Super- or Single Infection: *Wolbachia* Supergrouping of Wild Mosquito Populations from Varied Location Types in Peninsular Malaysia

(Jangkitan Super atau Tunggal: Superkumpulan *Wolbachia* Populasi Nyamuk Liar dari Pelbagai Jenis Lokasi di Semenanjung Malaysia)

NOOR SHAZLEEN HUSNIE MOHD MOHTAR, EMELIA OSMAN, MOHD FARIHAN MD YATIM & AISHAH HANI AZIL*

**ABSTRACT**

*Wolbachia* has the ability to cause reproductive abnormalities in infected hosts including cytoplasmic incompatibility (CI). CI is activated when there are multiple *Wolbachia* supergroups or strains infection present in insect populations. *Wolbachia*-transinfected mosquitoes have been used widely in some countries as a biological control agent. In order to ensure a successful *Wolbachia* establishment, it is important to determine the diversity of natural *Wolbachia* present in the wild mosquito populations. The adults and immature stages of mosquitoes were collected from urban, suburban and rural areas and were reared into adults and identified to species before being subjected to molecular analysis. We found that 22% out of 222 males and 34.6% of 543 females tested were carrying *Wolbachia* based on *PCR* amplification of the *Wolbachia* 16S rDNA genes technique. *PCR* digestion for *Wolbachia* supergrouping showed that most of the *Ae. albopictus* were superinfected with *Wolbachia* (52.41%), whereas 21% and 28% of the positive samples were singly infected with supergroup A and B, respectively. There is an indication that prevalence of *Wolbachia* varies between mosquito populations in different areas. However, further studies to incorporate both PCR amplification of the *Wolbachia* 16S rDNA and wsp genes with bigger sample size should be performed to measure exact infection of *Wolbachia* in Malaysia. The baseline data on diversity of *Wolbachia* supergroups is expected to facilitate *Wolbachia* strategy by helping us to better understand the patterns and impact of the bacteria’s transmission in the environment.

*Keywords: 16S rDNA; Culicidae; PCR digestion; Wolbachia supergroup*

**ABSTRAK**

*Wolbachia* berkebolehan menyebabkan keabnormalan reproduttif kepada perumah yang dijangkitinya, antaranya ketidakserasian sitoplasma (CI). CI diaktifkan apabila terdapat kepelbagaian jangkitan daripada superkumpulan atau strain Wolbachia yang hadir di dalam sesuatu populasi. Nyamuk transjangkitan Wolbachia ini telah digunakan secara meluas di sesetengah negara sebagai agen kawalan biologi. Namun bagi memastikan keberjayaan Wolbachia untuk bermandiri, adalah penting untuk mengenal pasti kepelbagaian Wolbachia yang hadir secara semula jadi di dalam populasi nyamuk liar. Nyamuk peringkat dewasa dan pra-matang disampel dari kawasan bandar, pinggir bandar dan pedalaman yang kemudiannya dibelah sehingga dewasa dan spesiesnya dikenal pasti sebelum diteruskan dengan analisis molekul. Berdasarkan kaedah amplifikasi *PCR* yang menyasarkan gen 16S rDNA, kajian mendapati 22% daripada 222 nyamuk jantan dan 34.6% daripada 543 betina membawa Wolbachia. Pencernaan produk *PCR* dilakukan bagi menentukan super-kumpulan Wolbachia dan hasilnya majoriti *Aedes albopictus* dijangkiti Wolbachia daripada kedua-dua superkumpulan A dan B (52.41%) manakala 21% dan 28% daripadanya masing-masing terjangkit secara tunggal, superkumpulan A dan B. Ini menandakan taburan kumpulan Wolbachia adalah herbeza antara populasi nyamuk di kawasan yang berbeza. Namun, kajian lanjutan yang melibatkan sampel saiz yang lebih besar serta gabungan penggunaan dua gen Wolbachia 16S rDNA dan wsp amat diperlukan bagi mengukur kadar jangkitan Wolbachia di Malaysia. Data garis dasar mengenai kepelbagaian superkumpulan Wolbachia yang hadir dijangka dapat membantu mempermudahkan untuk memahami taburannya dan kesan penyebarannya pada persekitaran.

*Kata kunci: 16S rDNA gen; Culicidae; pencernaan PCR; superkumpulan Wolbachia*
INTRODUCTION

Outbreaks of dengue and chikungunya are occurring in Malaysia for the past few years with a higher number of dengue cases reported in 2019 (130,101) compared to 2018 (80,615). However, the number of dengue cases reported until 7 November 2020 was 83,752, indicating a reduction of 25.5% compared to previous year (KKM 2020, 2019). These diseases are transmitted by *Aedes aegypti* and *Aedes albopictus*. Previously, *Ae. albopictus* is predicted to be the main vector responsible for transmission of DENV during the dengue outbreak in China mainland (Luo et al. 2017; Xu et al. 2007). Meanwhile, in Malaysia, *Ae. albopictus* was abundantly found in urban residential areas, for example Kampung Baru which are located at the centre of Kuala Lumpur (Chen et al. 2006; Rozilawati et al. 2015). Thus, this species could serve as a potential vector for the virus transmission in the areas. Due to increasing reported cases of the vector-borne diseases, extensive control must be planned and executed including those that target the vector. However, high usage of thermal spraying or fogging can result in the development of insecticide resistance in the vectors (Hamdan et al. 2005; Loke et al. 2010). Therefore, biological control which involves the release of *Wolbachia*-transinfected *Ae. aegypti* are suggested to be added to complement existing vector control methods. This method is predicted to limit the transmission of dengue viruses by manipulating the *Aedes* populations.

