Detection of Wolbachia in Aedes albopictus and Their Effects on Chikungunya Virus

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Abstract. Wolbachia-based vector control strategies have been proposed as a means to augment the currently existing measures for controlling dengue and chikungunya vectors. Prior to utilizing Wolbachia as a novel vector control strategy, it is crucial to understand the Wolbachia–mosquito interactions. In this study, field surveys were conducted to screen for the infection status of Wolbachia in field-collected Aedes albopictus. The effects of Wolbachia in its native host toward the replication and dissemination of chikungunya virus (CHIKV) was also studied. The prevalence of Wolbachia-infected field-collected Ae. albopictus was estimated to be 98.6% (N = 142) for females and 95.1% (N = 102) for males in the population studied. The Ae. albopictus were naturally infected with both wAlbA and wAlbB strains. We also found that the native Wolbachia has no impact on CHIKV infection and minimal effect on CHIKV dissemination to secondary organs.

INTRODUCTION

Aedes aegypti and Aedes albopictus are competent vectors for dengue virus (DENV) and chikungunya virus (CHIKV). The latter also known as Asian tiger mosquito always receive(s) lesser attention as it is considered the bridge vector to Ae. aegypti-dominated urban epidemics.1 Nonetheless, the Asian tiger mosquitoes can still act as the principle vector in epidemic areas where Ae. aegypti is present.1,2 The worldwide expansion of the geographic range of Ae. albopictus makes this invasive vector of human pathogenic viruses a major concern in many locations.1 In 2005, Ae. albopictus was incriminated as a sole vector responsible for causing chikungunya outbreak of unprecedented magnitude in the Indian Ocean.3 The outbreak continued to spread to central Africa,4 India,5 and then towards Europe,6 Asia,7–10 and North America.11 In Malaysia, a nationwide outbreak occurred in 2008, starting in Johor State, which later spread to other states and federal territories affecting about 10,000 people.9,12,13 Phylogenetic analysis of the viral sequence isolates revealed a point mutation of alanine to valine at point 226 (A226V) of the E1 gene of the polyprotein, enhancing the CHIKV replication and transmission efficacy in Ae. albopictus.14,15

Wolbachia species are obligate intracellular bacteria that infect a wide range of insects as well as some species of nematodes, making it the most ubiquitous bacteria yet described.16,17 Wolbachia infection has also been detected in mosquitoes including Ae. albopictus but is not found in Ae. aegypti. Wolbachia are vertically transmitted from infected females to their progeny. Wolbachia can alter the reproduction of its host in various ways, one such way is cytoplasmic incompatibility (CI). CI is a form of sterility in which if the same and compatible Wolbachia strain is not present in the egg during embryogenesis, embryonic development will be disrupted.18,19 CI phenomenon gives a reproductive advantage to the infected females, at which they can mate successfully with both infected and uninfected males and hence enhances Wolbachia invasion in a population.

Wolbachia has drawn much attention as some of the Wolbachia strains (e.g., wMelPop and wMel+wAlbB) have shown to reduce mosquito life span and/or induce pathogen blocking effects on the invertebrate hosts. These effects can substantially reduce the risk of pathogen transmission.20,21 Nonetheless, the Wolbachia-mediated viral blocking effect is not ubiquitous. Unlike the case for transfected hosts, effect of Wolbachia on virus replication in native hosts has been reported to be inconsistent. For instance, the naturally occurring Wolbachia of Aedes notoscriptus do not induce DENV interference within the native hosts22 contrary to the report on Ae. albopictus that demonstrated that native Wolbachia can limit transmission of DENV.23 Another study on Drosophila demonstrated that native Wolbachia render pathogen resistance toward the RNA viruses in their original hosts.24–26 The Wolbachia-based vector control strategies have taken the form of either population replacement or the incompatible insect technique (IIT) strategy. The population replacement strategy is highly dependent on the ability of the Wolbachia to invade and replace the target population with a population that cannot transmit virus.27,28 On the other hand, the IIT approach involves a continuous inundated release of males carrying an incompatible Wolbachia strain with that in the existing mosquito population, to suppress mosquito numbers below a threshold that enables continued virus transmission.29,30 In this study, we aim to determine the Wolbachia infection in field-collected Ae. albopictus from different geographical regions. This study is crucial to cater for the scarcity of information on Wolbachia infection status in field-collected Ae. albopictus population in Malaysia. Furthermore, we also investigated the effects of the naturally occurring Wolbachia on the replication of CHIKV in Ae. albopictus. These findings will help to facilitate the understanding of the Wolbachia–CHIKV–Ae. albopictus interaction, which will serve as a platform for the Wolbachia-based vector control approach to be conducted in Malaysia.

