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# Detection of *Wolbachia* from field collected *Aedes albopictus* Skuse in Malaysia

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***Background & objectives*: *Wolbachia*-based vector control strategies have been proposed as a mean to augment the existing measures for controlling dengue vector. Prior to utilizing *Wolbachia* in novel vector control strategies, it is crucial to understand the *Wolbachia*-mosquito interactions. Many studies have only focused on the prevalence of *Wolbachia* in female *Aedes albopictus* with lack of attention on *Wolbachia* infection on the male *Ae. albopictus* which also affects the effective expression of *Wolbachia* induced- cytoplasmic incompatibility (CI). In this study, field surveys were conducted to screen for the infection status of *Wolbachia* in female and male *Ae. albopictus* from various habitats including housing areas, islands and seashore.**

***Methods*: Adult *Ae. albopictus* (n=104) were collected using human landing catches and hand aspirator. Standard ovitraps were also set in the selected areas for five days and the larvae were identified to species level. All the collected *Ae. albopictus* were screened for the presence of *Wolbachia* using multiplex polymerase chain reaction (PCR) and gene sequencing of *Wolbachia* surface protein (*wsp*) gene.**

***Results*: A 100 per cent positivity of *Wolbachia* infection was observed for individual *Ae. albopictus* screened. For pooled mosquitoes, 73 of the 76 pools (female) and 83 of the 87 pools (male) were positive with *Wolbachia* infection. The *wsp* gene sequence of the *Wolbachia* strain isolated from individual and pooled mosquitoes showed a 100 per cent homology with *Wolbachia* sp. of *Ae. albopictus* isolated from various geographical regions. Phylogenetic analysis based on *wsp* gene fragments showed that the isolates were clustered into groups A and B, respectively.**

***Interpretation & conclusions*: The results indicated that *Wolbachia* infection was widespread in *Ae. albopictus* population both in female and male *Ae. 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.**

**Key words** *Aedes albopictus* - Malaysia - PCR - *Wolbachia*

*Wolbachia* species are obligate intracellular rickettsia-like bacteria belonging to the alpha subclass of Proteobacteria and the order of Rickettsiales that live inside the cells of various organs, but most frequently appear in ovaries and testes[1]. *Wolbachia* infects a wide range of arthropods including mosquitoes, ticks, flies and nematodes populations[2]. However, some of the major disease vectors are not naturally

infected, including the primary vector of dengue, *Aedes aegypti* and all anopheline mosquitoes sampled to date[3,4]. In arthropods, *Wolbachia* causes several host reproduction alterations, including cytoplasmic incompatibility (CI). In the simplest CI scenario, the mating between males and females that carry different and incompatible strains of *Wolbachia* will eventually result in a significant reduction in fecundity and egg hatching rate. Similar effects were also observed when mating occurred between uninfected females with infected males[5,6]. The introduction of *Wolbachia* from its native host into new hosts exhibited an interference with pathogens through several mechanisms including the upregulation of several immune genes[7,8]. The ability of a *Wolbachia* strain (*w*MelPop) to interfere with pathogens development and to cause reduction in adult mosquito longevity has also been reported[9,10]. From an applied perspective, *Wolbachia* utilizing strategies can be used for either population suppression or sweep desirable traits into pest populations such as the inability to transmit disease-causing pathogens[7,11], given that the *Wolbachia* infection must reach a stable equilibrium within the target population, at a rate that is high enough to cause a significant impact and eventually reducing disease transmission by the vector[12].

In Malaysia, *Ae. albopictus* Skuse and *Ae. (Stegomyia) aegypti* (Linnaeus) are the incriminated dengue vectors. The transovarial transmission of dengue virus has been proven for both species under both laboratory and field conditions[13,14]. Despite being the secondary vector for dengue virus, under some circumstances, the transovarial transmission of dengue virus was proven to be higher in larvae of *Ae. albopictus* compared to *Ae. aegypti* larvae[15]. *Ae. albopictus* adult was reported to be responsible for the viral transmission during dengue fever outbreaks not only in its native regions but also in introduced ranges like in Hawaii[16] and Mexico[17]. Thus, it is crucial to know the infection frequency and the types of native *Wolbachia* strain infections for both female and male *Ae. albopictus* mosquitoes, prior to exploring the potential use of CI-based strategies for dengue transmission control.

In the present study, field surveys were conducted to detect *Wolbachia* in both female and male field-collected *Ae. albopictus* mosquitoes from various habitats.