 Releases of females or males infected with selected strain of *Wolbachia* will play a major role in the successful of population replacement and suppression. The first release of *Ae. aegypti* transinfected *Wolbachia* (wMel strain) were conducted in Cairns region of northern Queensland, Australia and a successful of *Wolbachia* establishment in the released mosquito populations has been reported (Hoffman et al. 2011). Such releases were conducted via the World Mosquito Program involving several dengue-affected countries including Fiji which has undertaken wMel mosquito deployment in 2018 (WMP 2019). Meanwhile, the Wolbachia project in Malaysia involves the releases of *Ae. aegypti* carrying *Wolbachia* strain of wAlbB at six localities of dengue endemic areas (Nazni et al. 2019). Two mechanisms are predicted to occur. First mechanism is population replacement which takes place when infected females mate with uninfected males or infected males carrying same supergroup or strain in the field which will result in all progeny carrying *Wolbachia*. Second, population suppression which occurs when males infected with *Wolbachia* cross-mate with infected females carrying different strain or uninfected females in the field which will produce unhatched eggs. The successful establishments of introduced *Wolbachia* strains were recorded in Australia and Malaysia (Hoffmann et al. 2011; Nazni et al. 2019).

 However, the releases of *Wolbachia*-infected mosquitoes have been involving only *Ae. aegypti* thus far. It is more straightforward to introduce transinfected *Ae. aegypti* into the aposymbiotic mosquito populations compared to *Ae. albopictus* populations, which naturally carry *Wolbachia*. Many surveys have been conducted on the prevalence of natural *Wolbachia* strain present in various mosquito species and most of the *Ae. albopictus* samples collected were found to be positive with *Wolbachia* (Kittayapong et al. 2000; Nugapola et al. 2017; Rasgon & Scott 2004; Ricci et al. 2002; Zhou et al. 1998). *Wolbachia* does not induce pathogen interference when it naturally lives inside the host (Mousson et al. 2012). *Wolbachia* act as a potential gene-driving system by manipulating vector populations when they are artificially infected and cause a wide range of reproductive abnormalities called cytoplasmic incompatibility (CI) (Sinkins 2004). The CI activation enables *Wolbachia* to spread rapidly and replace the uninfected mosquito populations with introduced strain. Several studies have shown the successful development of *Wolbachia*-transinfected *Ae. albopictus* through the embryonic microinjection with the selected strains and the capability to suppress the population of *Ae. albopictus* was demonstrated in the laboratory (Calvitti et al. 2015; Fu et al. 2010; Zhang et al. 2016). The successful development of these strains is useful for future dengue vector control by targeting endemic areas which are abundant with *Ae. albopictus* populations.

 However, a better understanding on natural *Wolbachia* infection in the populations are needed before *Wolbachia*-transinfected *Ae. albopictus* are released. Therefore, our main objective was to determine the distribution of *Wolbachia* supergroups infection in the selected mosquito populations located in Peninsular Malaysia using a conventional method, PCR targeting 16Sr DNA gene and supergrouping by PCR-digestion. Based on study conducted by Marcon et al. (2011), both target gene 16S rDNA and *wsp* have been proved to be highly specific and sensitive in detection of *Wolbachia*. The primers designed targeting 16S rDNA have been proved as stand-alone primers which can be used both as detection and *Wolbachia* supergroup classification (Marcon et al. 2011) but *wsp* gene is more preferable
for the phylogenetic study. Furthermore, this study contributes to the current knowledge about Wolbachia strains prevalence in wild mosquito populations of a variety of species and locations.

MATERIALS AND METHODS

STUDY AREA AND MOSQUITO COLLECTIONS

This cross-sectional study was conducted in randomly selected areas involving several states in Peninsular Malaysia. Mosquitoes were sampled from March 2014 to May 2015 in several localities (Table 1), before the Wolbachia releases project by Ministry of Health which were conducted in another localities. Selected localities were classified as urban and rural areas. Mosquitoes from various species were collected using several collection methods; BG-Sentinel trap and human landing catch (HLC) for adults. Immature stages were collected using mosquito larvae trapping devices (MLTD) and larva survey. Most of the samplings were conducted during one-site-visit of each location such as BG-Sentinel, HLC and larva survey. MLTD or also known as autocidal trap have been used by Vector Unit of Kuala Lumpur City Hall (DBKL) as surveillance tools for dengue vector. MLTD is made from a cylindrical shape plastic container (24 x 13.5 cm), black funnel, cap and a jacket (black polybag) which used to cover the transparent container (Zainol-Ariffin et al. 2009). The MLTD was filled with approximately 1 L of dechlorinated water and monitored every week for the presence of larvae and eggs. All of these methods were used to maximize collections for both immature and adult stages from each location. The collected larvae and pupae were brought to the insectarium and reared into adults. Adult mosquitoes were identified using several keys of identification and were sorted according to species. Mosquitoes were kept in 95% ethanol and stored at -20 °C prior to DNA extraction.

Wolbachia DETECTION

Genomic DNA extraction procedure was conducted according to manufacturer’s protocol with several modifications as stated by Noor-Shazleen-Husnie et al. (2018). The DNA extractions were performed by homogenizing whole body of adult mosquito, individually in 100 μL of DNAzol® reagents (Life Technologies, USA). Subsequently, 50 μL of absolute ethanol AR (Ajax Finechem Pty. Ltd., Australia) were added to the supernatant to precipitate the DNA. The DNA was washed twice with 75% ethanol before been eluted with 50 μL of sterilized distilled water (ddH₂O) and stored at -20 °C. Wolbachia-infected Drosophila simulans was used as an internal control for the DNA extraction method and act as a positive control during Wolbachia screening.

