RESEARCH ARTICLE

Natural Wolbachia infections in malaria vectors in Kayin state, Myanmar [version 1; referees: 2 approved with reservations]

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Abstract
Background: Natural Wolbachia infections in malaria mosquitoes were recently reported in Africa, and negatively correlated with the development of Plasmodium falciparum in the vectors. The occurrence and effects of Wolbachia infections outside Africa have not been described and may have been underestimated.

Methods: Mosquitoes were collected by human-landing catch during May and June 2017 in ten villages in Kayin state, Myanmar. Closely related species of malaria vectors were identified with molecular assays. Wolbachia infection rates were assessed by quantitative real-time PCR.

Results: Malaria vectors were identified in the Funestus, Maculatus and Leucosphyrus Groups. Wolbachia were detected in 6/6 Anopheles species and in 5/10 villages. Mean prevalence of Wolbachia infection was 2.7% (95%CI: [1.3; 4.9]). The median Wolbachia load was seven orders of magnitude less in naturally infected malaria vectors than in artificially infected laboratory-reared Aedes aegypti. Phylogenetic analysis based on 16S rRNA sequences revealed a high diversity of Wolbachia strains and identified lineages different from those described in Africa.

Conclusion: Natural Wolbachia infections are common and widespread in malaria vectors in Kayin state, Myanmar. Their effects on Anopheles mosquitoes and malaria transmission is yet to be determined.

Keywords
Wolbachia, Anopheles, Plasmodium, 16S rRNA, entomological inoculation rate, Southeast Asia, Kayin state, wAnga

Open Peer Review

Referee Status: ? ?

Invited Referees
1
2

version 1 published
24 Jan 2019

1 Thomas Walker ¹, London School of Hygiene & Tropical Medicine, UK
2 Francesco Baldini ², University of Glasgow, UK

Any reports and responses or comments on the article can be found at the end of the article.
This article is included in the Mahidol Oxford Tropical Medicine Research Unit (MORU) gateway.

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Author roles: Sawasdichai S: Data Curation, Investigation, Supervision; Chaumeau V: Conceptualization, Data Curation, Formal Analysis, Funding Acquisition, Investigation, Methodology, Project Administration, Supervision, Visualization, Writing – Original Draft Preparation, Writing – Review & Editing; Dah T: Investigation; Kulabkeeree T: Investigation; Kajeechiwa L: Investigation; Phanaphadungtham M: Investigation; Trakoolchengkaew M: Investigation; Kittiphanakun P: Investigation; Akararungrot Y: Investigation; Oo K: Investigation; Delmas G: Funding Acquisition, Project Administration; White NJ: Conceptualization, Funding Acquisition; Nosten FH: Conceptualization, Funding Acquisition, Investigation, Project Administration, Writing – Original Draft Preparation, Writing – Review & Editing

Competing interests: No competing interests were disclosed.

Grant information: This work was supported by the Wellcome Trust [101148]; the Bill and Melinda Gates Foundation [GH OPP 1081420] and the Global Fund [THA-M-DDC]. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

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How to cite this article: Sawasdichai S, Chaumeau V, Dah T et al. Natural Wolbachia infections in malaria vectors in Kayin state, Myanmar [version 1; referees: 2 approved with reservations] Wellcome Open Research 2019, 4:11 (https://doi.org/10.12688/wellcomeopenres.15005.1

First published: 24 Jan 2019, 4:11 (https://doi.org/10.12688/wellcomeopenres.15005.1)
Introduction

Wolbachia are intracellular bacteria that infect a wide variety of arthropods and filarial nematodes. Symbiotic relationships that result from infection have a broad range of phenotypic effects on the infected hosts, from mutualism (beneficial) to commensalism (neutral) and parasitism (harmful). In mosquitoes, Wolbachia invades the germline and can induce cytoplasmic incompatibilities between the sperm from infected males and oocytes from uninfected females. This type of reproductive parasitism enhances the “vertical” transmission of Wolbachia, from infected females to the progeny. In addition, Wolbachia can interfere with the development of some pathogens, including dengue virus, Plasmodium malariae parasites and filarial nematodes. Therefore, the release of mosquitoes artificially infected with Wolbachia has been proposed as an intervention to control medically important mosquito vectors and the diseases they transmit.

