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Short Communication

Detection of Zika virus RNA in *Aedes aegypti* and *Aedes albopictus* larvae in Klang Valley, Peninsular Malaysia

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Abstract. We report the presence of Zika virus RNA in naturally infected field captured *Aedes aegypti* and *Ae. albopictus* mosquito larvae in Malaysia from May 2016 to April 2017. Zika virus RNA was detected (n = 30) in the larvae of both *Aedes* mosquito species. Phylogenetic analysis of the NS5 partial sequence of all positive samples shows that the circulating Zika virus in the field collected larvae are of the Asian lineage.

Zika virus (ZIKV) is an RNA flavivirus transmitted by *Aedes* (*Stegomyia* subgenus) mosquitoes present in nearly all tropical and subtropical regions worldwide. *Aedes* mosquitoes, particularly *Ae. aegypti* and *Ae. albopictus*, thrive in urban, highly populated environments and also transmit dengue, chikungunya and yellow fever (Baud *et al.*, 2017), placing approximately 3.6 billion people at risk of infection (Gubler, 2011).

The first known ZIKV outbreak was reported in Yap State in 2007 in the western Pacific (Duffy *et al.*, 2009) followed by a larger epidemic 6 years later in French Polynesia (Cao-Lormeau *et al.*, 2013). Previously, ZIKV had only caused sporadic infections in Africa and Asia. From 2015 to 2016, a large epidemic spread rapidly throughout the Americas and exhibited more severe pathogenicity and outcomes, especially a rise in neurological complications (World Health Organization, 2015).

ZIKV has been reported in Malaysia for decades, and was first isolated from *Ae. aegypti* in Pahang in 1969 (Marchette *et al.*, 1969). In that study, ZIKV was not detected in pools of field collected *Ae. albopictus* mosquitoes. Thus far there have been no further studies to assess the presence of ZIKV in *Ae. albopictus* mosquitoes. Compared to the primary *Ae. aegypti* vector, *Ae. albopictus* was reported in recent surveillance studies to be the predominant vector species in Malaysia (Faiz *et al.*, 2017; Rozilawati *et al.*, 2015). There have been reports of ZIKV seropositivity amongst nonhuman primates in Sabah (Wolfe *et al.*, 2001) and in residents from both Peninsular Malaysia and Borneo (Smithburn, 1954). However, since the study in 1969, there has been limited information on the presence and distribution of ZIKV in *Aedes* mosquitoes in Malaysia where dengue virus is endemic (Mohd-Zaki *et al.*, 2014). Thus, ZIKV infection could have been misdiagnosed as dengue infection due to similar clinical presentations and serological cross-reactivity. In this study, we sought to assess the presence of ZIKV RNA in field captured *Aedes* larvae in Malaysia and report the detection of ZIKV RNA in both *Aedes* mosquito species.

Field collections of *Aedes* mosquito larvae were carried out between May 2016 and April 2017 in two urban localities in the Klang Valley, Malaysia. Within each locality, nine sites comprising houses and residential parks at least 200 m apart were selected along a transect line. Mosquito larvae were collected at monthly intervals using ovitraps, of which 30 were placed at each site in shaded areas indoors and outdoors and collected within five days. In total, 270 ovitraps were collected monthly from each locality and transported back to the laboratory for processing. A trap was recorded as positive when it contained larvae or pupae of any species. Each larva was identified by species using keys by Rueda (2004), as well as by trap, site, and collection month. All samples were stored in -80°C freezers until analysed (Centers for Disease Control and Prevention, 2016). For *Ae. albopictus* larvae, due to the large numbers collected (N=7,819), one sample was selected randomly from each positive ovitrap at bimonthly intervals (N=1,025). All field collected *Ae. aegypti* from the same bimonthly intervals (N=364) were tested (Table 1).

Each larva was individually homogenized in 500 µL of TRIzol® reagent and RNA was extracted following the manufacturer's instructions (TRIzol® Reagent, Thermo Fisher Scientific, USA). cDNA synthesis was performed using ProtoScript® II Reverse Transcriptase (New England Biolabs, USA) and random primers, with 10 µL of RNA as template in a reaction volume of 20 µL according to the manufacturer's protocol. To detect ZIKV RNA in larvae, nested PCR was employed. The cDNA products were used directly for amplification by nested PCR using forward and reverse primers designed in-house (Table 2) to amplify a length of 364 nucleotides (nt) in the first step. To detect a highly conserved region (102 bp) within the nonstructural protein 5 (NS5) sequence, the PCR product from this reaction was used for the second round of PCR using primers as described (Faye *et al.*, 2013). All samples positive for ZIKV were sent for DNA sequencing to confirm the sequence. A phylogenetic tree was constructed based on the ZIKV NS5 partial sequence of all positive samples. The sequences have been deposited in GenBank (Accession numbers MG808108-137).