We used published primers targeting approximately 438 bp of 16S rDNA Wolbachia gene (Werren & Windsor 2000). Briefly, the PCR mixtures consisting 3 μL of extracted DNA, 2.5 μL of 10x PCR buffer (Invitrogen), 1 μL of MgCl₂ (50 mM), 0.8 μL of dNTPs (10 mM each), 1 μL of 20 pmol/μL forward and reverse primers and 0.3 μL of Taq DNA polymerase (5U/μL). The PCR was performed on an Eppendorf Mastercycler® Pro S (Eppendorf, Germany) with the following conditions: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, annealing at 57 °C for 45 s, extension at 72 °C for 1 min 30 s and final extension at 72 °C for 10 min. The PCR products were visualized on a 2.0% agarose gel and viewed under Gel Doc™ EZ System (Bio-Rad, USA). Negative controls containing ddH₂O were included in every run of PCR to exclude the possibility of contamination.

PCR DIGESTION

A total reaction of 25 μL containing 18 μL PCR product, 2.5 μL of CutSmart® buffer (10x), 1 μL restriction enzyme Rsal (New England Biolabs, USA) and water were added to the final volume. The reaction mixtures were incubated overnight or at least for 16 h at 37 °C. The digestion products were analyzed using 3% agarose gel electrophoresis. Restriction enzyme, Rsal were used to differentiate between Wolbachia supergroup A and B by cut the restriction sites GT^AC which are located on 16S rRNA gene which give supergroup A with three fragments (311, 83 and 46 bp) whereas supergroup B with five fragments (165, 146, 67, 46 and 16 bp) (Pourali et al. 2009).

ETHICAL APPROVAL

Ethical approval was obtained from the Research Ethics Committee, Universiti Kebangsaan Malaysia (Project code: FF-2014-074. Reference: UKM 1.5.3.5/244/FF-2014-074).
### TABLE 1. List of localities for mosquito sampling and the methods of collection used

| Location types | District/Parliament/ State | Localities | Coordinates | Collection methods |
|----------------|---------------------------|------------|-------------|--------------------|
| Urban          | Batu                       | Batu 5, Ipoh Road (DBKL Stor 220) | 3°11’47”N 101°40’44”E | MLTD               |
|                |                            | Sentul Pasar Road (DBKL Stor 225)  | 3°11’44”N 101°41’25”E | MLTD               |
|                | Bukit Nanas                | Forested area in Bukit Nanas         | 3° 9’7”N 101°42’17”E | BG-Sentinel, HLC   |
|                | Bandar Tun Razak          | Velodrome Cheras (DBKL Stor 215)    | 3° 6’37”N 101°43’42”E | MLTD               |
|                | Kampung Bharu             | UKM College 4, Raja Muda Abdul Aziz Road | 3°10’3”N 101°42’33”E | MLTD               |
|                |                            | UKM College 5, Raja Abdullah Road   | 3°10’2”N 101°42’11”E | MLTD               |
|                | Melaka                    | Bendahara Road                       | 2°11’55”N 102°15’08”E | HLC                |
|                | Seputeh                   | Klang Lama Road (DBKL Stor 200)      | 3° 6’21”N 101°40’40”E | MLTD               |
|                | Titiwangsa                | UKM College 1, Temerloh Road         | 3°10’27”N 101°42’38”E | MLTD               |
|                | Wangsa Maju               | Genting Kelang Road (Construction site) | 3°11’55”N 101°42’51”E | Larva survey       |
|                |                            | Seksyen 1 Block A8                   | 3°12’31”N 101°44’6”E  | MLTD               |
|                |                            | Taman Melati Flat                    | 3°13’33”N 101°43’29”E | MLTD               |
| Rural          | Gombak                    | Ulu Gombak Forest Reserve            | 3°19’28”N 101°45’1”E  | HLC                |
|                | Kuala Selangor            | Kilang Gula Lama Road, Tanjung Karang | 3°24’47”N 101°9’55”E  | BG-Sentinel, HLC, Larva survey |
|                | Kuantan                   | Panching Road                        | 3°47’57”N 103°9’58”E  | HLC                |
|                |                            | Lembing River                        | 3°54’56”N 103°1’54”E  | BG-Sentinel, HLC   |
|                |                            | Islam Cemetryy Sg. Mas               | 3°53’58”N 103°4’21”E  | HLC                |
|                |                            | Kg. Sg. Mas (Nearby school)          | 3°53’58”N 103°4’34”E  | HLC                |
|                |                            | Kg. Sg. Mas (Rubber Plantation)      | 3°53’57”N 103°4’21”E  | HLC                |
|                |                            | Deer Farm                            | 3°54’8”N 103°4’44”E   | HLC, Larva survey  |
|                | Perak                     | Batu Gajah, Kinta                    | 4°27’34”N 100°58’37”E | HLC                |
|                |                            | FELDA Gunung Besout, Sungkai         | 3°50’55”N 101°17’15”E | HLC                |
|                | Rawang                    | Hutan Lipur Kanching                 | 3°17’55”N 101°37’9”E  | BG-Sentinel, HLC   |
|                | Rompin                    | Muadzam Shah                         | 3°3’34”N 103°5’43”E   | BG-Sentinel, HLC   |
|                |                            | Jalan Muadzam                        | 3°3’26”N 103°5’24”E   | BG-Sentinel, HLC   |
|                | Serdang                   | Seksyen 7, Bandar Baru Bangi         | 2°58’7”N 101°46’42”E  | MLTD               |
|                | Shah Alam                 | Kg. Jalan Kebun                      | 2°59’40”N 101°30’0”E  | BG-Sentinel, HLC, Larva survey |
|                | Temerloh                  | Kuala Krau                           | 3°43’48”N 102°23’1”E  | BG-Sentinel, HLC   |
|                |                            | Kg. Felda Rumpun Makmur              | 3°46’2”N 102°14’27”E  | HLC                |
|                |                            | Kg. Lubok Wong                       | 3°43’31”N 102°19’32”E | HLC                |
|                |                            | Kg. Penderas                         | 3°43’49”N 102°17’2”E  | HLC                |
|                |                            | Kg. Terbol                           | 3°48’49”N 102°13’45”E | HLC                |
|                |                            | Gunung Senyum (rubber plantation)    | 3°41’41”N 102°25’53”E | HLC                |
RESULTS