Natural infections may have important effects on mosquito populations and dynamics of diseases transmission but they are not well described. Wolbachia has been detected in several mosquito species including Aedes spp., Armigeres spp., Coquillettidia spp., Culex spp., Caliseta spp., Hodgtesia spp., Mansonia spp., Ochlerotatus spp., Tripteroides spp. and Uranotaenia spp. Interestingly, this organism was not detected in malaria mosquitoes until recent observations of naturally infected anopheline vectors in Africa.

Only one study assessed the effects of natural Wolbachia infection on the reproductive fitness of the dominant African malaria vector Anopheles gambiae (s.s.)\(^1\). The authors did not observe cytoplasmic incompatibilities, difference in the number of eggs laid or progeny sex ratio, but infected females laid eggs more rapidly. Two studies demonstrated a negative effect of Wolbachia infections on the development of P. falciparum\(^1\).\(^2\). Shaw et al. observed a negative correlation between Wolbachia infection and the development of P. falciparum oocysts in naturally blood-fed females. Gomes et al. obtained similar results on the sporozoite stage by screening large numbers of mosquitoes. The results of Gomes et al. diverged from that of Shaw et al. regarding the effects of Wolbachia infection on the early stages of Plasmodium development. Gomes et al. infected a laboratory-adapted Anopheles colony with a local strain of Wolbachia, and performed artificial transmission studies with cultured gametocytes of P. falciparum strain NF54. They observed a moderate yet significant positive correlation between Wolbachia infection and oocyst development, and a negative correlation between Wolbachia infection and sporozoite development.

Natural Wolbachia infections in Southeast Asian malaria vectors have not been reported. Their effects on Anopheles mosquitoes and dynamics of malaria transmission are not known. The objective of this pilot study is therefore to assess the prevalence of natural Wolbachia infections in malaria vectors populations in Kayin state, Myanmar.

Methods

Study sites and entomological collections

Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1). Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1). Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1). Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1). Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1). Entomological surveys were conducted in May and June 2017 in ten villages in Kayin state, Myanmar (Figure 1).
and blasted against the National Center for Biotechnology Information nucleotide database in order to determine the corresponding species (accession numbers MK358471 - MK358807).

**Wolbachia detection**

DNA extracts were screened for the presence of *Wolbachia* using a quantitative real-time PCR (qPCR) assay that targets a conserved region of the 16S rRNA genes with the primer pair W-Specf/W-Specr (5’-CAT TAT TCG AAG GGA TAG-3’ and 5’- AGC TTC GAG TGA AAC CAA TTC-3’)

All experiments were performed on a CFX-96® (Biorad) machine. Reactions were conducted in 20μl of EVAGreen qPCR Mix Plus® (Euromedex); 5μl of DNA template was used in a total reaction volume of 25μl. The PCR mix was composed of 1X HOT FIREPol™ EvaGreen™ qPCR Mix Plus (Solis BioDyne, Tartu, Estonia) and 200 nM of each primer. The thermocycling protocol consisted in an activation step at 95°C for 15 minutes.
followed by 45 amplification cycles at 95°C for 15 seconds, 58°C for 15 seconds and 72°C for 20 seconds. Characterization of the PCR product was performed using the melt curve analysis of the amplicons (95°C for 15 seconds, 68°C for 1 minute, 80°C for 15 seconds, 60°C for 15 seconds, then 60°C to 90°C with an increment of 0.2°C per second). Specificity of the PCR was confirmed by Sanger sequencing and the sequences were used for phylogenetic analysis (Accession numbers MK336794 - MK336806).

Data analysis
For the quantitation of Wolbachia load in naturally infected samples, a standard curve was set-up by performing serial-dilution assays with laboratory-reared Aedes aegypti artificially infected with Wolbachia strain wMel as a reference material (data not shown). Crossing-point (CP) values were determined using the regression algorithm of the analysis software of the PCR device (CFX Biorad Manager version 3.01, Biorad). The best-fit-line and the subsequent values of the slope and y-intercept were obtained using least-square analysis of the linear portion of each curve (Pearson’s coefficient r²=0.990). The PCR efficiency (EFF) estimate was 110% with the formula EFF = 10(-1/slope)–1. The relative quantitation of Wolbachia load in natural samples was calculated with the formula 10(ΔCP/slope), with ΔCP defined as the difference in the mean CP values measured in the natural and reference DNA extracts respectively.

