

Detection of dengue viruses and *Wolbachia* in *Aedes aegypti* and *Aedes albopictus* larvae from four urban localities in Kuala Lumpur, Malaysia

Teo, C.H.J., Lim, P.K.C.*, Voon, K. and Mak, J.W.

School of Medicine, International Medical University, Jalan Jalil Perkasa 19, Bukit Jalil, 57000 Kuala Lumpur, Malaysia

*Corresponding author e-mail: kimchooi_lim@imu.edu.my

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Abstract. Dengue fever (DF) is currently one of the most important mosquito-borne diseases that affects humans. Dengue fever (DF) and dengue hemorrhagic fever (DHF) are caused by four serotypes of dengue viruses (DENV-1 to DENV-4). The main vector transmitting dengue is *Aedes aegypti* while *Aedes albopictus* acts as a secondary vector. As treatment is unavailable and the first dengue vaccine approved in Mexico, Dengvaxia® has yet to be accepted worldwide, prevention of the disease relies heavily on surveillance and control of mosquito vectors. A transgene driver, *Wolbachia* was found to limit the transmission of dengue virus in *Aedes* mosquitoes. *Wolbachia* alone was able to inhibit viral replication, dissemination and transmission in *A. aegypti* mosquitoes in experimental studies. In *A. albopictus*, *Wolbachia* did not affect the replication of dengue virus but was able to reduce the viral infection of mosquito salivary glands and limit transmission. Studies on *Wolbachia* have all been carried out in adult *Aedes* mosquitoes, hence this study was conducted to determine the presence of dengue virus serotypes and *Wolbachia* in *A. aegypti* and *A. albopictus* larvae collected from ovitraps in four localities in Kuala Lumpur viz. Happy Gardens, IMU Bukit Jalil, Ampang and Taman Yarl. Another objective of this study was to determine the association between dengue virus serotypes and the presence of *Wolbachia* in *A. aegypti* and *A. albopictus* larvae. A total of 300 mosquito larvae was collected; 99 (Happy Gardens), 85 (Bukit Jalil), 73 (Ampang) and 43 (Taman Yarl). Out of 300 larvae collected, 284 were identified as *A. albopictus* and 16 others were identified as *A. aegypti*. Of the 284 *A. albopictus* larvae collected, 211 (74.3%) and 73 (25.7%) were found to be negative and positive for dengue virus respectively. The dengue serotypes detected were 2 DENV-2 (2.7%), 58 DENV-3 (79.5%) and 13 DENV-4 (17.8%). DENV-1 was not detected in any of the *A. albopictus* larvae. For *A. aegypti*, out of 16 *A. aegypti* larvae collected, 12 (75%) were found to be negative and 4 (25%) were positive for DENV-2. For the detection of *Wolbachia* in *A. albopictus*, 71 out of 284 (25%) and 213 (75%) larvae were found to be positive and negative for *Wolbachia* respectively. For *A. aegypti*, 4 (25%) and 12 (75%) out of 16 larvae were positive and negative for *Wolbachia* respectively. This is the first report of *Wolbachia* in *A. albopictus* and *A. aegypti* larvae in Malaysia. A chi-square test analysis to determine the association between dengue virus and *Wolbachia* in *A. albopictus* and *A. aegypti* larvae collected from the four localities in Kuala Lumpur showed that there was no association ($\chi^2 = 3.080$; df = 1; P > 0.05).

INTRODUCTION

Dengue fever (DF) is one of the most important mosquito-borne diseases that affects humans in terms of morbidity and mortality (Tan *et al.*, 2011). The disease is caused by four dengue virus serotypes

(DENV 1 – DENV 4) belonging to the Flavivirus family. Dengue is predominantly found in urban and suburban areas, particularly within the tropical and subtropical regions around the world. An estimated 390 million people are infected every year (Bhatt *et al.*, 2015), more than

three times the figure predicted by the World Health Organization (WHO) in 2012 (World Health Organization, 2012). Malaysia experienced an unprecedented dengue outbreak beginning mid-2013, with incidence rates in 2014 exceeding 300 per 100,000 population which is more than double compared to 2013 (Ng *et al.*, 2013; Mudin, 2015). In 2014, Malaysia reported an incidence of 108,698 cases – the largest number of dengue cases reported in the country to date (Mudin, 2015).

In Southeast Asia, *Aedes aegypti* which breeds in stagnant water and commonly found indoors is the main vector for dengue virus, while *Aedes albopictus* which is commonly found outdoors acts as a secondary vector. As treatment is unavailable and the first dengue vaccine (Dengvaxia®) which was developed by Sanofi-Pasteur and approved in Mexico (<http://www.sanofipasteur.com/en/articles/dengvaxia-world-s-first-dengue-vaccine-approved-in-mexico.aspx>.) has yet to be accepted world-wide, prevention of the disease relies heavily on the surveillance and control of mosquito vectors (Tan *et al.*, 2011). Vector surveillance will contribute to timely implementation of control measures, such as adulticiding, habitat destruction, source reduction and community participation, which may reduce the occurrence of outbreaks. In addition to vector surveillance, active surveillance is another way of limiting the impact of dengue. This is because the detection of infected mosquitoes may be valuable in defining the spatial and temporal risks of acquiring dengue infection (Tan *et al.*, 2011).

One possible intervention would be the replacement of natural *A. aegypti* population with modified populations that are unable to transmit dengue virus by using transgene drivers (Chen *et al.*, 2007; Sinkins & Gould, 2006). A promising transgene driver is the maternally transmitted Gram-negative endosymbiotic bacterium, *Wolbachia*. *Wolbachia* is a genus of obligate, intracellular, maternally inherited bacteria that occurs in many insect species (Stouthamer *et al.*, 1999). Cytoplasmic incompatibility (CI), one of the several reproductive manipulations used by *Wolbachia* to reduce transmission

of dengue occurs in matings between individuals that differ in their *Wolbachia* infection type and results in early embryonic death (Xi *et al.*, 2005). Experimental studies to characterize the interaction of *Wolbachia* with dengue viruses in *A. aegypti* have shown that *Wolbachia* alone was able to inhibit viral replication, dissemination and transmission in these mosquitoes (Bian *et al.*, 2010). In addition, this *Wolbachia*-mediated viral interference was associated with an elevation of basal immunity and increase in longevity of the mosquitoes (Bian *et al.*, 2010). In another study conducted in La Reunion Island, the interaction of native *Wolbachia* symbionts in a naturally super-infected (with two strains *Wolbachia* strains, wAlbA and wAlbB) *A. albopictus* population was studied. It was reported that *Wolbachia* did not affect the replication of dengue virus in this strain of *A. albopictus* but was able to reduce the viral infection of mosquito salivary glands and limit transmission, thus suggesting the role of *Wolbachia* in naturally restricting transmission of dengue virus in *A. albopictus* from La Reunion Island (Mousson *et al.*, 2012).