METHODS

Mosquito collection. Aedes albopictus was collected from eight collection sites from five states in Malaysia as shown in Figure 1. A minimum of 50 ovitraps were set in each location for 5 days and were at least 150 m apart to minimize the probability of progeny from the same mother.

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Aedes ovitrapping was conducted following the guidelines of Ministry of Health, Malaysia, on ovitrap deployment. Ovitraps were brought back to the insectarium in the Medical Entomology Unit, Institute for Medical Research (IMR), Kuala Lumpur. Eggs collected were hatched and larvae (L3) recovered were individually identified to species level according to the key by Mahadevan and others. The identified *Ae. albopictus* larvae from each ovitrap were placed into a plastic container and were supplied with liver powder. Once the larvae reached pupal stage, the plastic containers were placed inside an adult cage (25 × 25 × 50 cm). Mosquito species were again confirmed. Adult mosquitoes were supplied with 10% glucose incorporated with liquid B-complex (Atlantic Laboratories Corp. Ltd, Bangkok, Thailand) and maintained using standard condition of 28°C with 70–80% of relative humidity.

**DNA extraction.** Adults (F0) aged between 7 and 10 days were subjected to DNA extraction. Briefly, 4–10 mosquitoes (males and females) recovered from each ovitrap were killed by placing them in freezer for an hour. The mosquitoes were individually homogenized in 180 μL cell lysis solution (ATL Buffer) and incubated in 20 μL proteinase K at 56°C in water bath for 3 hours. The subsequent procedures were performed according to the QIAamp® DNA Mini Kit protocol (Qiagen™, Hilden, Germany).

**Detection of Wolbachia.** Multiplex polymerase chain reaction (PCR) was carried out using a temperature profile of 95°C for 15 seconds, 57°C for 30 seconds, and 72°C for...
1 minute for 35 cycles using wsp primers. Primers used were 328F and 691R for wAlbA strain and 183F and 691R for wAlbB strain as described by Zhou33 (328F, 5′-CCA GCA GAT ACT ATT GCG-3′; 183F, 5′-AAG GAA CCG AAG TTC ATG-3′; 691R, 5′-AAA AAT TAA ACG CTA CTC CA-3′). The PCR mixture contained 5 μL of extracted DNA, 12.5 μL of MyTaq™ Mix (Bioline, Taunton, MA), 1 μL of each primer (10 μM), and 4.5 μL of ddH2O. Negative and positive controls for the PCR assay were included in each run. The positive control was obtained by screening the adult Ae. albopictus (resident strain) using PCR and sequencing of wsp gene to confirm that the amplified PCR product obtained was Wolbachia. The quality of the extracted DNA was checked using the 12S rRNA primer sets (12SA, 5′-AAA CTA GGA TTA GAT ACC CTA TTA T-3′; 12SB, 5′-AAG AGC GAC GGG CGA TGT GT-3′) to screen samples that were negative for wsp primers using the temperature profile of 95°C for 15 seconds for denaturation, 47°C for 30 seconds for annealing and 72°C for 1 minute for extension, conducted for 35 cycles. Samples that were negative for wsp primers but positive for 125 RNA primers were scored as uninfected. All the positive PCR products were visualized under 1.5% agarose gel electrophoresis.