For the phylogenetic analysis, sequenced PCR products were analyzed with the DECIPHER software version 2.0. Potential chimeras (4/17 samples) were removed from the dataset. Wolbachia sequences were blasted against the National Center for Biotechnology Information nucleotide database and the most similar sequence was downloaded. Reference Rickettsiales sequences were added and sequence alignment was performed using the CLUSTAL algorithm. A phylogenetic tree was reconstructed using a Tamura-Nei genetic distance model and neighbor joining with the ape package version 5.2 of the R software.

Ethical considerations
This project was approved through the ethics review committee on medical research involving human beings from Myanmar, Ministry of Health and Sports, Department of Medical Research (lower Myanmar): 73/Ethics 2014. All participants provided their written consent to participate in this study.

Results
Biodiversity of Anopheles mosquitoes
A total of 4743 Anopheles was collected during 500 person-nights of collection. We report the occurrence of 12 Anopheles taxa namely the Maculatus (45.5%), Funestus (36%), Jamesii (6.5%), Leucosphyrus (5.3%), Annularis (2.2%), Barbirostris (1.4%), Kochi (1.3%), Subpictus (0.7%), Tesselatus (0.4%), Hyrcanus (0.1%) and Asiaticus Groups (<0.1%), and the currently unclassified An. karwari (0.5%). In this area, primary malaria vectors belong to the Minimus Complex (Funestus Group), Dirus Complex (Leucosphyrus Group) and Maculatus Group. Therefore, we assessed species diversity, Plasmodium and Wolbachia infection rates in these Groups.

In total 1098 mosquitoes in the Maculatus, Funestus and Leucosphyrus Groups were identified at the species level with molecular assays. The Maculatus Group was mainly composed of An. maculatus (s.s.) (range= 50 – 88%), An. sawadwongporni (range= 5 – 41%) and An. pseudowillmori (range= 0 – 19%) (Table 1). The most frequent species in the Funestus Group were Anopheles minimus (s.s.) (range= 56 - 95%), An. culicifacies B (range= 0 - 41%) and An. jeyporiensis (range= 0 – 28%) (Table 2). The Dirus Complex represented >99% of the samples in the Leucosphyrus Group with 89 to 100% of the samples identified as An. baimaii (Table 3).

Table 1. Species diversity in the Maculatus Group determined by molecular assays.

<table>
<thead>
<tr>
<th>Village</th>
<th>An. maculatus (s.s.) n/N</th>
<th>p</th>
<th>95% CI</th>
<th>An. pseudowillmori n/N</th>
<th>p</th>
<th>95% CI</th>
<th>An. rampae n/N</th>
<th>p</th>
<th>95% CI</th>
<th>An. sawadwongporni n/N</th>
<th>p</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>HD-3634</td>
<td>75/90</td>
<td>83.3</td>
<td>74.9-90.4</td>
<td>2/90</td>
<td>2.2</td>
<td>0.3-7.8</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13/90</td>
<td>14.4</td>
<td>7.9-23.4</td>
</tr>
<tr>
<td>HG-369</td>
<td>42/59</td>
<td>71.2</td>
<td>57.9-82.2</td>
<td>4/59</td>
<td>6.8</td>
<td>1.9-16.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>13/59</td>
<td>22</td>
<td>12.3-34.7</td>
</tr>
<tr>
<td>LK-350</td>
<td>70/90</td>
<td>77.8</td>
<td>67.8-85.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>20/90</td>
<td>22.2</td>
<td>14.1-32.2</td>
</tr>
<tr>
<td>MK-3633</td>
<td>32/64</td>
<td>50</td>
<td>37.2-62.8</td>
<td>5/64</td>
<td>7.8</td>
<td>2.6-17.3</td>
<td>1/64</td>
<td>1.6</td>
<td>0-8.4</td>
<td>26/64</td>
<td>40.6</td>
<td>28.5-53.6</td>
</tr>
<tr>
<td>MK-3635</td>
<td>48/63</td>
<td>76.2</td>
<td>63.8-86</td>
<td>12/63</td>
<td>19</td>
<td>10.2-30.9</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>3/63</td>
<td>4.8</td>
<td>1-13.3</td>
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<td>MM-3631</td>
<td>63/81</td>
<td>77.8</td>
<td>67.2-86.3</td>
<td>1/81</td>
<td>1.2</td>
<td>0-6.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>17/81</td>
<td>21</td>
<td>12.7-31.5</td>
</tr>
<tr>
<td>NT-361</td>
<td>62/81</td>
<td>76.5</td>
<td>65.8-85.2</td>
<td>1/81</td>
<td>1.2</td>
<td>0-6.7</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>18/81</td>
<td>22.2</td>
<td>13.7-32.8</td>
</tr>
<tr>
<td>TG-357</td>
<td>58/77</td>
<td>75.3</td>
<td>64.2-84.4</td>
<td>1/77</td>
<td>1.3</td>
<td>0-7</td>
<td>2/77</td>
<td>2.6</td>
<td>0.3-9.1</td>
<td>16/77</td>
<td>20.8</td>
<td>12.4-31.5</td>
</tr>
<tr>
<td>TP-339</td>
<td>68/77</td>
<td>88.3</td>
<td>79-94.5</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>9/77</td>
<td>11.7</td>
<td>5.5-21</td>
</tr>
<tr>
<td>WM-367</td>
<td>39/51</td>
<td>76.5</td>
<td>62.5-87.2</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12/51</td>
<td>23.5</td>
<td>12.8-37.5</td>
</tr>
</tbody>
</table>
Table 2. Species diversity in the Funestus Group determined by molecular assays.