One *Wolbachia* strain (*wMelPop*) that shortens the lifespan of adult *Drosophila* by up to 50% was discovered from experiments on fruit-fly lifespan mutants (Moreira *et al.*, 2009). Recently, a study was carried out on the transfer of *wMelPop* from its natural host, *Drosophila melanogaster*, into the dengue fever vector *A. aegypti* (Iturbe-Ormaetxe *et al.*, 2011). *wMelPop*-infected mosquitoes showed approximately 50% reduction in adult lifespan, compared with their uninfected counterparts (Moreira *et al.*, 2009). The halving of adult mosquito lifespan and the high *Wolbachia* maternal transmission rates were also maintained in more genetically diverse outbred mosquitoes and larval nutrition did not affect the life-shortening ability of the *wMelPop* infected strain (McMeniman *et al.*, 2009). The *wMelPop* infection was widespread in *A. aegypti* tissues, with high bacterial densities in the head (brain and ommatidia), thorax (salivary glands, muscle) and abdomen (fat tissue, reproductive tissues and malpighian tubules) (McMeniman *et al.*, 2009).

To date, *Wolbachia* has been found to occur naturally in *A. albopictus* (Kittapayong *et al.*, 2002; Joanne *et al.*, 2015) but not in *A. aegypti*. A recent study on the distribution and reproductive dynamics of *Wolbachia* in *A. albopictus* conducted in Malaysia showed an infection rate from 60 to 100% (Joanne *et al.*, 2015). In another study, *A. albopictus* collected from different localities in peninsular Malaysia indicated that *Wolbachia* infection was widespread in *A. albopictus* population, both in female and male *A. albopictus*. All the infected females were superinfected with both A and B strains while the infected males showed a combination of superinfection of A and B strains and single infection of B strain (Noor Afizah *et al.*, 2015). All the studies on *Wolbachia* in *Aedes* have been carried out in the adult mosquito population, thus the objective of this study was to determine whether *Wolbachia* is present in both *A. aegypti* and *A. albopictus* larvae in Malaysia and to associate their occurrence with the presence of the various serotypes of dengue viruses in these vectors.

MATERIALS AND METHODS

Larvae Collection Sites

Mosquito larvae were collected from four localities in Kuala Lumpur namely, Happy Gardens (GPS: 3°4'53"N 101°41'6"E), Bukit Jalil (GPS: 3°3'35"N 101°41'14"E), Ampang (GPS: 3°9'00"N 101°46'12"E) and Taman Yarl (GPS: 3°4'21.9"N 101°40'14.2"E).

Larvae Collection Method

Ovitrap were used for the collection of *A. aegypti* and *A. albopictus* larvae. The ovitraps used were black plastic containers with an oviposition paddle (tongue depressor) and the containers were filled with tap water to a level of 5-6cm in height. They were placed indoors and outdoors mostly in shaded areas, so as to prevent from direct sunlight and heavy rain. For each sampling, thirty ovitraps were placed at each study site (15 indoors and 15 outdoors). They were collected after 7 days and replaced with fresh ovitraps for

the subsequent collection. This was carried out for a period of 4 months from November 2013 to February 2014. The collected ovitraps were brought back to the laboratory and the number and spp. (*A. aegypti* or *A. albopictus*) of larvae per ovitrap were identified and recorded.

Nucleic Acid Extraction (RNA/DNA)

The mosquito larvae were homogenized using glass beads (Tomy, Japan) in 2ml microsmash tubes. Each microsmash tube (Tomy, Japan) was filled to a quarter with microsmash glass beads. Thereafter, each mosquito larva was transferred into the respective labelled microsmash tubes and 0.5ml of TRIzol reagent was added. The larva was then homogenized using TOMY Micro Smash MS-100 machine with the speed of 5500rpm for 30 seconds. Immediately after homogenization, the microsmash tube was then put on ice for 5-10 minutes to prevent the nucleoprotein complex from denaturing due to the heat generated during the homogenization process.

Each homogenized sample was taken out from the ice bath and incubated at room temperature for 5 minutes to allow complete dissociation of the nucleoprotein complex in the homogenate. The homogenate was then transferred from the microsmash tube into a new sterile 1.5ml microtube and 0.1ml of chloroform was added into each microtube and the microtubes were capped securely. The microtubes were shaken by hand for approximately 15 seconds and were then incubated at room temperature for 2-3 minutes. Subsequently, the samples were centrifuged at 12,000xg for 15 minutes at 4°C using a refrigerated microcentrifuge (SIGMA 1-14K Model). After centrifugation, the mixture had separated into 3 phases; an upper colourless aqueous phase, a thin cloudy interphase and a lower red phenol-chloroform phase. The RNA remains exclusively in the upper aqueous phase while the DNA sample was present in the interphase and lower organic phenol-chloroform phase. The upper aqueous phase was then carefully withdrawn and transferred into a new sterile 1.5ml microtube for RNA isolation.

However, the interphase and lower organic phenol-chloroform phase were kept at 4°C for the isolation of DNA later on.

RNA Isolation

0.25ml of 100% isopropanol was added to the aqueous phase in each 1.5ml microtube. The samples were incubated at room temperature for 10 minutes and thereafter centrifuged at 12,000xg for 10 minutes at 4°C using a refrigerated microcentrifuge (SIGMA 1-14K). After centrifugation, the supernatant was removed from each microtube, leaving only the RNA pellet. The pellet was then washed with 0.5ml of 75% ethanol. Subsequently, the samples were vortexed briefly and centrifuged at 7500xg for 5 minutes at 4°C using a refrigerated microcentrifuge (SIGMA 1-14K). The wash was discarded, allowing the RNA pellet to air dry for 10 minutes.