Sequencing of Wolbachia endobacterium. The positive PCR product was purified using QiAquick™ Gel Extraction Kit (Qiagen™) before DNA sequencing. A minimum of 10 purified DNA extracts from individuals of each locality were outsourced for sequencing. All sequences were searched against the GenBank nucleotide database using the Basic Local Alignment Search Tool (BLAST®) provided by the National Center for Biotechnology Information (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Partial wsp gene sequences of Wolbachia were aligned using the Clustal-W algorithm and the evolutionary distances of Wolbachia isolates from Ae. albopictus was constructed using neighbor-joining tree, utilizing Kimura-2P analysis with 1,000 bootstrap replicates in MEGA 6.0 software.34 A Wolbachia sequence from Culex quinquefasciatus was included as an outgroup.

CHIKV production. CHIKV (Asian strain) was provided by the Virology Unit, IMR, Kuala Lumpur. The virus was isolated during the outbreak in Bagan Panchor, Perak, in 2006. The CHIKV was maintained in BHK-21 cell lines, Stock virus prepared by freeze-thawing the infected cells once, centrifuging the suspension at 40,000 x g and storing the filtered supernatants at 80°C. The infected cells were maintained in Virology Unit, IMR. The titer of the CHIKV stock was determined using the 50% cell culture infectious dose assay.

Mosquito samples for artificial oral infection. Mosquitoes from three localities were used for the oral infection experiment. Each locality was chosen to represent one habitat: 1) Besar Island (tourism island), 2) Tenggol Island (remote island), and 3) Bandar Rinching (urban residential area). Females from Besar Island were derived from the same island, and Bandar Rinching were derived from the existing colony in insectarium. All mosquitoes used for artificial oral infection were F6 generation.

Tetracycline treatment to clear Wolbachia. Adult mosquitoes were provided with a solution of 0.75 mg/mL tetracycline dissolved in 10% sucrose. After every treatment, 10 randomly selected treated mosquitoes from each generation were tested by PCR for Wolbachia infection. Treatment was continued if any randomly tested mosquitoes were positive for Wolbachia confirmed via PCR. This treatment was performed up to four generations of mosquitoes. Colonies of Wolbachia-free Ae. albopictus were maintained for a further two generations without tetracycline before experiments commenced to allow the reestablishment of beneficial microbiota.

Experimental oral infections. Experimental oral infection with CHIKV was conducted within an Arthropod Containment Level 2 insectarium. The artificial membrane feeding technique was performed using the Hemotek Feeding System (Discovery workshops, Accrington, United Kingdom) housed in an isolation glove box. Human blood used for artificial feeding was sent to Virology Unit, IMR, and confirmed to be negative by neutralization assay for CHIKV antibodies. Blood suspension containing 1:9 of CHIKV (titer 10⁷ plaque-forming units/mL) in human blood was used for artificial feeding. Uninfected samples (control group) were obtained by feeding the mosquitoes with human blood only. A total of 250 adult mosquitoes from each group Wolbachia infected (w+) and Wolbachia-free (w−) aged 3–5 days that have been starved overnight were subjected to artificial feeding. The blood was presented to the mosquitoes by placing the cups (containing 50 mosquitoes each) below the feeder with the surface of the nylon netting of the cup in contact with the membrane of the feeder. Mosquitoes were allowed to feed for approximately 20–30 minutes. The mosquitoes in cups were cold anesthetized by placing in –20°C freezer for 30 seconds. The mosquitoes were then sorted. All unfed mosquitoes were discarded. Engorged mosquitoes were placed in cups (10 per cup) and kept in incubator at 28°C and humidity of 80% for planned time points (days 0, 1, 2, 3, 5, 7, and 10) postinoculation (PI) studies. At each time point, mosquito samples from at least a single cup were cold anesthetized by placing them in –20°C for 30 seconds, before dissection to remove midguts, salivary glands, and ovaries. Individual mosquito was put on a glass slide. The thorax and head of the mosquito were first removed, followed by dissection of the salivary glands in a drop of saline. The abdomen was then dissected to remove midguts and ovaries in a drop of saline, respectively. Glass slide was replaced for each individual mosquito, and fresh drops of saline were used for each organ examined. It was ensured that the dissecting needles were rinsed in alcohol between each dissection to prevent contamination. A total of four engorged mosquitoes fed with clean human blood at days 0 and 10 PI were kept aside to serve as negative control. For each experimental time point, the infection rate is defined as the number of midguts with detectable virus titer divided by the number of mosquitoes sampled. The dissemination rate was defined as the number of salivary glands with detectable virus titer divided by the number of midguts with detectable virus titer.