A total of 1606 mosquitoes were collected and 765 of the mosquitoes belonging to 15 species and five genera were tested for Wolbachia detection by PCR. All samples (765 mosquitoes) were randomly chosen from the total of mosquitoes collected from each sampling sites with a minimum of 30 samples per localities. However, all samples were analysed if n≤30 for each locality. There are two types of location involved, urban (categorised into eight districts) and rural areas (nine districts) consisting of 32 localities (Table 1). As shown in Table 2, 49.4% (378/765) of the mosquitoes were caught by HLC followed with MLTD (34.6%; 265/765), BG-Sentinel (13.1%; 100/765) and larva survey (2.9%; 22/765). BG-Sentinel traps showed its capability to catch a variety of species (12 species), followed with HLC (7 species), larva survey (4 species) and MLTD (2 species). In this study, *Ae. albopictus* has the highest number of collections which were mostly caught using HLC and MLTD methods with a total of 266 and 251, respectively. Whereas, 22 of the *Ae. aegypti* tested were captured during larva survey and using MLTD method.

From the 765 mosquitoes tested across all study sites, 237 (31%) were positive for Wolbachia by PCR and more females were found to be infected with Wolbachia (34.6%; 188/543) compared to males (22.1%; 49/222). Out of 15 mosquito species tested, only five species were found to be positive for Wolbachia. The five species are *Aedes albopictus*, *Armigeres subalbatus*, *Armigeres* spp., *Culex mimeticus* and *Culex quinquefasciatus* (Table 2). For *Ae. albopictus*, out of 600 mosquitoes tested, 189 of the mosquitoes were positive for Wolbachia with 35.8% for females (145/405) and 22.6% for males (44/195). The absence of Wolbachia in *Ae. albopictus* populations were shown in mosquitoes collected from Bukit Nanas (n=19), Melaka (n=2) and Rawang (n=17). However, another species of mosquitoes collected from Bukit Nanas, *Armigeres subalbatus* was found to be positive for Wolbachia. Similar finding was also recorded by mosquitoes collected from Kg. Kg. Sg. Mas, Sg. Lembing (grouped into Kuantan) in which 10 out of 13 of *Ar. subalbatus* captured from these locations were carrying Wolbachia. Despite that, all the *Ae. albopictus* tested (n=10) from these areas were free from Wolbachia. Different findings were reported for Kg. Rumpun Makmur (grouped into Temerloh); only *Ae. albopictus* was positive with Wolbachia. Meanwhile, others mosquito species collected from Kg. Rumpun Makmur were all negative. The mosquito species were *Aedes* (Paraedes) collesi, *Ar. subalbatus*, *Coquillettidia crassipes*, *Culex gelidus*, *Cx. hutchinsoni*, *Cx. mimeticus*, *Mansonia annulata*, *Ma. indiana* and *Ma. uniformis*. Similarly, we also found that *Ae. albopictus* captured from Kg. Jalan Kebun (Shah Alam) were positive with Wolbachia and a total of 11 of *Ar. subalbatus* collected were tested negative for Wolbachia. However, Kg. Jalan Kebun recorded a very low infection rate of 8.5% for *Ae. albopictus* (4/47).

**TABLE 2.** List of mosquitoes species captured by four different collection methods and the status of Wolbachia infection