<table>
<thead>
<tr>
<th>Village</th>
<th>An. aconitus (s.s.)</th>
<th>An. culicifacies A</th>
<th>An. culicifacies B</th>
<th>An. harrisoni</th>
<th>An. jeyporiensis</th>
<th>An. minimus (s.s.)</th>
<th>An. varuna</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
<td>n/N p 95%CI</td>
</tr>
<tr>
<td>HD-3634</td>
<td>1/71 1.4 0-7.6</td>
<td>3/71 4.2 0.9-11.9</td>
<td>12/71 16.9 9-27.7</td>
<td>1/71 1.4 0-7.6</td>
<td>1/71 1.4 0-7.6</td>
<td>53/71 74.6 62.9-84.2</td>
<td>- - -</td>
</tr>
<tr>
<td>HG-369</td>
<td>2/86 2.3 0.3-8.1</td>
<td>- - -</td>
<td>11/86 12.8 6.6-21.7</td>
<td>- - -</td>
<td>2/86 2.3 0.3-8.1</td>
<td>71/86 82.6 72.9-89.9</td>
<td>- - -</td>
</tr>
<tr>
<td>LK-350</td>
<td>3/82 3.7 0.8-10.3</td>
<td>- - -</td>
<td>- - -</td>
<td>- - -</td>
<td>4/82 4.9 1.3-12</td>
<td>75/82 91.5 83.2-96.5</td>
<td>- - -</td>
</tr>
<tr>
<td>MK-3633</td>
<td>2/92 2.2 0.3-7.6</td>
<td>- - -</td>
<td>25/92 27.2 18.4-37.4</td>
<td>- - -</td>
<td>2/92 2.2 0.3-7.6</td>
<td>63/92 68.5 58-77.8</td>
<td>- - -</td>
</tr>
<tr>
<td>MK-3635</td>
<td>2/88 2.3 0.3-8</td>
<td>- - -</td>
<td>7/88 8 3.3-15.7</td>
<td>- - -</td>
<td>25/88 28.4 19.3-39</td>
<td>54/88 61.4 50.4-71.6</td>
<td>- - -</td>
</tr>
<tr>
<td>MM-3631</td>
<td>- - -</td>
<td>1/82 1.2 0-6.6</td>
<td>34/82 41.5 30.7-52.9</td>
<td>- - -</td>
<td>1/82 1.2 0-6.6</td>
<td>46/82 56.1 44.7-67</td>
<td>- - -</td>
</tr>
<tr>
<td>NT-361</td>
<td>- - -</td>
<td>2/83 2.4 0.3-8.4</td>
<td>32/83 38.6 28.1-49.9</td>
<td>- - -</td>
<td>1/83 1.2 0-6.5</td>
<td>48/83 57.8 46.5-68.6</td>
<td>- - -</td>
</tr>
<tr>
<td>TG-357</td>
<td>- - -</td>
<td>1/91 1.1 0-6</td>
<td>2/91 2.2 0.3-7.7</td>
<td>1/91 1.1 0-6</td>
<td>20/91 22 14-31.9</td>
<td>66/91 72.5 62.2-81.4</td>
<td>1/91 1.1 0-6</td>
</tr>
<tr>
<td>TP-339</td>
<td>- - -</td>
<td>1/84 1.2 0-6.5</td>
<td>1/84 1.2 0-6.5</td>
<td>- - -</td>
<td>2/84 2.4 0.3-8.3</td>
<td>80/84 95.2 88.3-98.7</td>
<td>- - -</td>
</tr>
<tr>
<td>WM-367</td>
<td>- - -</td>
<td>- - -</td>
<td>30/90 33.3 23.7-44.1</td>
<td>1/90 1.1 0-6</td>
<td>- - -</td>
<td>59/90 65.6 54.8-75.3</td>
<td>- - -</td>
</tr>
</tbody>
</table>
Table 3. Species diversity in the Leucosphyrus Group determined by molecular assays.

