

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 reproduktif 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 daripada kawasan bandar, pinggir bandar dan pedalaman yang kemudiannya dibiak 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 berbeza 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 mempermudah 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-Arifin 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 *RsaI* (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, *RsaI* were used to differentiate between *Wolbachia* supergroup A and B by cut the restriction sites GT[^]AC which are located on 16S rDNA 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	Larva survey
	Rural	Gombak	Ulu Gombak Forest Reserve	3°19'28"N 101°45'1"E
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
Lembing River				
Riverview Resort			3°54'56"N 103° 1'54"E	BG-Sentinel, HLC
Islam Cemetery 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		
		Jalan Muadzam	3° 3'34"N 103° 5'43"E	BG-Sentinel, HLC
		Desa Tanjung	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	
Kg. Felda Rumpun Makmur			3°43'48"N 102°23'1"E	BG-Sentinel, HLC
Kg. Lubok Wong			3°46'2"N 102°14'27"E	HLC
Kg. Paya Luas			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 $n \leq 30$ 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 *Cx. 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. 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 was 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) collessi*, *Ar. subalbatus*, *Coquillettidia crassipes*, *Culex gelidus*, *Cx. hutchinsoni*, *Cx. mimeticus*, *Cx. sinensis*, *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	<i>Wolbachia</i> Supergroup		Untested (But positive for <i>Wolbachia</i>)*		Uninfected Mosquitoes	
	BG Sentinel	HLC	Larva survey	MLTD	Male	Female		Male	Female	Male	Female	Male	Female
<i>Aedes aegypti</i>	0	0	8	14	7	15	-	-	-	-	-	7	15
<i>Aedes albopictus</i>	78	266	5	251	195	405	+	41 (A,B,AB)	106 (A,B,AB)	3	39	151	260
<i>Aedes (Paraedes) collessi</i>	0	1	0	0	0	1	-	-	-	-	-	0	1
<i>Armigeres subalbatus</i>	22	47	0	0	1	68	+	1 (A)	29 (A,AB)	0	6	0	33
<i>Armigeres</i> spp.	5	0	0	0	0	5	+	-	1 (A)	0	0	0	4
<i>Coquillettidia crassipes</i>	0	5	0	0	0	5	-	-	-	-	-	0	5
<i>Culex gelidus</i>	5	0	0	0	0	5	-	-	-	-	-	0	5
<i>Culex hutchinsoni</i>	16	0	0	0	12	4	-	-	-	-	-	12	4
<i>Culex mimeticus</i>	1	0	7	0	4	4	+	1 (B)	1 (B)	0	0	3	3
<i>Culex quinquefasciatus</i>	8	1	2	0	3	8	+	3 (B)	6 (B)	0	0	0	2
<i>Culex sinensis</i>	1	0	0	0	0	1	-	-	-	-	-	0	1
<i>Culex sitiens</i>	1	14	0	0	0	15	-	-	-	-	-	0	15
<i>Mansonia annulata</i>	2	0	0	0	0	2	-	-	-	-	-	0	2
<i>Mansonia indiana</i>	1	0	0	0	0	1	-	-	-	-	-	0	1
<i>Mansonia uniformis</i>	1	3	0	0	0	4	-	-	-	-	-	0	4
					222	543		46	143	3	45	173 (78%)	355 (65%)
Total	100 (13.1%)	378 (49.4%)	22 (2.9%)	265 (34.6%)	765			189 (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 Seputeh (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 <i>Wolbachia</i>)	<i>Wolbachia</i> Supergroup						Untested positive samples		Non- infected Mosquito	
			A		B		AB		Male	Female	Male	Female
			Male	Female	Male	Female	Male	Female				
Urban	Batu	27 (2)	0	0	0	1	0	1	0	0	7	18
	Bukit Nanas	19 (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
	Seputeh	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	1	0	0	7	4
	Kuala Selangor	25 (1)	0	0	0	0	0	0	0	1	17	7
	Rawang	17 (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	
			21 (11.23%)		28 (14.97%)		98 (52.41%)					
			147			42			411			

* Localities: Batu (DBKL MLTD Stor 220 Jalan Ipoh; DBKL MLTD Stor 225 Jalan Sentul 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), Seputeh (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 (Seksyen 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 Luas; Kg Lubok Wong; Kg Penderas; Kg Terbol; Taman Eko Rimba Gunung Senyum). DBKL = Kuala Lumpur City Hall, UKM = National University of Malaysia, Sg = Sungai or River, 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 (♂)					