DNA Isolation

0.15ml of 100% ethanol was added into each microtube containing the interphase and the phenol-chloroform phase. The microtubes were capped and inverted several times to mix. The sample was then incubated at room temperature for 2–3 minutes before centrifuging at 2000xg for 5 minutes at 4°C using a refrigerated microcentrifuge (SIGMA 1-14K). The phenol-ethanol supernatant was removed, leaving the DNA pellet in the microtube. The DNA pellet was washed with 0.5ml of sodium citrate/ethanol solution (0.1M sodium citrate in 10% ethanol, pH 8.5) and incubated at room temperature for 30 minutes with occasional mixing by gentle inversion. The sample was then centrifuged at 2000xg for 5 minutes at 4°C and the supernatant was removed. All these steps were repeated once for thorough wash. After that, 0.75ml of 75% ethanol was added into each microtube and the sample was incubated at room temperature for 10 minutes. The microtube was mixed occasionally by gentle inversion. After incubating at room temperature for 10 minutes, the sample was centrifuged at 2000xg for 5 minutes at 4°C using a refrigerated microcentrifuge (SIGMA 1-14K) and thereafter the supernatant was removed leaving the DNA pellet in the microtube. The

DNA pellet was then air dried for 10 minutes. The DNA pellet in each microtube was resuspended in 0.5ml of 8mM sodium hydroxide. Any insoluble material was removed by centrifuging the sample at 12,000xg for 10 minutes at 4°C. Following that, the supernatant of each microtube containing the DNA was transferred into a new sterile microtube. The DNA sample was stored at -30°C until used.

Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for detection of dengue viruses

Six ml of RNA from each sample were added with 1ml of deoxyribonucleotide triphosphate (dNTP), 1 µl of dengue forward primer (D1) and 1 µl of dengue reverse primer (D2) (Table 1) in a 0.2ml PCR tube. The mixture was incubated at 65°C for 5 minutes in the Bio-Rad MyCycler gradient PCR machine for heat denaturation, followed by immediate incubation on ice to prevent re-annealing of ssRNA. Thereafter, to complete the reverse transcription reaction, 4 µl of 5x Reverse Transcriptase Buffer, 2 µl of 0.1M dithiothreitol (DTT), 0.8 µl of Reverse Transcriptase (RT) Enzyme were added to the former mixture and topped-up with 4.2 µl of DEPC water, making up a 20 µl reaction mixture. It was then incubated at 38°C for 1 hour in the Bio-Rad MyCycler gradient PCR machine. The end product, complementary DNA (cDNA) was stored at -30°C before being further processed.

The cDNA from the previous step was used to perform uniplex and nested PCR for the detection of dengue virus serotypes. All primers used in this study are shown in Table 1. For the detection of dengue virus serotype 1 (DENV-1) and 2 (DENV-2), uniplex PCR was carried out using the Bio-Rad MyCycler gradient PCR machine. The polymerase chain reaction contained 3 µl of master mix (i-PCR 5x RED Master Mix, iDNA), 0.9 µl of 25mM MgCl₂, 0.5 µl of Dcon primer, 0.5 µl of TS1 (for DENV-1) or TS2 (for DENV-2) and topped-up with 9.7 µl of DEPC water to form a 14.6 µl reaction mixture. Following that, 0.4 µl of cDNA was then added into the mixture, forming a final volume of 15 µl reaction mixture. Separately, a negative

Table 1. List of primers used in PCR for the detection of dengue viruses and *Wolbachia* and the expected amplicon sizes

Organism	Primer name	Primer sequence	Amplicon size (bp)	Reference
Dengue viruses	D1 D2	5'-CAATATGCTGAAACGCGCGAGAAACCG-3' 5'-TTGCACCAACAGTCAATGTCTTCAGGTTTC-3'	511	Reynes <i>et al.</i> , 2003
Dengue 1	Dcon TS1	5'-AGTTGTTAGTCTACGTGGACCGACA-3' 5'-CGTCTCAGTGATCCGGGGG-3'	613	Yong <i>et al.</i> , 2007
Dengue 2	Dcon TS2	5'-AGTTGTTAGTCTACGTGGACCGACA-3' 5'-CGCCACAAGGGCCATGAACAG-3'	252	
Dengue 3	D1 TS3	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-TAACATCATCATGAGACAGAGC-3'	290	Lanciotti <i>et al.</i> , 1992
Dengue 4	D1 TS4	5'-TCAATATGCTGAAACGCGCGAGAAACCG-3' 5'-CTCTGTTGTCTTAAACAAGAGA-3'	392	
<i>Wolbachia</i>	wsp 81F wsp 691R	5'-TGG TCC AAT AAG TGATGA AGAAAC-3' 5'-AAA AAT TAA ACG CTA CTC CA-3'	600	Zhou <i>et al.</i> , 1998

and a positive control were also included. The PCR cycling conditions were as follows: 95°C for 3 minutes, followed by 35 cycles of (melting temperature of 95°C for 40 seconds, annealing temperature of 55°C for 45 seconds and extension temperature of 72°C for 1 minute) and lastly 72°C for 3 minutes. The PCR reaction was then terminated with a down regulation temperature of 10°C forever.

