained from four types of collection method, which include adult and larvae samples. Remarkably, only *Ae. albopictus* species was positively screened with ZIKV from the most sampling methods ie; resting catch, larval surveillance, and ovitrap methods. Distribution of the positive mosquitoes in Kg. Lopeng and Shang Ri La was mapped as in Figures 5 and 6, respectively.

DISCUSSION

After two confirmed human-infected cases of Zika were reported, mosquito composition in both localities was determined through the use of various collection techniques. In this study, *Ae. albopictus* was found to constitute the highest number of caught mosquito species for both adult and larvae stages. The abundance of *Ae. albopictus* indicates that the species is widespread and commonly found in urban and suburban areas of the sampling locations. Kg. Lopeng is categorized as a residential area located at the suburbs of the city and consists of traditional and terrace houses. This area includes workers' quarters that were built from wooden and cement walls with a poor drainage system. This sampling area is covered with natural vegetations and secondary forest. By contrast, Shang Ri La is considered as a new planned housing area comprising terrace and semidetached houses. Good drainage system at the area was observed during the visit. Similar findings were reported by another study¹² where the authors found high density of *Ae. albopictus* in urban areas in Sarawak using ovitrap surveillance. Furthermore, *Ae. albopictus* is well known as a semidomestic breeder that can be found in artificial and natural containers.^{13,14} Domestic animals were also observed at the surrounding area of both locations in Kg. Lopeng and Shang Ri La. Moreover, domestic animals such as cats, dogs, and chickens can be found at both locations, which could contribute as a potential optimal breeding habitat for *Ae. albopictus* as it is both zoophilic and anthropophilic.

Ae. aegypti and *Ae. albopictus* have been long incriminated as ZIKV vectors worldwide.^{15–17} A study by Marchette et al.⁴ has reported that ZIKV was successfully isolated from *Ae. aegypti* collected in Peninsular Malaysia resulting in the species being declared as a primary vector for ZIKV in Malaysia. However, in this study, we found that ZIKV was detected only in *Ae. albopictus* mosquitoes. Even though ZIKV transmission through *Ae. albopictus* is unknown in Malaysia, several studies^{18,19} have suggested that *Ae. albopictus* is a vector as

TABLE 2

Result of Zika virus by locality and mosquito species showing the maximum likelihood estimation (MLE) and minimum infection rate (MIR) based on the total number of pools and individuals tested

Stage	Collection sites	Mosquito species	No. of individuals	No. of positive pools	MLE	MIR	Type of methods
Adult	Kg. Lopeng	<i>Aedes albopictus</i>	161	5	33.22	31.06	Sticky trap and resting catch
		<i>Culex quinquefasciatus</i>	42	1	23.81	23.81	Sticky trap
		<i>Culex gelidus</i>	6	1	166.67	166.67	Sticky trap
	Shang Ri La	<i>Aedes albopictus</i>	25	1	40.83	40	Sticky trap
		<i>Culex quinquefasciatus</i>	25	2	153.85	153.85	Sticky trap
Larvae	Kg. Lopeng	<i>Aedes albopictus</i>	262	3	7.65	7.63	Larval surveillance
	Shang Ri La	<i>Aedes albopictus</i>	319	3	9.72	9.4	Ovitrap and larval surveillance

FIGURE 5. Geographical distribution of mosquito species with regard to Zika virus detection in Kg. Lopeng.

FIGURE 6. Geographical distribution of mosquito species with regard to Zika virus detection in Shang Ri La.

ZIKV has been detected from a pool of wild-caught mosquitoes in Gabon and Brazil. Moreover, laboratory research has showed that *Ae. albopictus* is a potential vector of ZIKV in Singapore.²⁰ Molecular analysis showed the association of ZIKV with high numbers of *Ae. albopictus* mosquitoes obtained in this study, whereas none of the *Ae. aegypti* caught was positive with the virus tested.

It was noteworthy that only *Ae. albopictus* species detected with ZIKV were caught and obtained from four types of collection methods including larval collection and ovitrapping. Detection of ZIKV from larval specimens also implies that there is a possibility that transovarian transmission occurred in *Ae. albopictus*. This mode of transmission has been demonstrated by another study²¹ where *Ae. aegypti* and *Ae. albopictus* were orally infected with ZIKV and subsequently reared until the fifth generation. Reverse transcription PCR analysis on adult mosquitoes showed that ZIKV was present in both species although the filial infection rate observed in *Ae. aegypti* was higher than that in *Ae. albopictus*. Furthermore, a laboratory study by Li et al.²² provided evidence of vertical transmission of ZIKV in two strains of *Ae. aegypti* from China. They showed that there was a high infection rate (100%) of ZIKV detected from the eggs of both *Ae. aegypti* strains after two gonotrophic cycles. Nevertheless, they also indicated that any infected larvae found from the field does not imply that the larvae would develop into infected adults due to possible transstadial loss of infection during the process of maturity to adult stage.

A study by Thangamranni et al.²³ also proved that ZIKV could be vertically transmitted in *Ae. aegypti*. Although previous studies have found other flaviviruses such as dengue, yellow fever, and West Nile viruses can use vertical transmission,^{14,18} laboratory experiments have yet to be conducted to determine the susceptibility status of Malaysian *Ae. albopictus* toward ZIKV and their vertical transmission status.

In this study, ZIKV was detected in adults of *Cx. quinquefasciatus* and *Cx. gelidus*, indicating that these mosquitoes could possibly be the potential vectors. This finding concurs with the study conducted by Phumee et al.,²⁴ which found *Cx. quinquefasciatus* and other species obtained from the field were positive with ZIKV through RT-PCR analysis. The study also detected ZIKV in different developmental stages, that is, larva, male, and female adults of *Cx. quinquefasciatus*. However, our study reported that ZIKV was only detected in the adults of *Cx. quinquefasciatus*. It is to be noted that the detection of ZIKV from non-*Aedes* mosquitoes could be due to their incidental infection from feeding on ZIKV patients and the presence of remnants of ZIKV RNA. Furthermore, the RT-PCR technique can only detect genetic materials of the ZIKV and cannot determine the viability of the virus, which will require culturing the virus in cell culture. To incriminate the vector status of *Cx. quinquefasciatus*, laboratory infection and epidemiological association studies need to be conducted to better understand the ZIKV vector status of *Cx. quinquefasciatus*.

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