<table>
<thead>
<tr>
<th>Village</th>
<th>An. baimaii</th>
<th>An. dirus (s.s.)</th>
<th>An. introlatus</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n/N</td>
<td>p</td>
<td>95%CI</td>
</tr>
<tr>
<td>HD-3634</td>
<td>52/56</td>
<td>92.9</td>
<td>82.7-98</td>
</tr>
<tr>
<td>HG-369</td>
<td>3/3</td>
<td>100</td>
<td>29.2-100</td>
</tr>
<tr>
<td>LK-350</td>
<td>41/41</td>
<td>100</td>
<td>91.4-100</td>
</tr>
<tr>
<td>MK-3633</td>
<td>16/18</td>
<td>88.9</td>
<td>65.3-98.6</td>
</tr>
<tr>
<td>MK-3635</td>
<td>20/20</td>
<td>100</td>
<td>83.2-100</td>
</tr>
<tr>
<td>MM-3631</td>
<td>29/32</td>
<td>90.6</td>
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<tr>
<td>NT-361</td>
<td>24/24</td>
<td>100</td>
<td>85.8-100</td>
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<td>TG-357</td>
<td>36/39</td>
<td>92.3</td>
<td>79.1-98.4</td>
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<td>TP-339</td>
<td>19/21</td>
<td>90.5</td>
<td>69.6-98.8</td>
</tr>
<tr>
<td>WM-367</td>
<td>10/10</td>
<td>100</td>
<td>69.2-100</td>
</tr>
</tbody>
</table>

Figure 2. Phylogenetic analysis based on the alignment of a conserved region of the 16S rRNA gene using Wolbachia-specific primers. Sequenced PCR products were blasted against the NCBI nucleotide database and the most similar result was downloaded. A phylogenetic tree was reconstructed using a Tamura-Nei genetic distance model and neighbor joining. Sequences from other non-Wolbachia proteobacteria were also included, and the sequence from Rickettsia japonica was used as the reference outgroup. Nodes with bootstrap support <50% were collapsed. Study samples were labeled with the host name and the study village into brackets. Formally named Wolbachia strains were labeled with their abbreviation: wPip is an endosymbiont of Culex pipiens, wNo of Drosophila simulans, wAlbB of Aedes albopictus, wAnga of An. gambiae, wMel and wMelCS of Drosophila melanogaster.
Natural Wolbachia infections in malaria vectors

Six Anopheles species were screened for Wolbachia infection including An. maculatus (s.s.), An. sawadwongporni, An. pseudowillmori (Maculatus Group), An. minimus (s.s.) (Funestus Group, Minimus Complex), An. dirus (s.s.) and An. baimaii (Leucosphyrus Group, Dirus Complex). Wolbachia DNA was detected in 13/370 samples. Six unique 16S rRNA sequences were identified. 16S rRNA sequences clustered with that of Wolbachia strains in the supergroups B, D and F (Figure 2). The detection of Wolbachia strain in the supergroup D suggests that some DNA extracts were contaminated with filarial nematodes carrying Wolbachia rather than actual natural Wolbachia infection in Anopheles.

After excluding Wolbachia that clustered in the supergroups D from the analysis, mean prevalence estimate of Wolbachia infections in malaria mosquitoes was 2.7% (95%CI=[1.3; 4.9]). Wolbachia were detected in 6/6 Anopheles species and in 5/10 villages (Figure 3). Crossing-point values ranged from 31.0 to 40.6 amplification cycles. Infected specimens were generally infected at a density close or below the limit of detection of the assay (only one sample gave 3/3 positive reactions). The median Wolbachia load in those naturally infected specimens was seven orders of magnitude less than that observed in laboratory-reared Aedes aegypti artificially infected with Wolbachia strain wMel.