	wAlbA	wAlbB	wAlbAwAlbB	wAlbAwAlbBwMel ^a	wMel ^b	wRi ^c
wAlbA	Fertile (♂♀A)	Bi-CI	Uni-CI	Bi-CI	Bi-CI	Bi-CI (Incomplete CI)
wAlbB	Bi-CI	Fertile (♂♀B)	Uni-CI	Bi-CI		Bi-CI (Incomplete CI)
wAlbAwAlbB	Fertile (♂♀AB)	Fertile (♂♀AB)	Fertile	Uni-CI (Complete CI)	Bi-CI ^{a,b} (Complete CI)	Bi-CI (Incomplete CI)
wAlbAwAlbBwMel ^a	Bi-CI	Bi-CI	Uni-CI (Incomplete CI)	Fertile (Low hatch rate)	Fertile	-
wMel ^b	Bi-CI	Bi-CI	Bi-CI (Complete CI)	Bi-CI (Complete CI)	Fertile (High hatch rate)	-
wRi ^c	Bi-CI (Incomplete CI)	Bi-CI (Incomplete CI)	Bi-CI (Incomplete CI)	-	-	Fertile (Half hatch rate)
Uninfected	Uni-CI	Uni-CI	Uni-CI ^b (Complete CI)	Uni-CI (Complete CI)	Uni-CI (Complete CI)	Uni-CI (Incomplete CI)

*Supergroup AB = strain wAlbA+ wAlbB; Supergroup A = strain wAlbA; Supergroup B = strain wAlbB; Several pattern 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-Husnie 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 (Wiwatanaratnabutr 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 *RsaI* 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 F_0 mother with *wAlbB* (supergroup B) did not produce F_1 (progeny) with a high-density of *wAlbB*. The variation of *Wolbachia* density may play a role in the CI activation which enables the spread 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 a 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* supergroups 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 absence of *Wolbachia* as both localities are categorized as natural rainforest which are located at Kuala Lumpur, Capital City of Malaysia. The geographical condition becomes the limitation for transportation to access thus, prevent the influx of outside mosquitoes (infected) into the population that is 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; Wiwatanaratnabutr 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*. Symbiont 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, *Ae. albopictus* microinjected with selected *Wolbachia* strain(s) might be used as vector and disease control. Therefore, baseline data on the distribution of natural *Wolbachia* in wild mosquito populations, including *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 *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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REFERENCES

- Afizah, A.N., Vythilingam, I., Lim, Y.A., Zabari, N.Z.A.M. & Lee, H.L. 2017. Detection of *Wolbachia* in *Aedes albopictus* and their effects on chikungunya virus. *The American Journal of Tropical Medicine and Hygiene* 96(1): 148-156.
- Afizah, A.N., Roziah, A., Nazni, W.A. & Lee, H.L. 2015. Detection of *Wolbachia* from field collected *Aedes albopictus* Skuse in Malaysia. *Indian Journal of Medical Research* 142(2): 205-210.
- Ahantari, A., Trinachartvanit, W. & Kittayapong, P. 2008. Relative *Wolbachia* density of field collected *Aedes albopictus* mosquitoes in Thailand. *Journal of Vector Ecology* 33(1): 173-177.
- Ant, T.H. & Sinkins, S.P. 2018. A *Wolbachia* triple-strain infection generates self-incompatibility in *Aedes albopictus* and transmission instability in *Aedes aegypti*. *Parasites and Vectors* 11(1): 295.
- Blagrove, M.S., Arias-Goeta, C., Failloux, A.B. & Sinkins, S.P. 2012. *Wolbachia* strain wMel induces cytoplasmic incompatibility and blocks dengue transmission in *Aedes albopictus*. In *Proceedings of the National Academy of Sciences*. pp. 255-260.
- Brelsfoard, C.L. & Dobson, S.L. 2009. *Wolbachia*-based strategies to control insect pests and disease vectors. *Asia-Pacific Journal of Molecular Biology and Biotechnology* 17(3): 55-63.
- Calvitti, M., Marini, F., Desiderio, A., Puggioli, A. & Moretti, R. 2015. *Wolbachia* density and cytoplasmic incompatibility in *Aedes albopictus*: Concerns with using artificial *Wolbachia* infection as a vector suppression tool. *PLoS ONE* 10(3): e0121813.