For the detection of dengue virus serotype 3 (DENV-3) and 4 (DENV-4), nested PCR was carried out using the Bio-Rad MyCycler gradient PCR machine. For the first round of PCR, the polymerase chain reaction contained 3 µl of master mix (i-PCR 5x RED Master Mix, iDNA), 0.9 µl of 25mM MgCl₂, 0.5 µl of dengue forward primer (D1), 0.5 µl of dengue reverse primer (D2) and topped-up with 9.1 µl of DEPC water to form a 14 µl reaction mixture. Following that, 1 µl of cDNA was then added into the mixture, forming a final volume of 15 µl reaction mixture. Separately, a negative and a positive control were included. The PCR cycling conditions were as follows: 95°C for 3 minutes, followed by 30 cycles of (melting temperature of 95°C for 40 seconds, annealing temperature of 55°C for 45 seconds and extension temperature of 72°C for 1 minute) and lastly 72°C for 3 minutes. The

PCR reaction was then terminated with a down regulation temperature of 10°C forever. The products from the first PCR were then used for the second round of PCR. The polymerase chain reaction contained 3 µl of master mix, 0.9 µl of 25mM MgCl₂, 0.5 µl of dengue forward primer (D1), 0.5 µl TS3 (for DENV-3) or TS4 (for DENV-4) and topped-up with 9.7 µl of DEPC water to form a 14.6 µl reaction mixture. Following that, 0.4 µl of cDNA was then added into the mixture. The PCR cycling conditions were as follows; 95°C for 3 minutes, followed by 35 cycles of (melting temperature of 95°C for 40 seconds, annealing temperature of 58°C for DENV-3 and 55°C for DENV-4 for 45 seconds respectively and extension temperature of 72°C for 1 minute) and lastly 72°C for 3 minutes. The PCR reaction was then terminated with a down regulation temperature of 10°C forever.

PCR for the detection of *Wolbachia*

For the detection of *Wolbachia*, a uniplex PCR was carried out using Bio-Rad MyCycler gradient PCR machine. The polymerase chain reaction contained 2 µl of 10x buffer without MgCl₂, 1.6 µl of 25mM MgCl₂, 0.45 µl of deoxyribonucleotide triphosphate (dNTP), 0.33 µl of Taq Polymerase, 1 µl of wsp 81F

forward primer, 1 µl of wsp 691R reverse primer and topped-up with 12.62 µl of ultrapure water to form a 19 µl reaction mixture. Following that, 1 µl of DNA was then added into the mixture, forming a final volume of 20 µl reaction mixture. Separately, a negative and a positive control were included. The PCR cycling conditions were as follows: 95°C for 3 minutes, followed by 40 cycles of (melting temperature of 95°C for 50 seconds, annealing temperature of 57°C for 50 seconds and extension temperature of 72°C for 2 minutes). The PCR reaction was then terminated with a down regulation temperature of 10°C forever.

***Wolbachia* Positive Control**

As no *Wolbachia pipientis* DNA was available commercially to serve as a positive control in PCR, we performed the PCR on 10 *A. albopictus* larval DNA to detect the *Wolbachia* wsp gene in these samples. After the PCR was performed, the PCR product of approximately 600bp from sample C1 was selected for further studies to determine whether it contained the wsp gene of *Wolbachia*. The PCR product was purified with GeneAll Expin Combo GP (GeneAll, Korea) according to manufacturer's protocol. The purified product was cloned in pCRII TOPO (Invitrogen, USA) according to the manufacture's protocol. Five µl of the ligated mixture were transformed into 100 µl of competent bacteria cells JM101 (Promega, USA) using the heat shock method: 42°C for 1 minute and immediately put on ice. The transformed JM101 cells were then added to 100 µl SOC medium in 1.5 µl tubes which was incubated at 200rpm for 1 hour at 37°C in an orbital shaker incubator (Yih Der LM-510, China). This was then plated onto agar with added kanamycin and left overnight at 37°C. One half of bacterial colonies were picked using sterile toothpicks and used as template DNA for colony PCR. Briefly, a mixture of 4 µl of 5x master mix, 1.5 µl of each 20uM M13 forward and reverse primers, and nuclease-free water were added to a total volume of 20 µl. The PCR was performed using the following protocol: 95°C for 13 minutes, 40 cycles of (denaturation at 94°C for 30 seconds, annealing at 55°C for 30

seconds, extension at 72°C for 1 minute) and lastly, 72°C for 3 minutes. Plasmid extraction was carried out to purify the plasmids using Nucleospin Plasmid Purification Kit (Machery Nagel, Germany) according to the manufacturer's centrifuge method protocol. The purified plasmids were then sent for gene sequencing to confirm the insert.

Agarose Gel Electrophoresis to detect PCR products

Ten µl of each PCR product was analyzed by running electrophoresis in 1.5% agarose gel containing ethidium bromide. Six µl of 100bp DNA ladder were also included into one well for each gel for comparison. The samples were run under the conditions of 180 volts for 25 minutes. The gels were then visualized under ultraviolet ray using Bio-Rad Gel EZ Imager and the images were captured.

RESULTS

Prevalence and distribution of dengue serotypes in *A. albopictus* and *A. aegypti* larvae by locality in Kuala Lumpur

A total of 300 mosquito larvae were collected from the 4 localities. Of the 300 mosquito larvae collected, 223 (78.5%) were collected outdoors and 77 (21.5%) were collected indoors. A total of 284 larvae (94.7%) were identified as *A. albopictus* whereas only 16 (5.3%) were identified as *A. aegypti* based on the morphology of the comb scales (Figures 1A and 1B).

Table 2 shows the prevalence of dengue virus in *A. albopictus* and *A. aegypti* larvae and the distribution of the dengue virus serotypes from the 4 localities in Kuala Lumpur collected from November 2013-February 2014. Of the 284 *A. albopictus* larvae collected, 211 (74.3%) and 73 (25.7%) were found to be negative and positive for dengue virus respectively. For *A. albopictus*, the dengue serotype distribution included 2 DENV-2 (2.7%), 58 DENV-3 (79.5%) and 13 DENV-4 (17.8%). For *A. aegypti*, of the 16 *A. aegypti* larvae collected, 12 (75%) and 4 (25%) were found to be negative and positive (DENV-2) for dengue virus respectively. The dengue serotypes were