Discussion

This study is the first report of natural Wolbachia infections in malaria vectors in Southeast Asia, including An. minimus (s.s.) (Minimus Complex, Funestus Group), An. maculatus (s.s.), An. sawadwongporni, An. pseudowillmori (Maculatus Group), An. dirus (s.s.) and An. baimaii (Dirus Complex, Leucosphyrus Group). Wolbachia-infected specimens were detected in 6/6 species and in 5/10 villages which suggests that Wolbachia infections are common and widespread in Kayin state. Our data and African studies confirm that the occurrence of natural Wolbachia infections has been underestimated in malaria mosquitoes.

Molecular phylogeny based on 16S rRNA sequences revealed a high diversity of Wolbachia strains, which belonged to different lineages than those recently reported in African malaria vectors. It was not possible to study more in detail the phylogeny of Wolbachia strain detected in this study by multi locus sequence typing because of the lack of DNA extracts after the screening. Prevalence estimates were in the lower range of those reported in Africa (1.3 to 78%) and in 5/10 villages which suggests that Wolbachia infections are common and widespread in Kayin state. Our data and African studies confirm that the occurrence of natural Wolbachia infections has been underestimated in malaria mosquitoes.
consistent with previous attempts to quantify Wolbachia in naturally infected malaria vectors\(^\text{16,17}\) and probably explains why Anopheles mosquitoes were repeatedly reported not to be infected with Wolbachia in natural settings. We did not assess the effects of Wolbachia infection on the phenotype of infected mosquitoes and dynamics of malaria transmission. In Kayin state, malaria transmission is low, seasonal and unstable, and Plasmodium infection rate is less than 1% and often nil in mosquito populations\(^\text{20}\). Therefore, it was not possible to establish direct correlations between Plasmodium and Wolbachia infection rates in naturally infected malaria vectors. In this setting, the effect of Wolbachia infections on malaria transmission may be better assessed by performing experimental infections of field-collected mosquitoes with Plasmodium malaria parasites.

**Conclusion**

Although the prevalence and bacterial load reported in this study were low, natural Wolbachia infections seem to be common and widespread in Southeast Asian malaria vectors. Their effects on Anopheles hosts and dynamics of malaria transmission are yet to be determined.

**Data availability**

The data is available upon request to the Mahidol Oxford Tropical Medicine Research Unit Data Access Committee (http://www.tropmedres.ac/data-sharing) and following the Mahidol Oxford Tropical Medicine Research Unit data access policy (http://www.tropmedres.ac/_asset/file/data-sharing-policy-v1-0.pdf).

**Grant information**

This work was supported by the Wellcome Trust [101148]; the Bill and Melinda Gates Foundation [GH OPP 1081420] and the Global Fund [THA-M-DDC].

The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Acknowledgments**

We thank communities from the study villages for their support to the study. Wolbachia-infected reference samples were kindly provided by Dr. Lauren Carrington from the Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam. SMRU is part of the Mahidol Oxford University Research Unit, supported by the Wellcome Trust of Great Britain.

References


Open Peer Review

Current Referee Status: ? ?

Version 1

Referee Report 04 March 2019

https://doi.org/10.21956/wellcomeopenres.16370.r34658

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This work identified Wolbachia strains in Anopheles species from Myanmar. To my knowledge this is the first evidence of identification of Wolbachia infection in Anopheles mosquito populations in Asia. This work opens novel questions on the potential role of these infections on the vectorial capacity of the vector host. Indeed, recently identified natural Wolbachia infections in Anopheles species in Africa have been shown to potentially influence the vectorial capacity of the infected vectors.

The authors state that Wolbachia infections were identified by using quantitative PCR approach followed by sequencing of the Wolbachia 16S gene, although some details are missing and the methodology should be clarified. Phylogenetic analysis is also not completely detailed. Specific comments to the manuscript are listed below.

Introduction:
The authors state that cytoplasmic incompatibility (CI) ‘enhances the “vertical” transmission of Wolbachia’. From my knowledge this is incorrect, as CI does not directly affect “vertical” transmission, but rather increases ‘indirectly’ the fitness of the progeny of Wolbachia infected mothers.