- Carvajal, T.M., Hashimoto, K., Harnandika, R.K., Amalin, D.M. & Watanabe, K. 2019. Detection of *Wolbachia* in field-collected *Aedes aegypti* mosquitoes in metropolitan Manila, Philippines. *Parasites and Vectors* 12(1): 361.
- Chen, C.D., Seleena, B., Nazni, W.A., Lee, H.L., Mohd Masri, S.M., Chiang, Y.F. & Sofian Azirun, M. 2006. Dengue vectors surveillance in endemic areas in Kuala Lumpur city centre and Selangor state, Malaysia. *Dengue Bulletin* 30: 197-203.
- de Albuquerque, A.L., Magalhães, T. & Ayres, C.F.J. 2011. High prevalence and lack of diversity of *Wolbachia pipiensis* in *Aedes albopictus* populations from northeast Brazil. *Memórias do Instituto Oswaldo Cruz* 106(6): 773-776.
- Dobson, S.L., Rattanadechakul, W. & Marsland, E.J. 2004. Fitness advantage and cytoplasmic incompatibility in *Wolbachia* single and superinfected *Aedes albopictus*. *Heredity* 93(2): 135-142.
- Fu, Y., Gavotte, L., Mercer, D.R. & Dobson, S.L. 2010. Artificial triple *Wolbachia* infection in *Aedes albopictus* yields a new pattern of unidirectional cytoplasmic incompatibility. *Applied and Environmental Microbiology* 76(17): 5887-5891.
- Gonçalves, D., Hooker, D.J., Dong, Y., Baran, N., Kyrillos, P., Iturbe-Ormaetxe, I., Simmons, C.P. & O'Neill, S.L. 2019. Detecting wMel *Wolbachia* in field-collected *Aedes aegypti* mosquitoes using loop-mediated isothermal amplification (LAMP). *Parasites & Vectors* 12(1): 1-5.
- Gulraiz, M., Alvi, F.M., Mustafa, T., Razzaq, A. & Latif, H.S. 2019. Distribution of *Aedes aegypti*, *Aedes albopictus* and *Culex* sp. and detection of *Wolbachia* among them in city district Lahore. *Journal of Fatima Jinnah Medical University* 13(2): 55-58.
- Hamdan, H., Sofian-Azirun, M., Nazni, W.A. & Lee, H.L. 2005. Insecticide resistance development in *Culex quinquefasciatus* (Say), *Aedes aegypti* (L.) and *Aedes albopictus* (Skuse) larvae against malathion, permethrin and temephos. *Tropical Biomedicine* 22: 45-52.
- Hoffmann, A.A., Montgomery, B.L., Popovici, J., Iturbe-Ormaetxe, I., Johnson, P.H., Muzzi, F., Greenfield, M., Durkan, M., Leong, Y.S., Dong, Y. & Cook, H. 2011. Successful establishment of *Wolbachia* in *Aedes* populations to suppress dengue transmission. *Nature* 476(7361): 454-457.
- Keller, G.P., Windsor, D.M., Saucedo, J.M. & Werren, J.H. 2004. Reproductive effects and geographical distributions of two *Wolbachia* strains infecting the Neotropical beetle, *Chelymorpha alternans* Boh. (Chrysomelidae, Cassidinae). *Molecular Ecology* 13(8): 2405-2420.
- Kittayapong, P., Baisley, K., Sharpe, R., Baimai, V. & O'Neill, S. 2002. Maternal transmission efficiency of *Wolbachia* superinfections in *Aedes albopictus* populations in Thailand. *American Journal Tropical Medicine Hygiene* 66(1): 103-107.

- Kittayapong, P., Baisley, K.J., Baimai, V. & O'Neill, S.L. 2000. Distribution and diversity of *Wolbachia* infections in Southeast Asian mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* 37: 340-345.
- KKM 2020. *Kenyataan Akhbar Ketua Pengarah Kesihatan Malaysia Mengenai Situasi Denggi, Zika dan Chikungunya di Malaysia - ME 45.2020*. Malaysia: Kementerian Kesihatan Malaysia (KKM). Accessed on 22 November 2020.
- KKM 2019. *I-Dengue: Statistik Denggi*. Malaysia: Kementerian Kesihatan Malaysia (KKM). Accessed on 22 November 2020.
- Kulkarni, A., Yu, W., Jiang, J., Sanchez, C., Karna, A.K., Martinez, K.J., Hanley, K.A., Buenemann, M., Hansen, I.A., Xue, R.D. & Ettestad, P. 2019. *Wolbachia pipientis* occurs in *Aedes aegypti* populations in New Mexico and Florida, USA. *Ecology and Evolution* 9(10): 6148-6156.