Nucleic acid extraction and quantitative PCR. Total nucleic acid was extracted from the dissected organs (midguts and salivary glands). Extraction was performed with innuPREP DNA/RNA Mini Kit (Analytik Jena AG, Jena, Germany) that enables the isolation of both RNA and DNA. RNA was used to determine viral load by real-time reverse transcription PCR (RT-PCR), and DNA to check for the presence of Wolbachia in ovaries using conventional PCR. For w+ group, individual mosquito that was
screened negative for Wolbachia (in ovaries) was excluded from the dataset. A minimum number of nine mosquitoes were used for each time point. A standard curve was generated using 10-fold serial dilutions of RNA synthetic transcript with known copy number. The new sequences of wsp gene using Kimura-2P analysis. GenBank sequences are shown with accession number. The new sequences of wsp gene were grouped into wAlbA and wAlbB clades, respectively (Figure 2). The size of the wsp fragment for wAlbA was 341 bp and wAlbB was 463 bp.

Table 1 shows the frequency of double and single infections of Wolbachia in the Ae. albopictus populations sampled from all the collection sites. For females, 97.2% (138/142) of the samples were superinfected with both wAlbA and wAlbB. Only 1.4% (2/142) of samples were singly infected with either wAlbA or wAlbB. Another 1.4% (2/142) samples were scored as uninfected. For males, 49.0% (50/102) was superinfected with wAlbA and wAlbB followed by 46.1% (47/102) infection with wAlbB only. The remaining 4.9% (5/102) samples were uninfected. None of the males

**RESULTS**

**Distribution of Wolbachia in Malaysian Ae. albopictus.** A total of 244 Ae. albopictus samples collected from eight sites in five states (Malacca, Selangor, Terengganu, Perak, and Pahang) in Malaysia were screened for Wolbachia infection using wAlbA- and wAlbB-specific wsp gene primers. Our results showed a high percentage of Wolbachia infection with 98.6% in females and 95.1% in males. For wsp gene, phylogenetic analysis revealed that Wolbachia isolates from the present study were closely related to Wolbachia isolates from different geographical regions, and the sequences were grouped into wAlbA and wAlbB clades, respectively (Figure 2). The size of the wsp fragment for wAlbA was 341 bp and wAlbB was 463 bp.

<table>
<thead>
<tr>
<th>Study sites</th>
<th>Types of habitat</th>
<th>Total female</th>
<th>Infected %</th>
<th>wAlbA and wAlbB</th>
<th>wAlbA</th>
<th>wAlbB</th>
<th>Uninfected</th>
<th>Total Male</th>
<th>Infected %</th>
<th>wAlbA and wAlbB</th>
<th>wAlbA</th>
<th>wAlbB</th>
<th>Uninfected</th>
</tr>
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<tr>
<td>Bukit Beruang, Malacca</td>
<td>Residential area</td>
<td>13</td>
<td>100</td>
<td>100 (13)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<td>0</td>
<td>18</td>
<td>100</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
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<td>0</td>
<td>5</td>
<td>100</td>
<td>20 (1)</td>
<td>0</td>
<td>80 (4)</td>
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<td>0</td>
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<td>6.7 (1)</td>
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<td>93.3 (14)</td>
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<td>Marang, Terengganu</td>
<td>Seashore</td>
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<td>0</td>
<td>19</td>
<td>100</td>
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<td>42.1 (8)</td>
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<td>Island</td>
<td>32</td>
<td>93.7</td>
<td>87.5 (28)</td>
<td>3.1 (1)</td>
<td>3.1 (1)</td>
<td>6.3 (2)</td>
<td>22</td>
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<tr>
<td>Kuantan, Pahang</td>
<td>Plantation</td>
<td>16</td>
<td>100</td>
<td>100 (16)</td>
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<td>0</td>
<td>0</td>
<td>16</td>
<td>87.5</td>
<td>87.5 (14)</td>
<td>0</td>
<td>0</td>
<td>12.5 (2)</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td></td>
<td>142</td>
<td>98.6</td>
<td>97.2 (138)</td>
<td>0.7 (1)</td>
<td>0.7 (1)</td>
<td>1.4 (2)</td>
<td>102</td>
<td>95.1</td>
<td>49.0 (50)</td>
<td>0</td>
<td>46.1 (47)</td>
<td>4.9 (5)</td>
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</table>
were positive for wAlbA only. The uninfected samples were confirmed by running the DNA with the 12S RNA primer set for mitochondrial DNA as a quality check.