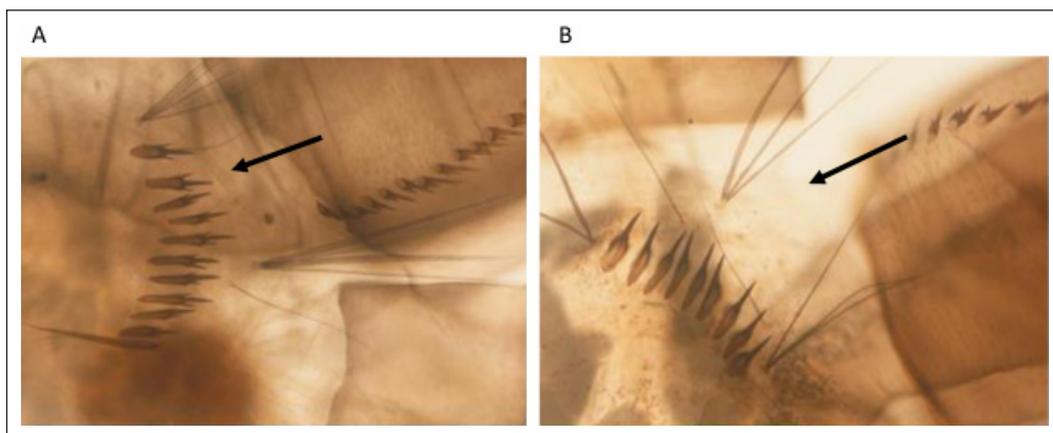


Figure 1. Microscopy identification of *Aedes* larva. A) *A. aegypti* larva: Comb scales with a distinct middle denticle and lateral denticles indicated by black arrow (200X magnification). Scale 1 cm=50 mm. B) *A. albopictus* larva: Comb scales with a single denticle indicated by black arrow (200X magnification). Scale 1 cm=50 mm.

Table 2. Prevalence and distribution of dengue serotypes in *A. albopictus* and *A. aegypti* larvae by locality in Kuala Lumpur

Locality and total no. of larvae collected	<i>A. albopictus</i> (n=284)				<i>A. aegypti</i> (n=16)			
	DENV-1	DENV-2	DENV-3	DENV-4	DENV-1	DENV-2	DENV-3	DENV-4
Happy Gardens N=99	0	0	34	13	0	0	0	0
IMU, Bukit Jalil N=85	0	0	9	0	0	0	0	0
Ampang N=73	0	2	7	0	0	2	0	0
Taman Yarl N=43	0	0	8	0	0	2	0	0
No. and % positive	73/284 (25.7%)				4/16 (25%)			

found to vary with the different localities; DENV-1 was not detected in any of the *A. albopictus* and *A. aegypti* larvae. DENV-2 was found in Ampang and Taman Yarl while DENV-4 (13 larvae) was only found in Happy Gardens. The most prevalent serotype was DENV-3 (58 larvae) which was found in all the 4 localities. Of the 4 localities, Happy Gardens was found to have the highest % positivity for dengue virus (47.47%) followed by Taman Yarl (23.25%), Ampang (12.33%) and Bukit Jalil (10.59%).

DNA sequence of cloned PCR product (*Wolbachia* *wsp* gene) from C1 sample to be used as positive control

From the PCR performed initially on 10 random *A. albopictus* DNA samples using primers for the *Wolbachia* *wsp* gene (wsp81F

and wsp691R) and it was observed that sample C1 from Happy Gardens had a PCR product of 600bp in size. The PCR for this sample was repeated and the PCR product was then cloned and sequenced to confirm the *Wolbachia* *wsp* gene. DNA sequencing showed that the C1 PCR product had 100% identity with *Wolbachia* endosymbiont of *Anastrepha fraterculus* (*South American fruit fly*) *wsp* gene (Figure 2A). Figure 2B shows the alignment scores for *Wolbachia* sequence which is more than 200 scores, indicating high similarity to *Wolbachia* sequences in database. The nucleotide sequence of the PCR product cloned in pCRII TOPO vector according to manufacturer's protocol is shown in Figure 2C. Subsequently C1 DNA was used as the positive control for *Wolbachia* PCR experiments.