- Lau, Y.L., Fong, M.Y., Mahmud, R., Chang, P.Y., Palaeaya, V., Cheong, F.W., Chin, L.C., Anthony, C.N., Al-Mekhlafi, A.M. & Chen, Y. 2011. Specific, sensitive and rapid detection of human *Plasmodium knowlesi* infection by loop-mediated isothermal amplification (LAMP) in blood samples. *Malaria Journal* 10(1): 1-6.
- Loke, S.R., Andy-Tan, W.A., Benjamin, S., Lee, H.L. & Sofian-Azirun, M. 2010. Susceptibility of field-collected *Aedes aegypti* (L.) (Diptera: Culicidae) to *Bacillus thuringiensis israelensis* and temephos. *Tropical Biomedicine* 27: 493-450.
- Luo, L., Jiang, L.Y., Xiao, X.C., Di, B., Jing, Q.L., Wang, S.Y., Tang, J.L., Wang, M., Tang, X.P. & Yang, Z.C. 2017. The dengue preface to endemic in mainland China: The historical largest outbreak by *Aedes albopictus* in Guangzhou, 2014. *Infectious Diseases of Poverty* 6(1): 148.
- Marcon, H.S., Coscrato, V.E., Selivon, D., Perondini, A.L.P. & Marino, C.L. 2011. Variations in the sensitivity of different primers for detecting *Wolbachia* in *Anastrepha* (Diptera: Tephritidae). *Brazilian Journal of Microbiology* 42(2): 778-785.
- Mousson, L., Zouache, K., Arias-Goeta, C., Raquin, V., Mavingui, P., Failloux, A.B. & Lambrechts, L. 2012. The native *Wolbachia* symbionts limit transmission of dengue virus in *Aedes albopictus*. *PLoS Neglected Tropical Diseases* 6(12): e1989.
- Nazni, W.A., Hoffmann, A.A., Noor Afizah, A., Cheong, Y.L., Mancini, M.V., Golding, N., Kamarul, G.M., Arif, M.A., Thohir, H., Nur Syamimi, H. & Zatil Aqmar, M.Z. 2019. Establishment of *Wolbachia* strain wAlbB in Malaysian populations of *Aedes aegypti* for dengue control. *Current Biology* 29(24): 4241-4248.
- Noor-Shazleen-Husnie, M.M., Emelia, O., Ahmad-Firdaus, M.S., Zainol-Arifin, P. & Aishah-Hani, A. 2018. Detection of *Wolbachia* in wild mosquito populations from selected areas in Peninsular Malaysia by loop-mediated isothermal amplification (LAMP) technique. *Tropical Biomedicine* 35(2): 330-346.
- Notomi, T., Mori, Y., Tomita, N. & Kanda, H. 2015. Loop-mediated isothermal amplification (LAMP): principle, features, and future prospects. *Journal of Microbiology* 53(1): 1-5.
- Nugapola, N.N.P., De Silva, W.P.P. & Karunaratne, S.P. 2017. Distribution and phylogeny of *Wolbachia* strains in wild mosquito populations in Sri Lanka. *Parasites and Vectors* 10(1): 230.
- Pourali, P., Roayaei, A.M., Jolodar, A. & Razi, J.M.H. 2009. PCR screening of the *Wolbachia* in some arthropods and nematodes in Khuzestan province. *Iranian Journal of Veterinary Research* 10(3): 216-222.
- Rasgon, J.L. & Scott, T.W. 2004. An initial survey for *Wolbachia* (Rickettsiales: Rickettsiaceae) infections in selected California mosquitoes (Diptera: Culicidae). *Journal of Medical Entomology* 41(2): 255-257.
- Ricci, I., Valzano, M., Ulissi, U., Epis, S., Cappelli, A. & Favia, G. 2012. Symbiotic control of mosquito borne disease. *Pathogens and Global Health* 106(7): 380-385.
- Ricci, I., Cancrini, G., Gabrielli, S., D'amelio, S. & Favia, G. 2002. Searching for *Wolbachia* (Rickettsiales: Rickettsiaceae) in mosquitoes (Diptera: Culicidae): Large polymerase chain reaction survey and new identifications. *Journal of Medical Entomology* 39(4): 562-567.
- Ross, P.A., Callahan, A.G., Yang, Q., Jasper, M., Arif, M.A., Afizah, A.N., Nazni, W.A. & Hoffmann, A.A. 2019a. An elusive endosymbiont: Does *Wolbachia* occur naturally in *Aedes aegypti*? *Ecology and Evolution* 10(3): 1581-1591.