## Material & Methods

*Mosquitoes collection*: *Ae. albopictus* mosquitoes were collected from five different localities ranging from housing areas (Malacca, Selangor), seashore (Terengganu) and islands areas (Ketam Island, Carey Island) in Peninsular Malaysia from March till September 2012. Adult *Ae. albopictus* were collected using human landing catches and hand aspirator and individually placed in glass vials. Standard ovitraps were also set in the selected areas for five days and the larvae were identified to species level using the *Aedes* sp. key[18]. The larvae from each ovitrap were placed inside an adult cage (25x25x50cm) for emergence. The adult mosquitoes emerging from each ovitrap were sexed and pooled together (2-10 mosquitoes/pool) at the age of 7-10 days. All mosquitoes were killed by keeping them in -20°C freezer for one hour just prior to pooling. The mosquitoes were homogenized with a clean pestle and incubated in 20 µl proteinase K at 56°C in a shaking water bath for three hours. The subsequent procedures were performed according to the QlAamp® DNA Mini Kit protocol (Qiagen™, Germany).

*Detection of Wolbachia*: Multiplex PCR was carried out using the temperature profile of 95°C for 1 min, 55°C for 1.5 min and 72°C for 2 min for 35 cycles using *wsp* primers. Primers used were 328F and 691R for *w*AlbA and 183F and 691R for *w*AlbB as described by Zhou[19] (328F, 5'-CCA GCA GAT ACT ATT GCG-3'; 183F,5'-AAG GAA CCG AAG TTC ATG-3'; 691R, 5'-AAA AAT TAA ACG CTA CTC CA-3'). A negative and positive control for the PCR assay were included in each run. The positive control was obtained by screening the adult *Ae. albopictus* (resident strain) using PCR and sequencing of *wsp* gene to confirm that the amplified PCR product obtained was *Wolbachia*. The quality of DNA extraction was checked by running all the negative samples using 12sRNA primer set (12SA, 5'-AAA CTA GGA TTA GAT ACC CTA TTA T-3'; 12SB, 5' - AAG AGC GAC GGG CGA TGT GT-3')[20]. Any sample that was negative for both *wsp* and 12sRNA primer sets was excluded from the data set. Samples that were negative for *wsp* primers but positive for 12sRNA primers were scored as uninfected. All the positive PCR products were visualized under 1.5 per cent agarose gel electrophoresis.

*Sequencing of Wolbachia endobacterium*: The positive PCR product was purified using QIAquick® Gel Extraction Kit (Qiagen, Germany) prior to DNA sequencing. One DNA extract each from individual and pooled mosquitoes for each locality (Malacca, Carey Island, Ketam Island, Terengganu and Selangor) was outsourced for sequencing. All sequences were subjected to run in Basic Local Alignment Search Tool

(BLAST®) (retrieved from: (*http://blast.ncbi.nlm.nih.gov/*) available in the GenBank.

Multiple sequence alignment was carried out using Clustal-W programme[21]. The evolutionary distances of *Wolbachia* isolates from *Ae. albopictus* based on *wsp* genes were constructed using Neighbour-Joining tree using Kimura-2P analysis with 1000 bootstrap replicates in MEGA 5.1 software[21]. A total of seven sequences of *Wolbachia* strains derived from GenBank were included in the analysis.

## Results

*Multiplex PCR of Wolbachia in adult mosquitoes*: Individual field-collected adults, as well as pools of mosquitoes recovered from positive ovitraps, were screened for the presence of *Wolbachia*. A total of 104 individual adult *Ae. albopictus* showed a 100 per cent positivity rate for *Wolbachia* infection; both males (n=46) and females (n=58) from the population were studied. For *Ae. albopictus* collected from ovitraps, it was decided to screen for the presence of *Wolbachia* from *Ae. albopictus* in pool due to the high numbers of samples recovered from positive ovitraps. A total of 73 of the 76 pools (female) and 83 of the 87 pools (male) of mosquitoes were positive for *Wolbachia* (Table I). To ensure that the negative pools (4 pools for female and 3 pools for male) were true negatives and not due to the poor quality of the extracted DNA, these samples were further analysed using 12sRNA primers. Based on PCR results, all the *wsp* negative pools were positive for 12sRNA primers, indicating the good quality of the extracted DNA and were scored as negative *Wolbachia* infection.

*Sequencing of Wolbachia endosymbiont DNA in Ae. albopictus*: For the *wsp* gene, all the individual females (n=58) tested were found to be superinfected with both strains A and B *Wolbachia.* On the other hand, the individual male mosquitoes showed a mix of either double or single infection; A and B (n=15) or B (n=31) strain only. None of the samples screened was infected nor infected with strain A only (Table I).

For the mosquitoes extracted in pool, the female pools were superinfected with both strains A and B in all instances. As none were found to be single infection with strain A or strain B only in individual females screened, it was inferred that the superinfection of strains A and B in the female pool was true. On the other hand, the pools for male mosquitoes showed a mix of

**Table I.** Infection status of *Wolbachia* from field collected *Ae. albopictus* as determined by standard multiplex PCR

| | | | Infection with *Wolbachia* strains | | |
|---|---|---|---|---|---|
| | | | A and B | A | B |
| **Screening of individual field-collected mosquitoes** | | | | | |
| Male (n=46) | | | 15 | 0 | 31 |
| Female (n=58) | | | 58 | 0 | 0 |
| **Screening of pools of field collected mosquitoes** | | | | | |
| Study sites | Types of habitat | | No. of pool tested | No. of positive pool | |
| Malacca | Housing area | Female | 5 | 5 | |
| | | Male | 6 | 6 | |
| Ketam Island | Island | Female | 9 | 9 | |
| | | Male | 12 | 12 | |
| Carey Island | Island | Female | 37 | 35 | |
| | | Male | 49 | 46 | |
| Terengganu | Seashore | Female | 8 | 8 | |
| | | Male | 9 | 9 | |
| Selangor | Housing area | Female | 17 | 16 | |
| | | Male | 11 | 10 | |

All females screened were superinfected with both strains A and B. In males, there was a combination of superinfection of A and B strains and single infected with strain B only

double and single infection of strains A and B or strain B only, similar to observation in individual male samples (Table I). A total of 59 pools were infected with only strain B *Wolbachia*, while only 24 pools of the remaining positive pools were infected with both strains A and B. However, with the pool extracted mosquitoes, the presence of A and B strains might also be contributed by strain B only from individual male mosquito in the pool and thus underestimating the presence of strain B only. The comparison between the proportion of A and B and B only infection (approximately 1:2 ratio) was relatively similar for individuals and pooled males and thus, giving the rough estimation of the types of *Wolbachia* infection in the population studied.

DNA of *Wolbachia* isolates in this study was successfully amplified and sequenced from 10 specimens (each isolate representing each locality) (Table II). The new sequences of *w*AlbA and *w*AlbB from individual samples obtained were deposited in GenBank (accession number KC004024 and KC004025). The sequencing results for *wsp* gene yielded fragments of 341and 463bp for strains A and B, respectively. The *Wolbachia* strains A and B of *Ae. albopictus* showed a 100 per cent homology with *Wolbachia* sp. in *Ae. albopictus* from different geographical regions. Phylogenetic analysis showed that *Wolbachia* isolate from the present study was closely related to *Wolbachia* isolated from *Ae. albopictus* collected from different locations and the sequences were grouping together according to the *wsp* strains A and B, respectively (Figure).

## Discussion

In arthropods, *Wolbachia* can cause numerous reproductive alterations. Among all the reproductive modifications caused by *Wolbachia*, CI has been utilized as one of the tools to control mosquitoes. CI is a form of sterility in which if the same and compatible *Wolbachia* strain is not present in the egg during embryogenesis, embryonic development will be disrupted[1]. Thus, the sustained repeated release of cytoplasmically incompatible *Wolbachia* infected mosquitoes will result in the increasing rate of incompatible mating and hence, lead to suppression of the vector population[22].

In our study, a 100 per cent positivity for *Wolbachia* was observed in field-collected individual male and female *Ae. albopictus*. Both theoretical and empirical data have suggested that *Wolbachia* are expected to rapidly spread to fixation once a *Wolbachia* infection enters a population[7,23,24]. The *wsp* sequencing indicated that all the female (individuals and pools) *Ae. albopictus* tested were superinfected with both strains A and B. This result was similar with many studies which stated that the superinfection with both strains A and B in field population of female *Ae. albopictus* was common and virtually fixed in a population, suggesting efficient vertical transmission of both strains A and B[25,26]. In addition, the presence of both strains A and B in this mosquito species has been claimed to contribute to high fidelity of maternal transmission of *Wolbachia*[27].

In most instances, the male *Ae. albopictus* were infected with strain B only and in some instances,

**Table II.** Voucher no., accession no. and collection location for the *Wolbachia* strain A and B of *Ae. Albopictus*

| No | Voucher no. | GenBank accession no. | Strain | Locality | |
|---|---|---|---|---|---|
| 1. | 34Fa | KC004024 | *wAlb*A | Malacca | |
| 2. | 97Fa | | | Ketam Island | |
| 3. | 57Fa | | | Carey Island | |
| 4. | 26Fa | | | Terengganu | |
| 5. | 11Fa | | | Selangor | |
| 6. | 34Fb | KC004025 | *wAlb*B | Malacca | |
| 7. | 97Fb | | | Ketam Island | |
| 8. | 57Fb | | | Carey Island | |
| 9. | 26Fb | | | Terengganu | |
| 10. | 11Fb | | | Selangor | |
| 11. | | AY462864 | *wAlb*A | Taiwan | * |
| 12. | | DQ842453 | | USA | |
| 13. | | EU727139 | | UK | |
| 14. | | AF020058 | | USA | |
| 15. | | AY462863 | *wAlb*B | Taiwan | |
| 16. | | AF020059 | | USA | |
| 17. | | KC242223 | | China | |