In total, ZIKV RNA was detected in 30 samples with a total prevalence of 2.16%. Phylogenetic analysis (Figure 1) shows that the circulating ZIKV in larvae collected from May 2016 to April 2017 are of the Asian lineage and appears to be nearest neighbours with recent ZIKV outbreak isolates in South America – KU729217 (Brazil, 2015), KU926310 (Brazil, 2016) and KU820897 (Colombia, 2016). During the large epidemic in 2015-2016, two different strains from the Asian lineage were reported in Malaysia. In an imported case from Singapore, the infecting strain was closely related to those from French Polynesia and in a locally transmitted case in Sabah, the strain was similar to strains from Micronesia (Bernama, 2016). We can conclude that the strains detected in this study are of the Asian lineage, but due to the short sequence length we are unable to infer their relationship to previously and currently circulating ZIKV strains. Nevertheless, the NS5 partial sequence detected is highly divergent from other flaviviruses, confirming the presence of ZIKV RNA in *Aedes* mosquito larvae (Faye *et al.*, 2013). The presence of ZIKV RNA in field collected *Ae. albopictus* larvae demonstrates the potential for *Ae. albopictus* to transmit the virus in Malaysia, along with *Ae. aegypti*.

The detection of viral RNA in field collected larvae and the lack of reported Zika outbreaks in Malaysia likely indicates that immunity may already be present in the community, or it has been misdiagnosed as the more prevalent dengue infection because of similar clinical symptoms or serological cross-reactivity (Dejnirattisai *et al.*, 2016). Other possible explanations include the absence of clinical symptoms in the majority of infections or the nature of the circulating strain of the virus in Malaysia that may be less virulent. Further studies to assess the transmission potential of the virus in adult mosquitoes and comprehensive sero-

Table 1. Number of *Aedes aegypti* and *Ae. albopictus* mosquito larvae collected from Selangor and Kuala Lumpur in the Klang Valley, Malaysia and analysed for the presence of Zika virus (ZIKV) RNA, June 2016–April 2017

Year	2016				2017		Total
Month	June	Aug	Oct	Dec	Feb	April	
Selangor							
Ae. aegypti							
No. collected	8	0	34	58	7	27	134
No. tested*	8	0	34	58	7	27	134
No. positive (%)	0	0	2 (5.9)	2 (3.5)	0	0	4 (3.0)
Ae. albopictus							
No. collected	455	368	144	935	464	539	2,905
No. tested*	57	49	44	131	63	81	425
No. positive (%)	0	1 (2.0)	0	12 (9.2)	0	1 (1.2)	14 (3.3)
Kuala Lumpur							
Ae. aegypti							
No. collected	41	63	41	40	19	26	230
No. tested*	41	63	41	40	19	26	230
No. positive (%)	0	0	0	0	2 (10.5)	1 (3.9)	3 (1.3)
Ae. albopictus							
No. collected	866	692	607	897	747	1,105	4,914
No. tested*	136	102	59	117	66	120	600
No. positive (%)	0	0	0	9 (7.7)	0	0	9 (1.5)

* ZIKV RNA was detected via reverse transcription polymerase chain reaction (RT-PCR).

Table 2. PCR amplification primers used for Zika virus detection by RT-PCR

Primer	Sequence (5' – 3')	Size (bp)
Forward primer	TTG AAG GGC TRG GAY TRC	364
Reverse primer	TCC TCA GCC TCC ATR TTC	

prevalence studies are warranted. Sampling in this study was also limited to two areas in the Klang Valley and hence molecular surveillance needs to be expanded to determine the presence and distribution of ZIKV in the country.

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There are no conflicts of interest.