- Ross, P.A., Ritchie, S.A., Axford, J.K. & Hoffmann, A.A. 2019b. Loss of cytoplasmic incompatibility in *Wolbachia*-infected *Aedes aegypti* under field conditions. *PLoS Neglected Tropical Diseases* 13(4): e0007357.
- Rossi, P., Ricci, I., Cappelli, A., Damiani, C., Ulissi, U., Mancini, M.V., Valzano, M., Capone, A., Epis, S., Crotti, E. & Chouaia, B. 2015. Mutual exclusion of *Asaia* and *Wolbachia* in the reproductive organs of mosquito vectors. *Parasites and Vectors* 8(1): 278.
- Rozilawati, H., Tanaselvi, K., Nazni, W.A., Masri, S.M., Zairi, J., Adanan, C.R. & Lee, H.L. 2015. Surveillance of *Aedes albopictus* Skuse breeding preference in selected dengue outbreak localities, Peninsular Malaysia. *Tropical Biomedicine* 32(1): 49-64.
- Sinkins, S.P. 2004. *Wolbachia* and cytoplasmic incompatibility in mosquitoes. *Insect Biochemistry and Molecular Biology* 34(7): 723-729.
- Teo, C.H.J., Lim, P., Voon, K. & Mak, J.W. 2017. Detection of dengue viruses and *Wolbachia* in *Aedes aegypti* and *Aedes albopictus* larvae from four urban localities in Kuala Lumpur, Malaysia. *Tropical Biomedicine* 34(3): 583-597.
- Tortosa, P., Charlat, S., Labbe, P., Dehecq, J.S., Barré, H. & Weill, M. 2010. *Wolbachia* age sex-specific density in *Aedes albopictus*: A host evolutionary response to cytoplasmic incompatibility? *PLoS ONE* 5(3): e9700.
- Werren, J.H. & Windsor, D.M. 2000. *Wolbachia* infection frequencies in insects: Evidence of a global equilibrium? In *Proceedings of the Royal Society of London. Series B: Biological Sciences*. pp. 1277-1285.
- Wiwatanaratnabutr, I. 2013. Geographic distribution of wolbachial infections in mosquitoes from Thailand. *Journal of Invertebrate Pathology* 114(3): 337-340.

- WMP 2019. *The World Mosquito Program's Wolbachia Method is Helping Communities Around the World Prevent the Spread of Mosquito-Borne Disease*. Sri Lanka: World Mosquito Program (WMP).
- Wong, M.L., Liew, J.W.K., Wong, W.K., Pramasivan, S., Hassan, N.M., Sulaiman, W.Y.W., Jeyaprakasam, N.K., Leong, C.S., Low, V.L. & Vythilingam, I. 2020. Natural *Wolbachia* infection in field-collected *Anopheles* and other mosquito species from Malaysia. *Parasites and Vectors* 13(1): 1-15.
- Xu, G., Dong, H., Shi, N., Liu, S., Zhou, A., Cheng, Z., Chen, G., Liu, J., Fang, T., Zhang, H. & Gu, C. 2007. An outbreak of dengue virus serotype 1 infection in Cixi, Ningbo, People's Republic of China, 2004, associated with a traveler from Thailand and high density of *Aedes albopictus*. *The American Journal of Tropical Medicine and Hygiene* 76(6): 1182-1188.
- Zainol, A.P., Ahmad, Z.Z., Norhayati, S., Umi, A., Osman, H., Awaluddin, M.A., Abdul, H., Omar, H. & Mohd, H.M. 2009. Using mosquito larvae trapping device as an additional tool for dengue fever control in Kuala Lumpur. *Malaysian Journal of Public Health Medicine* 9(Suppl. 2): 34.
- Zhang, D., Lees, R.S., Xi, Z., Bourtzis, K. & Gilles, J.R.L. 2016. Combining the sterile insect technique with the incompatible insect technique: III-robust mating competitiveness of irradiated triple *Wolbachia* infected *Aedes albopictus* males under semi-field conditions. *PLoS ONE* 11(3): e0151864.
- Zhou, W., Rousset, F. & O'Neil, S. 1998. Phylogeny and PCR-based classification of *Wolbachia* strains using wsp gene sequences. *Proceedings of the Royal Society of London. Series B: Biological Sciences* 265: 509-515.
- 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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