| Mosquito species | Method of Collections | Total mosquitoes tested | Status of infection | Wolbachia Supergroup | Uninfected Mosquitoes |
|------------------|-----------------------|-------------------------|--------------------|----------------------|-----------------------|
|                  |                       |                         | Male | Female | Male | Female | Male | Female | Male | Female | Male | Female |
| *Aedes aegypti*  | BG Sentinel | 0 | 0 | 8 | 14 | 7 | 15 | - | - | - | - | 7 | 15 |
| *Aedes albopictus* | HLC       | 78 | 266 | 5 | 251 | 195 | 405 | + | 41 | (A,B,AB) | 106 | (A,B,AB) | 3 | 39 | 151 | 260 |
| *Aedes (Paraedes) collesi* | Larva survey | 0 | 1 | 0 | 0 | 0 | 1 | - | - | - | - | - | 0 | 1 |
| *Armigeres subalbatus* | Larva survey | 22 | 47 | 0 | 0 | 1 | 68 | + | 1 | (A) | 29 | (A,B,AB) | 0 | 6 | 0 | 33 |
| *Armigeres* spp. | Larva survey | 5 | 0 | 0 | 0 | 0 | 5 | + | - | 1 | (A) | 0 | 0 | 0 | 4 |
| *Coquillettidia crassipes* | Larva survey | 0 | 5 | 0 | 0 | 0 | 5 | - | - | - | - | - | 0 | 5 |
| *Culex gelidus* | Larva survey | 5 | 0 | 0 | 0 | 0 | 5 | - | - | - | - | - | 0 | 5 |
| *Culex hutchinsoni* | Larva survey | 16 | 0 | 0 | 0 | 12 | 4 | - | - | - | - | - | 12 | 4 |
| *Culex mimeticus* | Larva survey | 1 | 0 | 7 | 0 | 4 | 4 | + | 1 | (B) | 1 | (B) | 0 | 0 | 3 | 3 |
| *Culex quinquefasciatus* | Larva survey | 8 | 1 | 2 | 0 | 3 | 8 | + | 3 | (B) | 6 | (B) | 0 | 0 | 0 | 2 |
| *Culex sinensis* | Larva survey | 1 | 0 | 0 | 0 | 0 | 1 | - | - | - | - | - | 0 | 1 |
| *Culex sitiens* | Larva survey | 1 | 14 | 0 | 0 | 0 | 15 | - | - | - | - | - | 0 | 15 |
| *Mansonia annulata* | Larva survey | 2 | 0 | 0 | 0 | 0 | 2 | - | - | - | - | - | 0 | 2 |
| *Mansonia indiana* | Larva survey | 1 | 0 | 0 | 0 | 0 | 1 | - | - | - | - | - | 0 | 1 |
| *Mansonia uniformis* | Larva survey | 1 | 3 | 0 | 0 | 0 | 4 | - | - | - | - | - | 0 | 4 |

| Total | 100 | 378 | 22 | 265 | (13.1%) | 49 (4.4%) | (2.9%) | 34.6% | 765 | 237 (31%) | 48 | 528 |

* Mosquitoes were positive for Wolbachia but supergrouping was not performed.
The prevalence of *Wolbachia* were focused on the *Ae. albopictus* populations categorised in two types of location, urban and sub-urban or rural areas (Table 3). The higher prevalence of *Wolbachia* were observed from Serdang with 95% (19/20) of the mosquitoes collected were carrying *Wolbachia* followed by Perak (73.3%; 22/30), Bandar Tun Razak (69.4%; 25/36), Wangsa Maju (68.4%; 26/38) and Muadzam Shah (50%; 20/40). Other locations showed a lower rate of infection within the range of 4 to 40%. We also found that 4 out of 14 *Wolbachia* positive locations showed the presence of all three *Wolbachia* supergroups. As shown in Table 3, Bandar Tun Razak recorded almost equal number of *Wolbachia* infection among both males and females. Meanwhile, *Ae. albopictus* from Titiwangsa and Kuantan was predominantly superinfected with both A and B; and followed by single infection. Meanwhile, mosquitoes collected from other three locations were only found to be superinfected with *Wolbachia* (AB), which were the Sepetuh (2/5), Gombak (1/12) and Muadzam Shah (20/40) groups. Interestingly, only three locations showed males with supergroup A single infection of *Wolbachia* which were Bandar Tun Razak (3/25), Titiwangsa (3/16) and Kuantan (1/9). In addition, five locations have showed single infection of females with *Wolbachia* from supergroup A. Meanwhile, single infection of males with supergroup B were also found at five locations and females at seven locations. Nonetheless, more males were singly infected by supergroup B with 60.7% (17/28) infection rate as compared to females (39.3%; 11/28).

### TABLE 3. Status of *Wolbachia* supergroup infection of *Aedes albopictus* in several districts in Malaysia

| Location types | District/Parliament/State | Total mosquito tested (Positive *Wolbachia*) | *Wolbachia* Supergroup | Untested positive samples | Non-infected Mosquito |
|----------------|--------------------------|---------------------------------------------|------------------------|--------------------------|-----------------------|
| Urban          | Batu                     | 27 (2)                                      | 0 0 0 0 1 0 1 0 0 0 7 18 |
|                | Bukit Nanas              | 19 (0)                                      | 0 0 0 0 0 0 0 0 0 0 1 18 |
|                | Bandar Tun Razak         | 36 (25)                                     | 3 3 5 3 2 7 0 2 0 11  |
|                | Kampung Bharu            | 53 (17)                                     | 0 0 1 1 1 14 0 0 27 9 |
|                | Sepetuh                  | 5 (2)                                       | 0 0 0 0 0 2 0 0 2 1  |
|                | Titiwangsa               | 75 (16)                                     | 3 2 0 1 2 5 1 2 35 24 |
|                | Wangsa Maju              | 38 (26)                                     | 0 3 5 1 6 3 1 7 3 9  |
|                | Melaka                   | 2 (0)                                       | 0 0 0 0 0 0 0 0 0 2  |
| Rural          | Gombak                   | 12 (1)                                      | 0 0 0 0 0 0 1 0 0 7 4  |
|                | Kuala Selangor           | 25 (1)                                      | 0 0 0 0 0 0 0 0 0 1 17 7 |
|                | Rawang                   | 17 (0)                                      | 0 0 0 0 0 0 0 0 0 4 13 |
|                | Serdang                  | 20 (19)                                     | 0 0 3 1 5 10 0 0 1 0  |
|                | Shah Alam                | 47 (4)                                      | 0 0 0 0 0 3 0 1 15 28 |
|                | Perak                    | 30 (22)                                     | 0 5 0 0 0 8 1 8 2 6  |
|                | Kuantan                  | 27 (9)                                      | 1 1 0 2 0 3 0 2 3 15  |
|                | Rompin                   | 40 (20)                                     | 0 0 0 0 1 18 0 1 11 9 |
|                | Temerloh                 | 127 (25)                                    | 0 0 3 1 0 6 0 15 16 86 |
| Total          |                          | 600 (189)                                   | 7 14 17 11 17 81 3 39 151 260 |