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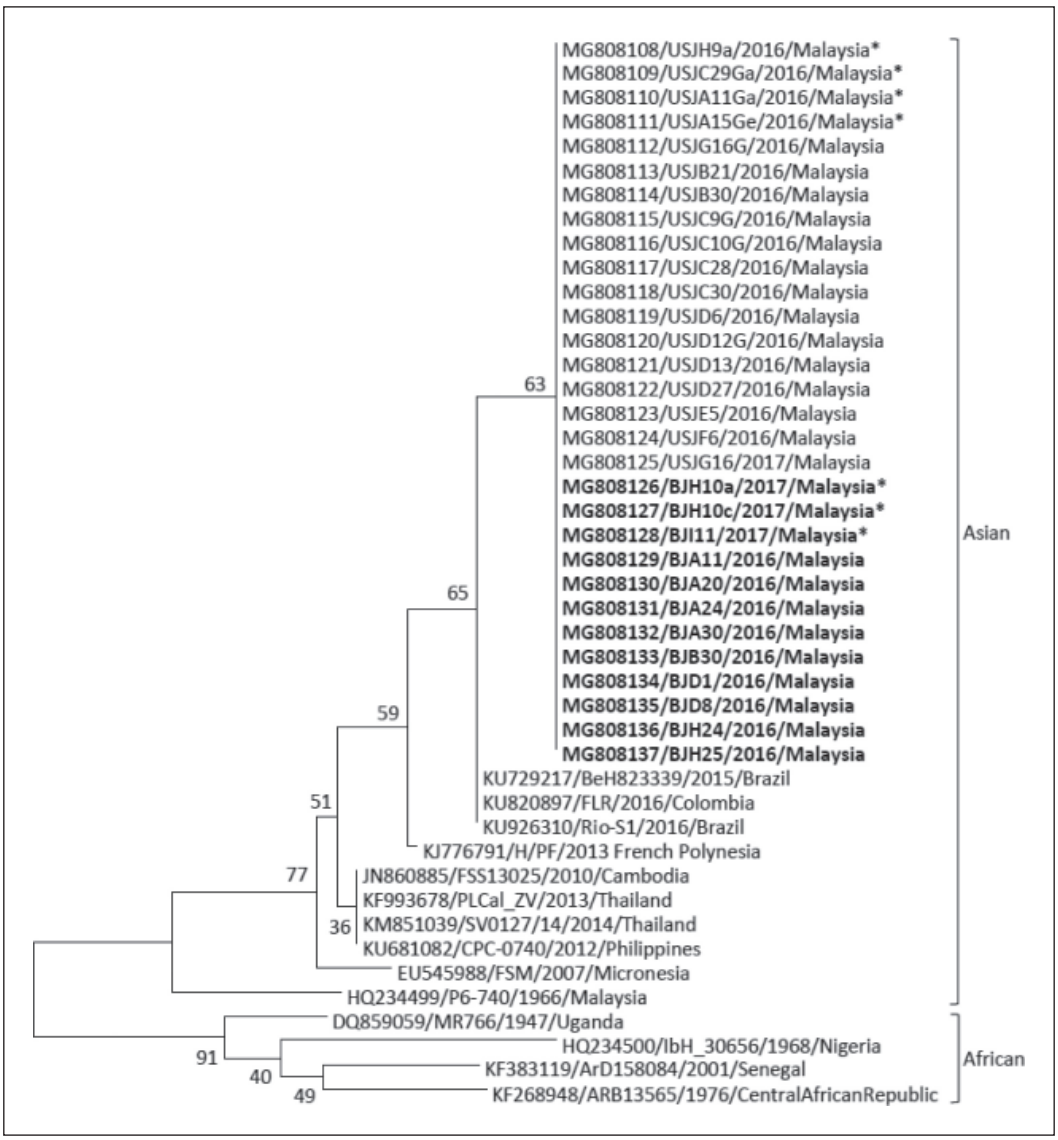

Figure 1. Phylogenetic tree of the Zika virus nonstructural protein 5 (NS5) gene of 30 positive samples (GenBank accession numbers MG808108-137) detected from *Aedes aegypti* and *Ae. albopictus* mosquitoes in Klang Valley, peninsular Malaysia. The alignment includes 14 reference NS5 partial sequences from both Asian and African lineages which were available in GenBank. The partial NS5 gene sequences (102 nt) generated from PCR products obtained for each sample were aligned and assembled using ClustalW. MEGA 10.0.1 (www.megasoftware.net/) was used to perform phylogenetic analyses using the neighbor-joining (NJ) method and the p-distance model with 1000 bootstrap re-sampling. Each sequence is labelled with the GenBank accession numbers, sample name, year of collection and sampling location. Sequences labelled with an asterisk in the sample group indicate those from *Ae. aegypti*, and those without an asterisk from *Ae. albopictus*. Bold indicates samples collected from the Federal Territory of Kuala Lumpur and those in regular font from the state of Selangor. Virus lineages are shown on the right.

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