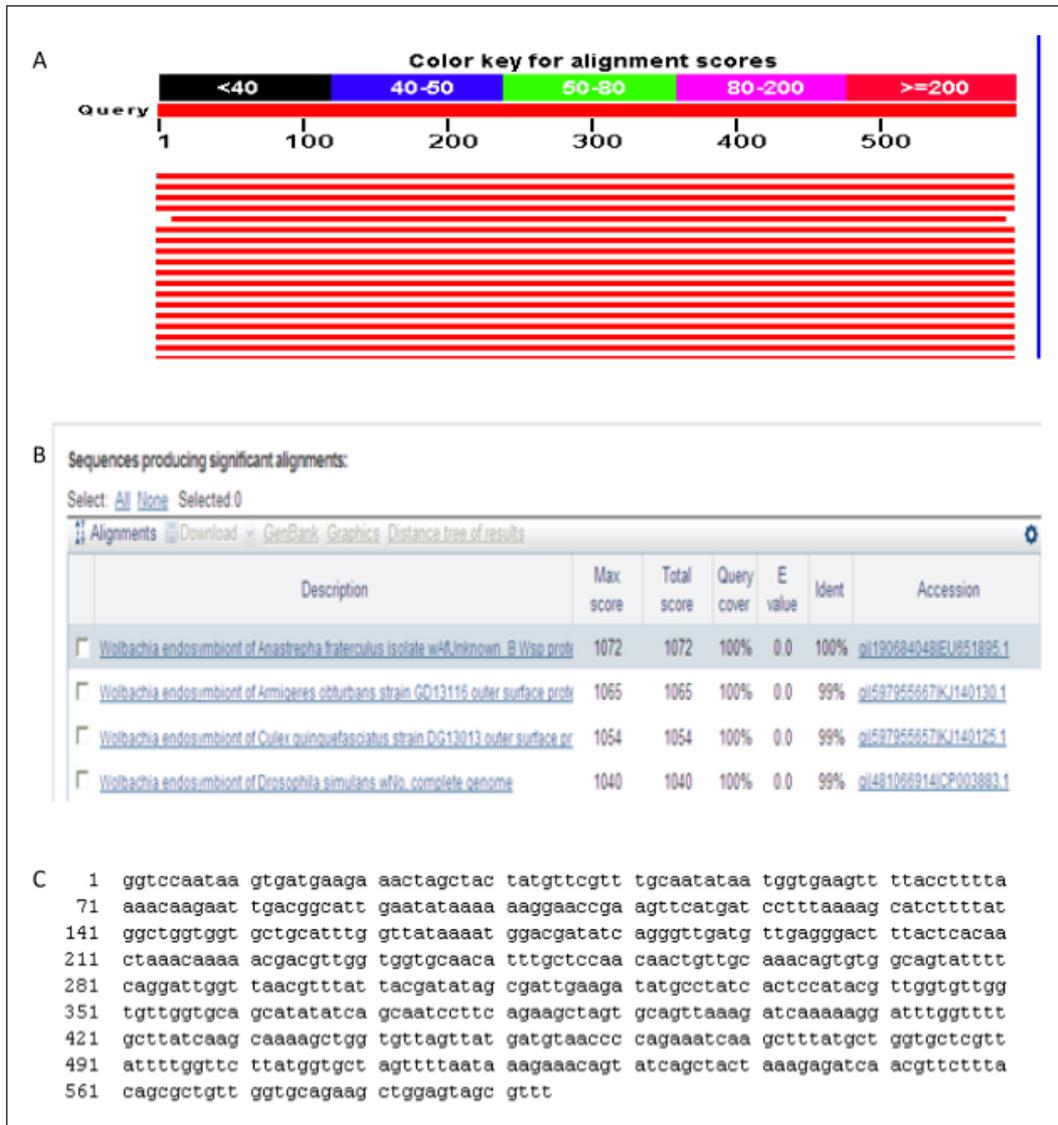


Figure 2. BLAST result of *Wolbachia* sequence. A) Alignment scores for *Wolbachia* sequence. B) Deposited sequences in GenBank producing significant alignments with detected *Wolbachia* sequence in this study. C) Nucleotide sequence of the *Wolbachia* PCR product cloned in pCRII TOPO vector.

Table 3 shows the number of *A. albopictus* and *A. aegypti* larvae that were positive for *Wolbachia* with and without dengue virus from the 4 localities. For *A. albopictus*, a total of 71 out of 284 (25%) were found to be positive for *Wolbachia*. Of these, 57/71 (80.28%) of them were negative for dengue virus while 14/71 (19.71%) were positive for DENV-3 virus. The highest % positivity for *Wolbachia* in *A. albopictus* larvae was seen in Ampang

(49.32%) followed by Taman Yarl (48.83%), Bukit Jalil (10.59%) and Happy Gardens (5.05%). For *A. aegypti*, 4 out of 16 larvae (25%) from Taman Yarl were positive for *Wolbachia*. Of these 3 (75%) were negative for dengue virus and 1 (25%) was positive for DENV-2 virus.

A chi-square test analysis to determine the association between dengue virus and *Wolbachia* in *A. albopictus* and *A. aegypti* collected from the four localities in Kuala

Table 3. Detection of *Wolbachia* and dengue virus in *A. albopictus* and *A. aegypti* larvae in the four localities in Kuala Lumpur

Locality and total no. of larvae	<i>A. albopictus</i> (n=284)					<i>A. aegypti</i> (n=16)				
	Wol + DENV -ve	Wol + and DENV +ve				Wol + DENV -ve	Wol + and DENV +ve			
		DENV-1	DENV-2	DENV-3	DENV-4		DENV-1	DENV-2	DENV-3	DENV-4
Happy Gardens N=99	0	0	0	5	0	0	0	0	0	0
IMU, Bukit Jalil N=85	9	0	0	0	0	0	0	0	0	0
Ampang N=73	30	0	0	6	0	0	0	0	0	0
Taman Yarl N=43	18	0	0	3	0	3	0	1	0	0
Total N=300	57	0	0	14	0	3	0	1	0	0

Wol +: Positive for *Wolbachia* by PCR
DENV -ve: Negative for dengue virus by RT-PCR
DENV +ve: Positive for dengue virus by RT-PCR

Lumpur showed no association between dengue virus and *Wolbachia* in *A. albopictus* and *A. aegypti* larvae ($\chi^2 = 3.080$; df = 1; $P > 0.05$).

DISCUSSION

Dengue is a significant public health problem in Malaysia and the reported dengue cases have generally been increasing in recent years (Annual reports, MOH 2011; 2012). In 2012, the four states showing the highest Incidence Rate (IR) of dengue cases (per 100,000 population) were Selangor, Federal Territory of Kuala Lumpur, Kelantan and Perlis. Also, in terms of fatal cases, Selangor contributed to the highest number followed by Federal Territory and Putrajaya. Based on these statistics, we selected four urban sites in Kuala Lumpur for the collection of *Aedes* larvae using ovitraps for our study on dengue and *Wolbachia*, a common endosymbiont reported in *A. albopictus*. Currently, the primary preventive measure for dengue is the control of mosquito populations. A practical

and recommended environmental control strategy is source reduction whereby unnecessary container habitats that collect water where *A. aegypti* and *A. albopictus* can lay their eggs are eliminated. Another method of reducing *A. aegypti* and *A. albopictus* is to use ovitraps as they appear to be ideal locations for the container breeding mosquitoes (*Aedes* spp.) to lay their eggs on the paddles. Ovitrap are also useful for mosquito surveillance as when sufficient numbers of ovitraps are used and maintained frequently, the vector population can be diminished (<http://www.nature.com/scitable/topicpage/controlling-dengue-outbreaks-22403714>). A successful example of the use of ovitraps was reported in Singapore where ovitraps were used to eliminate mosquitoes at the international airport (Chan, 1973).

From the mosquito larvae collected in the ovitraps in the four localities in Kuala Lumpur, *A. albopictus* was found to be abundant both outdoors as well as indoors while *A. aegypti* was only found indoors, in 2 out of the 4 sampling sites (Ampang and Taman Yarl). In this study, the sample size of

300 *Aedes* larvae is rather small, thus a larger sample size may give more conclusive results on whether *A. aegypti* occurs only indoors in the four study sites. Studies have shown that *A. aegypti* is one of the most efficient mosquito vectors for arboviruses, because it is highly anthropophilic and it thrives in close proximity to humans, preferring to live indoors. It is commonly found in urban areas especially in the most densely populated districts (Jansen & Beebe, 2010). On the other hand, *A. albopictus* is a highly adaptable species that is found mostly outdoors. The highly adaptable nature of *A. albopictus* makes it hard to control, as despite being transferred to a new region regardless of whether in the temperate or northern latitudes, they are still able to recolonize tree holes in the forests (Hawley, 1988). *A. albopictus* predominates in countries of South East Asia and it has been found in all types of country, urban, suburban, rural, farmland and even deep forest (Hawley, 1988). Based on the results of premise inspections carried out in 2012 in Malaysia, it was reported that 58,690 out of 3,959,258 (1.5%) premises inspected were found to be positive for *Aedes* breeding and the highest *Aedes* index was at construction sites followed by factories, vacant land, recreation areas and schools (Annual Report MOH, 2013). From the larvae collections in the ovitraps placed outdoors, it was observed that the number of ovitraps positive for *Aedes* larvae was highest in Happy Gardens. Besides that, the average number of mosquito larvae per ovitrap was highest in those collected from the playground in Happy Gardens. *A. albopictus* larvae were collected in all 4 localities while *A. aegypti* larvae were collected from Ampang and Taman Yarl.