* Localities: Batu (DBKL MLTD Stor 220 Jalan Ipoh; DBKL MLTD Stor 225 Jalan Semul Pasar), Bukit Nanas, Bandar Tun Razak (DBKL MLTD Stor 215 Velodrome Cheras), Kampung Bharu (UKM Residential 4 Jalan Raja Muda Abdul Aziz; UKM Residential 5 Jalan Raja Abdullah, now known as PICOMS Residential), Sepetuh (DBKL MLTD Stor 200 Jalan Klang Lama), Titiwangsa (UKM Residential 1 Jalan Temerloh), Wangsa Maju (Taman Melati Apartment; DBKL houses Seksyen 1), Melaka (Jalan Bendahara), Gombak (Hutan Simpan Hulu Gombak), Kuala Selangor (Jalan Kilang Gula Lama, Tanjung Karang), Rawang (Hutan Lipur Kanching), Serdang (Sekayu 1 Bandar Baru Bangi), Shah Alam (Kg Jalan Kebun), Perak (Batu Gajah; Felda Gunung Besout), Kuantan (Sg Lembing; Sg Panching), Rompin (Muadzam Shah), Temerloh (Kg Rumpun Makmur; Kg Paya Luar; Kg Lubok Wong; Kg Penderas; Kg Terbol; Taman Eko Rimba Gunung Senyum). DBKL = Kuala Lumpur City Hall, UKM = National University of Malaysia, Kg = Kampung or Village
TABLE 4. Possible crossing patterns between wild mosquitoes from Bandar Tun Razak and (hypothetically) released mosquitoes containing introduced Wolbachia strain(s)

| Female (♀) | Male (♂) | Uninfected | wAlbA | wAlbB | wAlbAwAlbB | wAlbAwAlbBwMel⁺ | wMel⁺ | wRe⁺ |
|------------|----------|------------|-------|-------|------------|------------------|-------|-------|
| wAlbA      | Fertile  | Bi-CI      | Uni-CI| Bi-CI | Bi-CI      | Bi-CI            | Bi-CI | Bi-CI |
| wAlbB      | Bi-CI    | Fertile    | Uni-CI| Bi-CI | Bi-CI      | Bi-CI            | Bi-CI | Bi-CI |
| wAlbAwAlbB | Fertile  | Fertile    | Uni-CI| Bi-CI | Bi-CI⁺⁺⁺    | Bi-CI            | Bi-CI | Bi-CI |
| wAlbAwAlbBwMel⁺ | Bi-CI | Bi-CI      | Bi-CI | Bi-CI | Bi-CI      | Fertile          | Fertile| -     |
| wMel⁺      | Bi-CI    | Bi-CI      |       |       | Bi-CI⁺⁺⁺    |                  |       | -     |
| wRe⁺       | Bi-CI⁺⁺⁺ | Bi-CI⁺⁺⁺   |       |       | Bi-CI⁺⁺⁺    |                  |       | Fertile|       |
| Uninfected | Uni-CI   | Uni-CI     | Uni-CI| Uni-CI| Uni-CI⁺⁺⁺   | Uni-CI⁺⁺⁺        | Uni-CI| Uni-CI|

*Supergroup AB = strain wAlbA+ wAlbB; Supergroup A = strain wAlbA; Supergroup B = strain wAlbB; Several patterns of crosses have been tested as annotation as (a) study by Ant and Sinkins (2018), (b) study by Blagrove et al. (2012), (c) study by Xi et al. (2006); The results of crosses were described as Complete CI which means eggs fail to hatch and Incomplete CI which means ability of eggs to hatch (but shows reduction in hatching rates)

**DISCUSSION**

This current paper extends from our previous article (Noor-Shazleen-Hus nie et al. 2018). Here, we discussed in greater details on the types of Wolbachia infection occurred in males and females captured from different populations of mosquito. As previously reported, a lower rate of infection was recorded with 31% out of 765 mosquitoes molecularly tested were positive with Wolbachia. This study exhibited that more females (34.6%) were infected with Wolbachia compared to males (22.1%) (Table 2). Lower rate of infection was also reported from Thailand and Sri Lanka with positivity of 28.1% and 26.4%, respectively (Kittayapong et al. 2000; Nugapola et al. 2017). Out of 13 mosquito species collected from seven provinces of Sri Lanka, only four species were detected with Wolbachia which were Ae. albopictus, Ar. subalbatus, Cx. quinquefasciatus, and Ma. uniformis. Meanwhile, a study conducted in other regions of Thailand showed a high prevalence of Wolbachia, 61.6% (n=1622 tested) and 28 species out of 74 species screened were infected (Wiwananaratanabutr et al. 2013). Several reasons can be associated with these variations of the infection rate observed. For instance, different Wolbachia detection method employed and genes selection for testing could contribute to the variability of the results. A study has shown an increase in the number of Wolbachia-positive samples after they changed into a new target DNA, wsp gene from previously used, ftsZ (de Albuquerque et al. 2011). Previously, Marcon et al. (2011) have suggested that the combination of 16S rDNA and wsp targets genes is the best molecular method for Wolbachia detection that could prevent false negative results. In our study, we used 16S rDNA as the target gene and Rsal digestion to class the Wolbachia into supergroup.