The authors state that ‘Shaw et al. observed a negative correlation between Wolbachia infection and the development of P. falciparum oocysts in naturally blood-fed females’. This is not completely correct, as in this work the authors have quantified the prevalence of P. falciparum in resting blood fed females inside house 5 days after collection/blood feeding, without any prior knowledge on the infectious status of the mosquitoes; thereby, the stage of parasite infection (oocyst or sporozoite) was not investigated.

Methods:
Authors indicate Shaw et al. and Gomes et al. as references for the qPCR using W-Spec primers against Wolbachia 16S. This is where I am getting confused, as Shaw et al. did not use qPCR and Gomes et al. use a different primer set for qPCR. The methodology should be clarified.

If W-Spec primers were used, the expected product size is >400 base pairs (bp); this bp size is often too large for qPCR, as large amplicons tend to produce secondary structures during the dissociation steps, thus resulting in multiple melting peaks. If possible, it would be informative to provide more details on the optimization of this assay (in case additional reagents were added, for example) and to show the dissociation profile of the obtained amplicons, as this would enable troubleshooting of the technique if others will try to replicate the work and/or use the same methodology. Also, it should be specified how sequencing was performed, e.g. direct purification after qPCR (how?), which primer was used, etc.
In the phylogenetic analysis the authors should state what was the sequence size used to build the tree. Novel obtained sequences should also be uploaded and their unique identifier indicate in the article.

Statistical analysis used in Table 1-3 should be indicated (if any).

**Results:**
The authors state the ‘we assessed species diversity, Plasmodium and Wolbachia infection rates in these Groups’. This is incorrect as Plasmodium infection rates are not shown.

Captions in Table 1-3 are missing information on what each column indicates. Although these tables can be generally informative, I wonder if showing species diversity using pie-charts (for each species group) over imposed on the map in Figure 1 would provide a more direct illustration of the species composition and abundances of the Anopheles species in the study area.

As indicated in the methods, the size of the sequence used for Figure 2 should be indicated. It would be informative to include the alignment use for the tree figure.

Figure 3b shows the overall prevalence of Wolbachia in different villages without specifying the species, so I am not sure what is the purpose of illustrating the result in this way. If this is too show that some villages have higher prevalence over others this should be indicated only if statistical analysis supports it (although I doubt this is the case if species distribution is included as a variable).

The authors state that ‘Crossing-point values ranged from 31.0 to 40.6 amplification cycles. Infected specimens were generally infected at a density close or below the limit of detection of the assay (only one sample gave 3/3 positive reactions).’ More details should be given regarding the rational for inclusion (or exclusion) of an infected/amplified sample; it is not clear to me if ‘reactions’ refers to technical replicates in the same qPCR run or in different qPCR assays. This should be described with more details. It would also be informative to normalize the quantity of the amplified Wolbachia 16S using a mosquito housekeeping gene, for example. Indeed, as ‘density [was] close or below the limit of detection of the assay’ normalization would provide information on the likelihood of false negatives in samples, as you would expect if the total DNA is very low (for example due to inefficient DNA extraction).

**Discussion:**
The authors state that ‘Our data and African studies confirm that the occurrence of natural Wolbachia infections has been underestimated in malaria mosquitoes.’ As direct assessment of Wolbachia prevalence on samples previously identified as uninfected was not performed here (nor in African samples) it cannot be ruled out that previous Wolbachia negative samples were not true negatives, so this work (and others) only suggest possible underestimation in previous works, as they have not directly confirmed it.

The authors state that ‘It was not possible to study more in detail the phylogeny of Wolbachia strain detected in this study by multi locus sequence typing because of the lack of DNA extracts after the screening.’ It is not clear to me if any attempts were made at all or not. If so, please give more details on the targeted genes and discuss why these could not be amplified.

**Is the work clearly and accurately presented and does it cite the current literature?**
Partly

**Is the study design appropriate and is the work technically sound?**
Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

**Competing Interests:** No competing interests were disclosed.

**Reviewer Expertise:** I believe that I have an appropriate level of expertise to assess the submitted article. I have expertise in identification of natural Wolbachia infections in natural populations of Anopheles and other vectors; I have also expertise in the methods used, mainly quantitative PCR and phylogenetic analysis.