From the results of PCR for the dengue serotypes, DENV-1 was not detected in any of the *Aedes* larvae from the four localities. The 3 dengue serotypes detected in this study were DENV-2, DENV-3 and DENV-4 with DENV-3 being the most common. Dengue is hyperendemic in Malaysia and all four DENV serotypes are known to co-circulate at any one time (AbuBakar & Shafee, 2002; Mohd-Zaki *et al.*, 2014). From 2011 to 2014, the predominant serotypes

circulating in Malaysia each year were DENV1, DENV3, DENV2 and DENV1/2 respectively (Mohd-Zaki *et al.*, 2014; Mudin, 2015).

The fact that some of the *Aedes* larvae collected in this study were infected with dengue virus indicates that transovarial transmission occurred in both *A. albopictus* and *A. aegypti*. Transovarial transmission of all the four dengue serotypes has been reported in *A. albopictus* mosquitoes and the rates of transmission varied with the serotype and strain of virus (Rosen *et al.*, 1983). Transovarial transmission has also been reported in *A. aegypti* in a number of countries. DENV-2 was recovered from 3 out of 123 pools of naturally infected *A. aegypti* collected from water containers in Rangoon (Khin & Than, 1983) while DENV-4 was isolated from *A. aegypti* adult mosquitoes in Trinidad (Hull *et al.*, 1984). In a prospective study conducted in an urban area in Thailand, transovarial dengue transmission of all four dengue serotypes were detected in two different forms of *A. aegypti* with DENV-4 being predominant, followed by DENV-3, DENV-1 and DENV-2 respectively (Thongrungrat *et al.*, 2011). It has also been reported for the first time that dengue-3 virus can persist for generations through transovarial transmission passage in successive generations of *A. aegypti* mosquitoes reared in the laboratory (Joshi *et al.*, 1996). These observations suggest that the *Aedes* vector mosquitoes may play an important role in maintaining the virus in nature and serve as reservoirs of dengue viruses. In Malaysia, Lee and Rohani have reported that transovarial transmission of dengue virus in *A. albopictus* and *A. aegypti* occurred prior to the reporting of human cases (Lee & Rohani, 2005). The intervals between detecting the transovarial transmission and occurrence of the first dengue case ranged between 7–41 days and transovarial transmission appeared to maintain or enhance the dengue epidemic. Dengue serotype 1 was however, not found in the study sample; its absence may be due to its low prevalence at the time of sampling and also due to the limited sample size. When using PCR to evaluate detection rate, it is

extremely important to eliminate laboratory contamination in the process.

The prevalence rates of dengue virus in *A. albopictus* and *A. aegypti* larvae in this study was 25.7% and 25% respectively. This rather high prevalence rate is not surprising based on the high number of dengue cases reported so far for the study period (18,165 dengue cases reported from 1st January to 3rd March 2014) (Sipalan, 2014). In addition, the World Health Organization has reported that in 2014, trends “indicate increases in the number of cases in the Cook Islands, Malaysia, Fiji and Vanuatu with Dengue Type 3 (DENV-3) affecting the Pacific Island countries after a lapse of over 10 years” (WHO, 2014).

DENV-2 was found in both *A. albopictus* and *A. aegypti* collected indoors. The Vector Borne Diseases Division of the Ministry of Health Malaysia has reported that from the analysis of blood samples from dengue patients in 44 clinics and hospitals nationwide for the first two months of 2014, most of the patients were infected with DENV-2 followed by DENV-1, DENV-4 and DENV-3 (Arumugam & Shah, 2014). In this study, DENV-3 was the most common virus found in mosquitoes outdoors while DENV-2 was found indoors. So a high number of cases of DENV-2 in patients may be due to a high indoor transmission rate by *A. aegypti*. Also, patients with DENV-2 virus were reported to show more severe effects, therefore leading to more DENV-2 cases in hospitalized dengue patients ((Arumugam & Shah, 2014).

An important objective of this study was to determine whether *Wolbachia* was present in both *A. albopictus* and *A. aegypti* larvae in Malaysia as no studies have yet been reported to-date. It was discovered that all known wild populations of *A. albopictus* are naturally infected with two strains of the maternally inherited bacterium *Wolbachia pipientis*, known as *wAlbA* and *wAlbB*; whilst *A. aegypti* is naturally uninfected with the bacterium (Sinkins & Gould, 1995; Zhou *et al.*, 1998). For our study, we amplified the *wsp* gene. The sequences from this gene were highly variable which could be used to resolve the phylogenetic relationships of different *Wolbachia* strains. The *wsp* gene

is a very useful tool for typing different *Wolbachia* strains. The large variability in the gene makes it possible to design specific PCR primers which can recognize both individual strains of *Wolbachia* or groups of *Wolbachia* strains. By using a combination of these primers in PCR reactions, an unknown *Wolbachia* strain could be easily assigned to a particular group. This approach is very effective and efficient as it enables different *Wolbachia* strains to be rapidly typed without the need to individually clone and sequence genes from all new isolates (Zhou *et al.*, 1998).