Our surveillance on Ae. albopictus populations found that most of the localities were predominantly superinfected with Wolbachia by which females (55.9%; 81/145) and males (38.6%; 17/44). Both rural and urban
areas showed the presence of all three types of *Wolbachia* supergroup with a high number of supergroup single infections recorded in this study. Two previous studies conducted in Malaysia reported a very low or no-single infection recorded, albeit higher rate (almost 100%) of *Wolbachia* superinfection of *Wolbachia* were recorded from *Ae. albopictus* populations (Afizah et al. 2017, 2015). Although the mothers carried high density of *Wolbachia*, it is not confirmed that all its progeny will carry the same density of this endosymbiotic bacteria, *Wolbachia*. A study conducted by Ahantarig et al. (2008) showed a high-density infection of F1 mother with *wAlbB* (supergroup B) did not produce F1 (progeny) with a high-density of *wAlbB*. The variation of *Wolbachia* density may plays role in the CI activation which enables the spreads of introduced strain (*Wolbachia*-transinfected mosquito) and *Wolbachia* is randomly passed through generations from mothers to male and female offspring (Ahantarig et al. 2008). However, in this study, we found a low infection rate was recorded in males as compared to females. This low infection of *Wolbachia* detected in males could be due to low *Wolbachia* density presence inside the mosquitoes making it difficult to be detected by conventional PCR method. Previously, reduction of *wAlbA* density in males at day 5 of post-emergence has been shown, whilst the density of *wAlbA* infection in females were found to increase throughout maturation. Mosquitoes age, sex, and different populations play role in *Wolbachia* distribution (Tortosa et al. 2010).

Our study has shown a variation of *Wolbachia* infections detected from different localities. The variation of *Wolbachia* supergroup detected in populations could be due to the activation of CI that changed the female fitness (Sinkins 2004). Superinfected females of *Ae. albopictus* have the advantages of having the compatibility to mate with all types of males (A, B, AB or non-infected) and all offspring will be carrying both supergroup A and B (Dobson et al. 2004; Kittayapong et al. 2002). Kittayapong et al. (2002) showed that superinfected mother from field collection can produce progeny carrying single infection of *Wolbachia* either supergroup A or B (12.5%; 10 out of 80 mothers). In our study, half of the mosquitoes collected from Rompin district were infected with both *Wolbachia* supergroups without the presence of single infection. This is different from Bandar Tun Razak which recorded almost equal number of *Wolbachia* supergroups infection in both males and females. In this condition, various possible cross-mating are predicted to happen involving the activation of two types of CI which are unidirectional (Uni-CI) and bi-directional CI (Bi-CI). However, disadvantages will happen when the infected males do not harbor the same *Wolbachia* supergroup as in females which results in no offspring and suppresses the populations. The modification of sperm by infected *Wolbachia* cannot be rescued in embryo of infected females which then will activate the CI (Brelsfoard & Dobson 2009; Dobson et al. 2004). Due to the high prevalence of superinfected *Ae. albopictus* in natural environment, several studies have developed the artificial *Wolbachia* triple-strain superinfection in *Ae. albopictus*. Theoretically, this will enable the activation of Uni-CI that might increase the possibility of population replacement (Ant & Sinkins 2018; Fu et al. 2010).

We have predicted possible cross-mating that might occur in the case of Bandar Tun Razak if these artificially infected *Ae. albopictus* are introduced (illustrated in Table 4). All males at the Bandar Tun Razak were infected with *Wolbachia*. Therefore, population replacement with the introduced strain might be harder to achieve because the compatible crosses between male and female of artificially generated strains of *wAlbAwAlbBwMel* will result in low number of eggs hatched (Ant & Sinkins 2018). Meanwhile, a study by Fu et al. (2010) have successfully developed males *Ae. albopictus* carrying three *Wolbachia* strains which are *wAlbA*, *wAlbB* and *wRi*, and the cross-mating with natural superinfected females (*wAlbA*, *wAlbB*) showed a new pattern of Uni-CI but still able to produce eggs hatching rate of 16%.

Three localities of *Ae. albopictus* were free with *Wolbachia* which are mosquito collected from forested area in Bukit Nanas, Jalan Bendahara in Melaka and Hutan Lipur Kanching located at Rawang. It is difficult to conclude that Melaka group was entirely free from *Wolbachia* due to the low number of samples tested (n=2). Therefore, a further study is needed in order to confirm this. However, *Ae. albopictus* from Bukit Nanas and Rawang were free of *Wolbachia* and the location types may play roles in the absent of *Wolbachia* as both localities are categorized as natural rainforest which are located at Kuala Lumpur, Capital City of Malaysia. The geographical condition become the limitation for transportation to access thus, prevent the influx of outside mosquitoes (infected) into the population that free of *Wolbachia*. Similar finding was also reported in a study carried out in Lahore, Pakistan which showed that out of 24 *Ae. albopictus* tested, none of them were positive for *Wolbachia* (Gulraiz et al. 2019). The study has postulated that high temperature condition during the samplings had
caused the *Wolbachia* density inside mosquito to reduce which made detection difficult. Furthermore, a previous study in Panama indicated that extreme dry season had an effect towards the natural *Wolbachia* densities inside the beetle *Chelymorpha alternans* (Keller et al. 2004).

In addition, the effect of constant temperatures (up to 40 °C) on *Wolbachia*-infected eggs have been tested and reduction of *Wolbachia* density in adult mosquitoes was shown (Ross et al. 2019a).