**Analysis of linearity and LOD determination.** The linear dynamic range for the multiplex RT-PCR assay was 100% at the range of 100 to $10^2$ RNA copies per reaction but decreased to 88.8% at 10 copies. The LOD was set at 100 copies, which correspond to the mean Cq of 28. Samples with Cq value > 28 was scored as uninfected. Only mosquitoes that score above the LOD was reported to infer

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**FIGURE 3.** The percentage of Wolbachia infection status in ovaries.

**FIGURE 4.** The percentage of chikungunya virus (CHIKV) infection in (A) midguts and (B) salivary glands for Wolbachia infected (w+) and Wolbachia-free (w−) for all localities.
For mosquitoes sampled from Bandar Rinching, the viral CHIKV infection and dissemination in midguts and salivary glands, respectively.

Wolbachia infection status in ovaries of mosquitoes used in the experimental oral infection study. The presence of Wolbachia in females used in the experimental oral infection study was confirmed by screening the ovaries. It was noticed that ovaries for females were highly infected with at least 90% infection and above. There was no significant difference in the percentage of wild-type (w+) and w− groups at all the time points for the three localities (P > 0.05, Fisher’s exact test) (Figure 3).

Laboratory infection of Wolbachia infected (w+) and uninfected (w−) Ae. albopictus with CHIKV. CHIKV midgut infections were observed for w+ and w− groups for each time point, the midgut infection rate was consistent with percentage of at least 60% for w+ and 70% for w− for all localities (Figure 4A). There was no significant difference in the infection and dissemination rate between w+ and w− groups for all the time points tested for three localities (P > 0.05, Fisher’s exact test). It was noticed that CHIKV was detected in salivary glands as early as day 2 PI (Figure 4B).

The number of CHIKV genome copies in midguts was not significantly different between w+ and w− groups at any time point tested. CHIKV titer reached the peak as early as day 2 or day 3. For Besar Island, the peak titer for w+ and w− groups (median = 10^5.9 versus 10^7.3 viral copies/midgut, P = 0.05) achieved at day 3 PI. For Tenggol Island, the virus replication reached its peak at day 3 for w+ (median = 10^5.9 viral copies/midgut) and day 2 PI for w− (median = 10^1.3 viral copies/midgut), respectively. For mosquitoes sampled from Bandar Rinching, the viral copies were at the peak of viral infection for w+ and w− at day 3 PI (median = 10^7.8 versus 10^7.2 copies/midgut, P = 0.143) (Figure 5A).

For salivary glands, the amount of CHIKV copies ranged from below the LOD (100 CHIKV copies) to 10^6 CHIKV copies in both w+ and w− groups. Although there was a variation in median CHIKV titer among the groups, these differences were not statistically significant at other time points, except for day 2 PI (Figure 5B). For Besar Island, CHIKV copy number of w+ group was almost one log lower than for w− group for salivary glands (medians = 10^1.8 versus 10^2.4, P < 0.05, Mann–Whitney U tests) (Figure 5B). Similarly, for Bandar Rinching, CHIKV copies in salivary glands for w+ was lower than w− group (median = 10^2.1 versus 10^3.3, P < 0.001, Mann–Whitney U tests) (Figure 5B).

The total CHIKV titer in different organs was compared between localities as shown in Table 2. The statistical analysis using Kruskal–Wallis test showed that total CHIKV titer in midguts were significantly different from at least one locality (P < 0.05). Subsequent post hoc analysis using the Bonferroni correction substantiated the difference. For w+, a significantly lower overall CHIKV titer in midguts was observed in Besar Island compared with Bandar Rinching and Tenggol Island. For w− group, a significantly higher overall CHIKV titer in midguts was observed in Tenggol Island compared with Bandar Rinching and Besar Island. No significant difference for CHIKV titer in salivary glands was observed among the three localities for w+ (P = 0.805) and w− (P = 0.431).