To date there have been no reports of natural *Wolbachia* infections in *A. aegypti*. It has been reported that *A. aegypti* has no native *Wolbachia* infection and *Wolbachia* is naturally absent in this species of mosquitoes (Bull & Turelli, 2013). In this study, *Wolbachia* was detected in both *A. albopictus* and *A. aegypti* larvae at a prevalence rate of 25%. Compared to other reports (Sinkins *et al.*, 1995; Zhou *et al.*, 1998; Kittayapong *et al.*, 2002), the percentage of *Wolbachia* infection of 25% in *A. albopictus* in this study is rather low. A recent study (Joanne *et al.*, 2015) using the PCR to detect the *Wolbachia* *wsp* gene reported that *A. albopictus* from various districts in Malaysia had *Wolbachia* infection rates ranging from 60% to 100%. This lower percentage prevalence of *Wolbachia* in *A. albopictus* may be due to the fact that the mosquito stage used in this study is different from the other reported studies. In all the other studies reported, *Wolbachia* was detected in the adult mosquitoes rather than the larvae whereas in this study, PCR was performed on DNA extracted from individual *Aedes* larva. The *Wolbachia* load in the larval stage may be much lower as compared to adult mosquitoes, thus this may cause a lower prevalence to be detected in this study. The common sites reported in the adult mosquitoes for *Wolbachia* include the malpighian tubules, thoracic ganglia, thoracic muscle, ovaries, head and salivary glands (Moreira *et al.*, 2009); these organs are still immature in the larval stage, thus this may result in a lower detection rate of *Wolbachia* in the mosquito larvae. However, in a recent study, *Wolbachia* in *A. albopictus* from Malaysia were only found in the ovaries

and midguts of the mosquitoes but absent in the salivary glands (Joanne *et al.*, 2015).

The results of this study showed for the first time the presence of *Wolbachia* in *A. aegypti* larvae collected in Kuala Lumpur, Malaysia. This novel finding is interesting as so far, *A. aegypti* adult mosquitoes have never been reported to harbour *Wolbachia* and therefore further work should be carried out to confirm the type of *Wolbachia* strain(s) present in *A. aegypti* larvae. One of the *Wolbachia* PCR products from *A. albopictus* (Sample C1 collected from Happy Gardens) was determined to be the *wsp* gene of *Wolbachia* with 100% homology to *Wolbachia b* isolated from *Anastrepha fraterculus* (South American fruit fly). This *Wolbachia* sample was subsequently used as a positive control in the PCR to detect *Wolbachia* in the study. *A. albopictus* that are superinfected with both *Wolbachia a* and *b* strains have been reported in a study conducted in Thailand and a female adult mosquito that is PCR tested as single infection will be considered to be actually superinfected if any of her progeny were superinfected (Kittayapong *et al.*, 2002). In the study conducted in Thailand, after combining the PCR data of both parents and F1 offspring, the superinfection prevalence in *A. albopictus* population was estimated to be 99.41%. In our study, PCR was used to detect the presence or absence of *Wolbachia* but not the bacterial load. Other methods such as real time PCR could be used to determine the *Wolbachia* load in the mosquito larvae (Mousson *et al.*, 2012; Lu *et al.*, 2012). Information of the *Wolbachia* load in individual mosquitoes may help in understanding the dynamics of interaction between *Wolbachia* and dengue virus in the mosquito vector.

A. albopictus and *A. aegypti* larvae were identified using morphological method in this study. To further confirm the identity of *A. aegypti*, molecular methods can be used such as PCR. In a study done in the United States, specific fluorogenic probe hydrolysis (TaqMan) PCR assay was developed for real-time screening using a field-deployable thermocycler to identify *A. aegypti* (McAvin *et al.*, 2005). With real-time TaqMan PCR

assays for rapid identification of *A. albopictus*, it was possible to identify all the stages, including eggs and first instar larvae of each mosquito (McAvin *et al.*, 2005; Hill *et al.*, 2008).

This study showed that there was no association between *Wolbachia* and presence of dengue virus in *Aedes* larvae. *Wolbachia* presence in mosquitoes may inhibit dengue virus but in this study there were 14 *A. albopictus* larvae that were found to have both *Wolbachia* and DENV-3 virus. The occurrence of both *Wolbachia* and dengue virus may be due to strain differences of *Wolbachia* as experimental studies have shown that certain *Wolbachia* strains are not as effective as others in inhibiting dengue virus replication (Osei-Poku *et al.*, 2012). Sequence analysis of the *wsp* gene has shown variations between and within the *Wolbachia* supergroup, some being more dominant than others and phylogenetics has demonstrated that the *wsp* gene is able to diverge between *Wolbachia* supergroups A and B (Osei-Poku *et al.*, 2012). Therefore, further analysis could be done to determine whether *A. albopictus* and *A. aegypti* are infected with a single supergroup strain or superinfected with both supergroups. In addition, it is interesting to note in this study that for *A. aegypti*, one of the mosquito larvae had both *Wolbachia* and DENV-2 virus. The *Wolbachia* strain in this *A. aegypti* larva should be further characterized to provide more information as it appeared to be not effective in inhibiting the dengue virus replication. This has significant implications in the choice of *Wolbachia* strains to be used as a transgene driver for *A. aegypti*. Another reason may be the load of *Wolbachia* in *Aedes* larvae was too low to have an inhibitory effect on dengue virus.

While this study was able to demonstrate for the first time that *Wolbachia* was present in *A. aegypti* larvae, there are some limitations. The sample size for *A. aegypti* larvae in the study was very small, therefore this may affect the prevalence data for dengue virus and *Wolbachia* obtained in this study. PCR is a qualitative study, so it could only detect the presence or absence of dengue virus and *Wolbachia* but not the viral or

bacterial load. Real-Time Quantitative PCR would have been a better method but due to budget constraints this was not carried out. Mosquito larvae were used in this study whereas other studies used adult mosquitoes; hence there is a sample difference and the *Wolbachia* load in the larvae stage may not reflect that found in adult mosquitoes.

In conclusion, this study has shown for the first time that *Wolbachia* is present in *A. albopictus* and *A. aegypti* larvae from Kuala Lumpur, Malaysia. Further studies should be done on a larger sample size and the *Wolbachia* strains found in both these *Aedes* spp. should be characterized using molecular methods. The mosquito larvae were identified based on morphology of the comb scales of the larvae and molecular methods could be used to confirm *A. aegypti*. Also, it was interesting to note that *Wolbachia* and dengue virus were found to be present in some of the *Aedes* larvae. Follow up studies could be conducted to determine whether larvae positive for both *Wolbachia* and dengue virus will continue to have both when they become adult mosquitoes to further understand the mechanisms of transmission of *Wolbachia* in *Aedes* mosquitoes.

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