Other than *Ae. albopictus*, *Ar. subalbatus*, and *Cx. quinquefasciatus*, we have found that *Cx. mimeticus* captured from Sg. Lembing were positive with *Wolbachia* from supergroup B. This study is first to report *Wolbachia*-positive *Cx. mimeticus*, after negative infection status had been reported from previous studies (Kittayapong et al. 2000; Wiwatanaaratanabutr et al. 2013).

Nonetheless, *Ae. aegypti* collected from urban study areas showed negative infection of *Wolbachia* which are in line with most studies in other countries (Gulraiz et al. 2019; Kittayapong et al. 2000). Rossi et al. (2015) have postulated that the absence of *Wolbachia* in *Ae. aegypti* is associated with the presence of other types of bacteria in mosquito reproductive system known as *Asaia*. Symbiotic bacteria, *Asaia* have the potential as biological control agent for vector borne diseases (Ricci et al. 2012). Previously, a Malaysian study reported the presence of *Wolbachia* in 25% of *Ae. aegypti* larvae collected from a collection site (Teo et al. 2017). Meanwhile, several studies recently have also reported the presence of natural *Wolbachia* from the screened *Ae. aegypti* (Carvajal et al. 2019; Kulkarni et al. 2019). Higher rate of infection was showed by *Ae. aegypti* collected from New Mexico, in which 57.4% out of 148 was found to be infected with *Wolbachia* from supergroup B (Kulkarni et al. 2019). In 2019, Ross et al. (2019b) conducted cross-mating experiment involving *Ae. aegypti* originated from the study sites of Kulkarni et al. (2019) and detection of *Wolbachia* was conducted using highly sensitive molecular methods. However, the results are contrary with the findings as none of the sample was positive with *Wolbachia*. They postulated that cross-contamination between positive mosquitoes in previous study may contributed to the false positive results. Our study had taken several protective measures to prevent the cross contamination such as by taking extra precaution while opening the sample tubes when doing the DNA extraction. In addition, negative and positive control were always included as internal control either during PCR or DNA extraction (Noor-Shazleen-Husnie et al. 2018). Furthermore, all mosquitoes were individually tested instead of pool in group to prevent misdetection in low infected mosquito population (Kulkarni et al. 2019).

From our study, we have successfully detected *Wolbachia* in various species of mosquitoes such as *Ae. albopictus*, *Ar. subalbatus*, *Cx. quinquefasciatus*, and *Cx. mimeticus* by targeting 16S rDNA gene. A study conducted by Wong et al. (2020) showed that most of the mosquito tested were found positive when using 16S rRNA primers compared to *wsp* primers especially in *Anopheles* genera. Meanwhile, their study did not detect *Wolbachia* in *Ar. subalbatus* and only detected it in a low number of *Ae. albopictus* using 16S rRNA. However, our study found that *Wolbachia* was able to be detected using 16s rDNA primers in both of the mosquito species. Therefore, we believed that 16S rDNA could be used as target gene if we would like to conduct the *Wolbachia* detection when involving various species of mosquitoes as first screening molecular method.

Therefore, to overcome problems of low-density detection of *Wolbachia* in the infected mosquito, a highly specific and sensitive molecular technique such as LAMP is required. According to Gonçalves et al. (2019), the analytical sensitivity and specificity of the LAMP assay reached 99.6% and 92.2%, respectively, with a positive predictive value of 97.08% and a negative predictive value of 99.30%. In fact, several studies have reported that LAMP assay can be applied as an alternative technique to replace the gold standard, PCR for *Wolbachia* detection when involving large-scale screening (Gonçalves et al. 2019; Noor-Shazleen-Husnie et al. 2018). We recommend this assay as a rapid, cost-effective and simple method that could be applied within the field at short notice and utilised by users with limited training. All the equipment that would be required would be a hot-block or water bath (Lau et al. 2011). Reagent-wise, the costs would be similar to that of PCR, but the real advantage of this would be the rapidity of this assay, yielding results within an hour of testing, compared to 4-8 hours taken with the PCR method (Notomi et al. 2015).

**CONCLUSION**

*Wolbachia*-infected mosquito is one of the potential control approaches that would enable reduction of the use of chemical application and our reliance on insecticide. This promising approach has been used in several dengue-endemic areas in Malaysia by releasing *Aedes aegypti* carrying selected strain of *Wolbachia*. Meanwhile, *Ae. albopictus* must not be forgotten as they also play role in the transmission of vector-borne
diseases especially dengue and chikungunya. In the next few years, \textit{Ae. albopictus} microinjected with selected \textit{Wolbachia} strain(s) might be used as vector and disease control. Therefore, baseline data on the distribution of natural \textit{Wolbachia} in wild mosquito populations, including \textit{Ae. albopictus}, presented in our manuscript will help to predict and provide better understanding on the outcomes of progeny when CI is activated in the wild mosquito populations. However, further studies are needed to understand the distribution of natural \textit{Wolbachia} infection in Malaysia mosquito populations using molecular technique that incorporated amplification of both 16S rDNA and wsp genes with large scale of mosquito screening before the application of this biological control can be implemented widely.

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Noor Shazleen Husnie Mohd Mohtar, Emelia Osman & Aishah Hani Azil* Department of Parasitology and Medical Entomology Faculty of Medicine Universiti Kebangsaan Malaysia 56000 Cheras, Kuala Lumpur, Federal Territory Malaysia

Mohd Farihan Md Yatim Institute for Public Health Centre for Communicable Diseases Research National Institutes of Health Ministry of Health 40170 Shah Alam, Selangor Darul Ehsan Malaysia

*Corresponding author; email: aishah.azil@ppukm.ukm.edu.my

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