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Thomas Walker
Department of Disease Control, London School of Hygiene & Tropical Medicine, London, UK

The authors present an interesting study in which Wolbachia strains were detected in Anopheles species from Myanmar. This study is particularly timely given the recent discoveries of natural Wolbachia strains in Anopheles malaria vectors in Sub-Saharan Africa and evidence that these natural endosymbiotic bacteria could be influencing malaria parasite infection prevalence in wild mosquito populations. The study provides evidence for Wolbachia infections using amplification and sequencing of the Wolbachia 16S gene although more clarity is needed on which primer set was used given the authors report undertaking qRT-PCR and sequencing of PCR products. The manuscript would be significantly improved with additional Wolbachia gene analysis and to provide the quantitative PCR data. This would provide more information on the Wolbachia strain infections being presented and allow these strains to be put into context with recent discoveries in other Anopheles species.

**Introduction**
The introduction needs significant improvement in the referencing. For example, the sentence ‘In addition, Wolbachia can interfere with the development of some pathogens, including dengue virus’ contains a reference to a publication that only describes Wolbachia establishment and invasion in an Aedes aegypti laboratory population (not virus inhibition).
Furthermore, including references 5 & 6 in the context of pathogen blocking is not appropriate given this was work which was proposing to use cytoplasmic incompatibility to reduce Culex mosquito populations and artificial Wolbachia-infected mosquito lines were only established in the mid 2000s.

The paragraph describing natural Wolbachia infections in mosquitoes also needs further references.

The final paragraph in the introduction presents the fact that Natural Wolbachia infections in Southeast Asian malaria vectors have not been reported. However, the authors should reference the studies in which screening of Anopheles species for Wolbachia was undertaken despite finding no evidence of natural infections.

**Methods**

The primers used for Wolbachia detection W-Specf (CATACC TATTCGAGGGGATAG) and W-Specr (AGCTTCGAGTGAAAACCAATTC) produce a product size of 438 bp and this (to my knowledge) would not be possible or has not been reported using a qrtPCR format. The authors also reference Gomes et al. which used a different reverse primer (5′-TTGGGGACTTAACCCAACA-3′) that results in amplification of a smaller fragment of the 16S rRNA gene for qrtPCR. The accession numbers MK336794 - MK336806 refer to sequences with >400 bases indicating W-Specf/W-Specr was used. The authors need to clarify if W-Specf/W-Specr was used on a qrtPCR format or if both were used independently and report the differences in prevalence rates using these two primer sets.

**Results**

The inclusion for analysis of only what would be considered ‘primary malaria vectors’ needs more explanation if the authors overall aim was to provide evidence for natural Wolbachia infections in Anopheles species given the mosquitoes were not screened for Plasmodium infection.

Tables 1-3 provide a breakdown of the species composition collected at the different villages but I think it would be more informative to have all the different species grouped according to villages. Currently it’s difficult to determine mosquito species prevalence on a village level.

The statistics used in tables 1-3 don’t appear to be explained either in the manuscript methods or in the table legends. For example, I am assuming ‘n/N’ means the species/total number collected but again this would be much easier to understand if species were grouped by villages.

**Wolbachia infections**

With reference to my previous point raised in the methods, which 16S PCR primer set and format was used to determine the prevalence rates and to generate sequences for Figure 2?

Figure 2 needs to have more details included such as the number of nucleotide sequences used in the analysis and the total positions in the final dataset.

The authors provide the overall prevalence rate (13/370) and then have Figure 3 to show the individual species. Figure 3a I don’t feel is needed because plotting 1/11 (PSE) and 1/12 (DIR) seems unnecessary and could be in a table that incorporates prevalence rates by species and village. Having an overall village prevalence rate (Fig 3b) has little biological relevance given you have variable Anopheles species containing what appears to be different Wolbachia strains based on 16S analysis.

A major limitation of the phylogenetic analysis (and even the prevalence rates) is only using a single
Wolbachia gene (16S) but I appreciate that Cp values ranging from 31-40.6 are at the limit of detection. The authors should provide these 16S Cp values to allow the reader to see the variation both between technical and biological replicates.

Could the authors also provide the rationale for concluding that samples were positive where not all technical replicates produced positive amplification given ‘only one sample gave 3/3 positive reactions’? How do these results fit with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines?

Could the authors not have used another Wolbachia qPCR assay based on a second gene that targets a broad range of strains?

The density comparison to laboratory-reared Aedes aegypti artificially-infected with the wMel Wolbachia strain is not particularly informative for several reasons. