Asian Pacific Journal of Tropical Biomedicine

journal homepage: www.apjtb.com

Document heading doi:10.12980/APJTB.4.2014APJTB-2014-0020

Modified technique of *Wolbachia* removal from Malaysian *Aedes albopictus*

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PEER REVIEW

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Comments

The study has provided a viable method to produce *Wolbachia* free *Ae. albopictus* which is increasingly implicated as a vector of dengue transmission in many endemic countries. Susceptibility studies with dengue serotypes in *Wolbachia* positive or negative mosquitoes will be easily available now.
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ABSTRACT

Objective: To develop an artificial and modified *Wolbachia* removal technique using tetracycline from naturally *Wolbachia* infected *Aedes albopictus* (*Ae. albopictus*) so as to be able to produce generations of *Wolbachia* free offsprings.
Methods: In this study, seven different tetracycline treatment methods were conducted to obtain the best removal method. Four methods focused on larvae tetracycline treatment, one method on both larvae and adult tetracycline treatment and the last two methods on adult mosquito sucrose treatment.
Results: All larval tetracycline treatments resulted in either high larvae mortality, sterile F_0 adult mosquitoes or unsuccessful *Wolbachia* removal. Treatment of both larvae and adults resulted in reduced larvae mortality, successful *Wolbachia* removal but slow mosquito fecundity. As for the adult treatment, 1.0 mg/mL as previously published was not able to completely remove *Wolbachia* in F_1 generation whereas 1.25 mg/mL successfully removed *Wolbachia* from F_1 and F_2 mosquitoes in 2 weeks.
Conclusions: This method is different from the previously published methods as it provides an improved *Wolbachia* removal technique from *Ae. albopictus* with high egg hatchability, low larvae mortality, increased fecundity and better *Wolbachia* removal rate.

KEYWORDS
Wolbachia, tetracycline, *Aedes albopictus*

1. Introduction

Wolbachia pipientis is an intracellular bacteria found in most of the arthropods, nematodes and isopods[1,2]. They are vertically transmitted rickettsia endosymbiont bacteria[3]. In order to ensure the parasite being successfully transmitted maternally, *Wolbachia* tend to alter reproduction properties of their host[4]. Common alteration that have been reported are male killing, feminization, parthenogenesis and cytoplasmic incompatibility (CI)[5,6]. *Wolbachia* modifies the spermatogenesis causing no viable offspring to be produced when infected male mates with uninfected female or female infected differently from the male[7]. Well understood CI can be used to reduce population of the host.

Aedes albopictus (*Ae. albopictus*) is an arthropod known to be naturally infected with *Wolbachia pipientis* bacteria. Most strains of *Ae. albopictus* screened in Malaysia were superinfected with both two *Wolbachia* strains (*w*AlbA and *w*AlbB). *Aedes aegypti* and *Ae. albopictus* are the major vectors for dengue in Malaysia. They are lethal vectors which transmit many deadly pathogens including dengue fever virus, chikungunya fever virus and West Nile Virus[8]. The combination of dengue blocking activity and rapid spread due to CI has led researchers to suggest that

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Foundation Project: Supported by University Malaya Research Grant (UMRG) with grant NO.: RG372/11HTM.

Article history:
Received 1 May 2014
Received in revised form 12 May, 2nd revised form 15 May, 3rd revised form 29 May 2014
Accepted 23 Jun 2014
Available online 28 Jul 2014

Wolbachia can be used to develop public health strategies to reduce dengue incidence in human[9,10].

In order to study the CI and effect of *Wolbachia* on Malaysian *Ae. albopictus*, it is necessary to have both *Wolbachia* infected and uninfected strains.

Ae. albopictus is a species naturally infected with *Wolbachia* therefore obtaining a natural strain without *Wolbachia* would be very rare[11]. Therefore an artificial *Wolbachia* removal technique is needed.

Previous studies have suggested treatment of larvae with tetracycline antibiotic to remove *Wolbachia* from *Ae. albopictus*[3,11]. However, reduced fecundity and egg viability was observed when the above mentioned method was implemented. Another study proposed treatment of only the adult mosquito with tetracycline antibiotic[11]. This managed to overcome the reduced fecundity and egg viability issue. However, when the method was implemented, the resulting offsprings were found not to be totally free from *Wolbachia*.

In this study, a modified *Wolbachia* removal method from *Ae. albopictus* is reported. It has minimal effect on the mosquito fecundity and egg viability and was able to produce generations of *Wolbachia* free offsprings.

2. Materials and methods

2.1. Mosquito strain

A strain of *Ae. albopictus* obtained from Bukit Lagong, Selayang, Kuala Lumpur, Malaysia in August 2013 was used in this study. Mosquitoes were maintained in cages with 10% sucrose with 100 mg B-Complex solution. They were blood fed and eggs were collected weekly. Mosquito infection status was confirmed using polymerase chain reaction (PCR) amplification and sequencing.

2.2. Infection status

A minimum of 30 blood fed mosquitoes were randomly caught for each new generation, blood fed, allowed to lay eggs and then extracted using Dneasy Blood and Tissue Extraction Kit according to the protocol provided by the manufacturer (Qiagen, CA, USA). Extracted DNA were stored at −20 °C until needed. All samples were screened for the presence of *Wolbachia* using multiplex PCR with Promega (Promega, Madison, WI) reagents for amplification of the wsp gene with diagnostic primers (Genomics BioSci & Tech, China).

The *w*AlbA strain gene was amplified with the wsp 328F and 691R primer pair whereas *w*AlbB strain gene was amplified with the wsp 183F and 691R primer pair. PCR was conducted in a 20 μL reaction per individual. This consisted of 10 μL ddH_2O, 4 μL 5X Green GoTaq® Flexi Buffer, 1.6 μL magnesium chloride, 0.4 μL dNTPs, 0.6 μL of each primer (183F, 328F and 691R), 0.2 μL of GoTaq® Flexi DNA polymerase and 2 μL template. Samples were denatured for 5 min at 94 °C, followed by 35 cycles of 1 minute at 94 °C, 1 min at 55 °C and 1 min at 72 °C. A negative control was run along with each batch of PCR amplification by substituting 2 μL of sample with 2 μL of ddH_2O[12].

A total of 8 μL of each sample was run in 1% agarose gel to detect the presence of amplified DNA fragments. One hundred kilobyte ladder (Promega, Madison, WI) was used to confirm presence of *w*AlbA (363 bp) and *w*AlbB (508 bp) genes[12] (Figure 1).

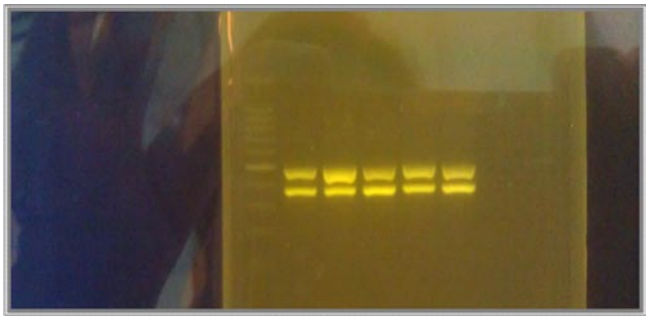

Figure 1. Gel electrophoresis result image. Lane 1 is the 100 kB ladder. Lane 2-6 are my samples.
The upper row are *w*AlbB amplified gene at 508 bp and the lower row bands are *w*AlbA amplified gene at 363 bp.

2.3. Tetracycline treatment

All *Wolbachia* removal studies were conducted on strains with confirmed *w*AlbA and *w*AlbB superinfection. Studies were conducted as stated in Table 1. Treatment 1 was conducted as previously described by Otsuka and Takaoka in 1997[3]. Treatment 5 and 6 were conducted as described previously by Dobson and Rattanadechakul in 2001[11]. Treatments 2, 3, 4 and 7 consisted of a modified technique were conducted by this group. Larvae after the treatment period in treatment 1, 2, 3 and 4 were transferred back into water without tetracycline and reared to adulthood. In each treatment, randomly caught 25 blood fed adult mosquitoes were allowed to lay eggs first and then tested for presence of *Wolbachia* using PCR method as mentioned above. If no *Wolbachia* infection was found in all tested mosquitoes, the eggs obtained were hatched. Larvae after 24 h treatment in treatment 5 was transferred back into water without tetracycline and reared to adulthood.

Table 1
Tetracycline treatment design.

Treatment	Life cycle stage	Treatment period (h)	Concentration of tetracycline
1[a]	24th-48th hour larvae	24	5.00 mg/mL in 2 L overnight water
2[c]	24th-72nd hour larvae	72	1.25 mg/mL in 2 L overnight water
3[c]	48th-120th hour larvae	72	1.25 mg/mL in 2 L overnight water
4[c]	48th-72th hour larvae	24	1.25 mg/mL in 2 L overnight water
5[b]	48th-72th hour larvae	24	1.25 mg/mL in 2 L overnight water
	Newly emerged adult mosquito	Continuous	0.50 mg/mL in 10% sucrose solution with 100 mg B-Complex
6[b]	Newly emerged adult mosquito	Continuous	1.00 mg/mL in 10% sucrose solution with 100 mg B-Complex
7[c]	Newly emerged adult mosquito	Continuous	1.25 mg/mL in 10% sucrose solution with 100 mg B-Complex

[a]: Conducted as previously described by Otsuka and Takaoka in 1997[3].
[b]: Conducted as described previously by Dobson and Rattanadechakul in 2001[9].
[c]: Modified techniques conducted by this group.

Adult mosquitoes in treatment 5, 6 and 7 were blood fed

after two weeks and one month for egg collection. Twenty five mosquitoes from which eggs were collected were tested for presence of *Wolbachia* using PCR method as mentioned above. Eggs collected from the treatment 5, 6 and 7 were allowed to hatch in 2 L overnight water. Egg hatching rate were calculated to determine egg viability for each treatment. Once the F_1 generation mosquitoes were obtained, 25 blood fed adult mosquitoes were randomly caught from each colony, allowed to lay egg first and then tested for presence of *Wolbachia* using PCR method as mentioned above. Average *Wolbachia* infectivity of F_1 for treatment 6 and 7 was obtained by calculating the mean infected mosquito numbers for three replicates of *Wolbachia* testing. The same calculation was done for average *Wolbachia* infectivity of F_2 for treatment 7.

3. Results

The strain of *Ae. albopictus* used in this study had 100% both *w*AlbA and *w*AlbB infection. Figure 1 shows the result of PCR amplification when both *w*AlbA and *w*AlbB is present. The F4 eggs were used in this *Wolbachia* removal study.

Percentage of eggs hatched that survived to pupation, percentage of adult mosquitoes emerged, percentage of *Wolbachia* infection status of the F_0 and percentage of F_1 eggs hatched were calculated for all treatments 1-7. Results are shown in Table 2.

Table 2

Percentage eggs hatch in F_0 and F_1 tetracycline treated strains.

Treatment	No. of eggs (F_0)	Pupae after 5 d	Adult mosquito (F_0)	*Wolbachia* infection status	No. of eggs obtained (F_1)	Eggs hatch
1	132	4 (3.00%)	4 (100.00%)	All 4 *Wolbachia* free (100.0%)	0	NA
2	111	6 (5.41%)	5 (83.30%)	All 5 *Wolbachia* free (100.0%)	0	NA
3	122	42 (34.43%)	32 (76.19%)	All 25 tested *Wolbachia* free (100.0%)	5	0 (0.00%)
4	142	88 (61.97%)	83 (94.32%)	13 out of 25 *Wolbachia* free (52.0%)	29	0 (0.00%)
5[x]	145	82 (56.55%)	78 (95.12%)	All 25 tested *Wolbachia* free (100.0%)	230	41 (17.83%)
6[y]	153	107 (70.00%)	105 (98.13%)	All 25 tested *Wolbachia* free (100.0%)	249	142 (57.03%)
7[z]	143	92 (64.34%)	88 (95.65%)	All 25 tested *Wolbachia* free (100.0%)	189	98 (51.85%)

[x]: Emerged mosquitoes were treated with 0.5 mg/mL tetracycline treated sucrose solution. F_0 adult mosquitoes were only *Wolbachia* free after 1 month of treatment. [y]: Emerged mosquitoes were treated with 1.0 mg/mL tetracycline treated sucrose solution. F_0 adult mosquitoes were only *Wolbachia* free after 1 month of treatment. [z]: Emerged mosquitoes were treated with 1.25 mg/mL tetracycline treated sucrose solution. F_0 adult mosquitoes were *Wolbachia* free after 2 weeks of treatment. NA: Not applicable.

Treatment 5, 6 and 7 had F_1 eggs therefore studies were continued to obtain the percentage of F_1 adult mosquitoes, *Wolbachia* infectivity status of F_1 colony and *Wolbachia* infectivity status of F_2 colony (only treatment 7). Results for this continuation studies are shown in Table 3.

4. Discussion

Tetracycline is a group of broad-spectrum antibiotics. Its overall usage has been reduced with the increasing bacterial resistance[13]. Since *Wolbachia* is an endosymbiotic bacteria, tetracycline at the right concentration and delivery method should be able to remove them from their respective hosts. This concurs with previous studies conducted[11].

Treatment 1 which was conducted based on Otsuka method was not effective in this study as it caused low egg viability, high larval mortality and sterile adult mosquitoes[3]. Same issue have been reported by Dobson and Rattanadechakul in 2001[11]. This may have been due to the high concentration of the tetracycline used to treat the larvae.

Similar larval treatments were carried out with reduced concentration to 1.25 mg/mL in treatment 2, 3 and 4 at different exposure periods.

High larval mortality was observed when larvae were treated for more than 24 h. However, improved larval mortality was observed when the 48 h larvae were treated instead of the 24 h larvae. This may be because 24 h larvae are too young to withstand the tetracycline treatment.

Treatment 4 was designed to expose 48 h larvae for 24 h which gave lower larval mortality and a higher percentage of adults.

Although low larval mortality was observed, the treatment failed to remove *Wolbachia* completely from all surviving adults. Therefore it can be concluded that perhaps the period of treatment or tetracycline concentration was not sufficient.

Treatment 5 was conducted based on Dobson report in 2001 which subjects both larvae and adult mosquitoes tetracycline[11]. This method had low larval mortality and was able to completely remove *Wolbachia* from all surviving F_0 adults. A good number of F_1 eggs were obtained but the hatching rate of the F_1 eggs was very low compared to untreated strains.

Treatment 6 was conducted based on the final method from Dobson paper in 2001 which treats only the adult with 1.0 mg/mL[11]. No alternative food source was provided for the mosquitoes. F_0 Adult mosquitoes were tested for *Wolbachia* after 2 weeks exposure to tetracycline sucrose treatment. Mosquitoes were not found to be completely free of *Wolbachia*. F_0 adult mosquitoes were again tested for *Wolbachia* after 1 month tetracycline treatment and all were *Wolbachia* free. Eggs were collected and F_1 mosquitoes were obtained. Although the experiment was repeated three times, we failed to obtain entirely *Wolbachia* free F_1 adult mosquitoes. Therefore treatment 6 as proposed by Dobson was not effective in this study.

Treatment 7 was designed exactly as treatment 6 with a

Table 3

Wolbachia infectivity status of F_1 and F_2 tetracycline treated strains.

Treatment	No. of larvae	No. of adults (F_1)	Average *Wolbachia* infectivity of F_1	Average *Wolbachia* infectivity of F_2
5	41	32 (78.05%)	All 25 tested *Wolbachia* free (100.0%)	Not applicable because no eggs was obtained
6	142	130 (91.55%)	18 out of 25 *Wolbachia* free (72.0%)	Not applicable
7	98	92 (93.88%)	All 25 tested *Wolbachia* free (100.0%)	All 25 tested *Wolbachia* free (100.0%)

slight increment of the concentration of tetracycline in the sucrose solution. Complete *Wolbachia* removal from the F_0 adult mosquitoes was observed in two weeks tetracycline treated mosquitoes. This was confirmed with two replicates. Egg hatching rate was slightly lower than treatment 6 and 93.88% became F_1 adults. In contrast to treatment 6, F_1 adults were 100% *Wolbachia* free. Average was obtained from three replicates. All F_2 adults was also found to be *Wolbachia* free.

Tetracycline treatment of only adult mosquitoes simplifies the process, improves the egg hatchability, reduces larval mortality and increases adult fecundity. The best concentration for the adult treatment is concluded to be 1.25 mg/mL in sucrose solution with no alternative food source. This method is able to remove both *w*AlbA and *w*AlbB completely in just two weeks and gives subsequent generations free of *Wolbachia*.

This self-sustaining *Wolbachia* free *Ae. albopictus* colony developed can be used to study the effect of *Wolbachia* on Malaysian *Ae. albopictus*. Future research may be conducted to develop a singly infected *Ae. albopictus* strain with a modified antibiotic treatment as none has been established so far.

Conflict of interest statement

We declare that we have no conflict of interest.

Acknowledgements

This project have been financially supported by University Malaya Research Grant (UMRG) with grant NO.: RG372/11HTM.

Comments

Background

There is increasing interest on *Wolbachia* endosymbionts in *Aedes* vectors as they are related to fecundity and to dengue transmission. There is a need to obtain strains of *Ae. albopictus* free from endosymbionts but existing methods through treatment of larvae with tetracycline have not been satisfactory.

Research frontiers

An improved method to obtained *Wolbachia* free and viable *Ae. albopictus* mosquitoes for further studies.

Related reports

Although the use of tetracycline to obtain *Wolbachia* free *Aedes* has been previously studied, the dosage and methods used did not produce satisfactory results.

Innovations and breakthroughs

An improved method of using tetracycline in obtaining subsequent generations of *Wolbachia* free *Ae. albopictus*.

Applications

This study is important for research on *Wolbachia* and dengue susceptibility.

Peer review

The study has provided a viable method to produce *Wolbachia* free *Ae. albopictus* which is increasingly implicated as a vector of dengue transmission in many endemic countries. Susceptibility studies with dengue serotypes in *Wolbachia* positive or negative mosquitoes will be easily available now.

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