*Wolbachia* strains A and B of *Ae. albopictus* from which DNA was extracted

*No 11-17; Sequences obtained from GenBank (*http://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch@LINK_LOC=blasthome*) used for comparison in phylogenetic analysis

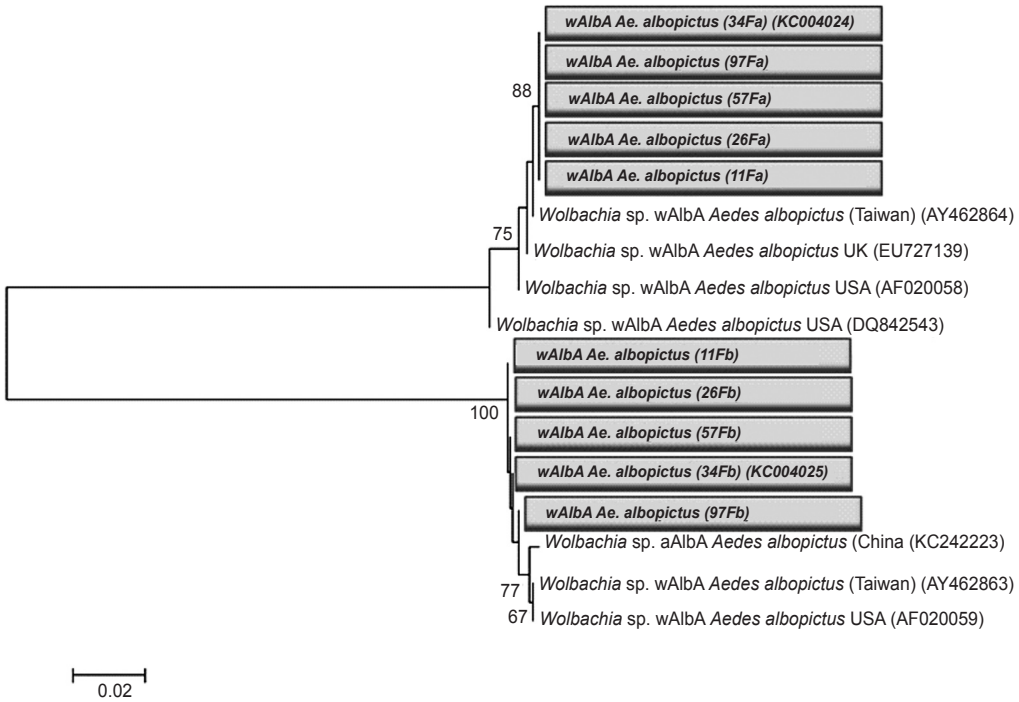


**Figure.** Neighbour-joining phylogenetic tree of *Wolbachia* strain, isolated from *Ae. albopictus* based on partial sequence of *wsp* gene using Kimura-2P analysis. Figures in parentheses are GenBank accession numbers.

they were superinfected with both strains A and B. The efficient transmission of both strains A and B observed in female *Ae. albopictus* advocates that the loss of *w*AlbA is hardly adaptive and, therefore, the same vertical transmission of both strains are expected to be seen in male mosquitoes as well. The density of type A *Wolbachia* has been shown to be significantly decreased with age in male *Ae. albopictus* population[28]. In some cases, the density of strain A was reduced towards a complete loss within 5-day period post-emergence. In other instances, all tested males showed no strain A infection regardless of age, suggesting that the loss of *w*AlbA might have taken place earlier before the emergence[28]. This could be the reason for the lack of strain A infection in the male population aged 7-10 days (pooled mosquitoes) as observed in this study. This observation was further supported by another study that showed a reduction in *w*AlbA CI in 10-days old *w*AlbA mono-infected laboratory reared *Ae. albopictus* which possibly correlated with the lack of *w*AlbA strain in aged *Ae. Albopictus*[29].

*Wolbachia*-based vector control strategy to complement the existing method for vector control has been investigated in several laboratories[11,30,31]. Though the impact of *Wolbachia* on mosquito vector in arbovirus transmission has been reported[9,11,31], this was not examined in our study. Our data on *Wolbachia* natural infection provide important initial baseline information in developing potential strategies prior to exploring the possibility of utilizing this strategy for dengue transmission control.

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