DISCUSSION

Our study on the distribution of Wolbachia in the field-collected Ae. albopictus showed a high infection rate of 98.6% in females and 95.1% in males. Females showed a common superinfection of wAlbA and wAlbB, occurred at a high prevalence of 97.2%. This is in accordance to other studies, showing superinfection percentage is common in field-collected Ae. albopictus infecting at least 96% of mosquitoes. 35–39 Wolbachia are expected to rapidly spread to fixation once a Wolbachia infection enters a population. 40,41 As females that carry both wAlbA and wAlbB strain can successfully mate with males that are either singly or superinfected, the superinfection of Wolbachia in females may explain the high fidelity of maternal transmission of Wolbachia of the mosquito species in the wild. 42 The males were also most commonly superinfected with wAlbA and wAlbB at a percentage of 49.0% followed by 46.1% of wAlbB-only infection. No single infection of wAlbA was detected in males. Apart from the maternal transmission efficacy of Wolbachia, the geographical populations are reported to be strong predictors affecting Wolbachia infection rate and pattern. 38 For example, a single-strain (wAlbA) infected population has been described in Koh Samui and Mauritius Islands that are geographically distinct from the superinfected populations. 39,44,45 A study by Tortosa and others demonstrated that the density of wAlbA significantly decreased with age in male Ae. albopictus population in which a complete loss was observed within 5-day period postemergence. 46 Therefore, this finding may serve as a plausible explanation for the lack of single wAlbA strain infection in the males despite having females that were
**CONCLUSION**

Our results suggest a high prevalence of *Wolbachia* infection in the wild-caught *Ae. albopictus*. In accordance to other studies, the *Ae. albopictus* are naturally infected with wAlbA and wAlbB. Our data showed that the presence of *Wolbachia* do not pose any significant impact in the CHIKV infection in the midguts and dissemination to salivary glands in its native host, *Ae. albopictus*. The presence of *Wolbachia* does not interfere with the extensive CHIKV replication in midguts. Nonetheless, the native *Wolbachia* has a minimal effect on the CHIKV titer in the salivary glands, explaining why *Ae. albopictus* is a competent vector for CHIKV despite naturally infected with *Wolbachia*.

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**TABLE 2**

<table>
<thead>
<tr>
<th>Locality</th>
<th>Sample size</th>
<th>Median CHIKV copy number (IQR)</th>
<th>CHIKV titer</th>
<th>Median copy number (IQR)</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>w+</td>
<td>w−</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Midguts</td>
<td>Salivary glands</td>
<td>Midguts</td>
</tr>
<tr>
<td>Besar Island, Malacca</td>
<td>55</td>
<td>6.07 (5.21, 6.93)</td>
<td>2.38 (1.63, 4.63)</td>
<td>7.02 (4.01, 7.80)</td>
</tr>
<tr>
<td>Tenggol Island, Terengganu</td>
<td>58</td>
<td>7.44 (5.20, 8.09)</td>
<td>2.70 (1.45, 6.36)</td>
<td>7.83 (6.10, 8.53)</td>
</tr>
<tr>
<td>Bandar Rinching, Selangor</td>
<td>60</td>
<td>7.01 (6.64, 7.89)</td>
<td>2.93 (1.73, 5.39)</td>
<td>6.98 (6.02, 7.50)</td>
</tr>
</tbody>
</table>

Data are presented as median CHIKV copy number (IQR) and were compared among localities by Kruskal–Wallis test and Mann–Whitney U test for post hoc pairwise comparisons.

*Wolbachia* = chikungunya virus; IQR = interquartile range.

*Median with different superscripts (i.e., a and b) within the same column indicates significant difference (P < 0.05).
identifying, and dissecting the mosquitoes. This work formed a part of the graduate study (MSc) of the first author at University of Malaya, Kuala Lumpur, Malaysia.

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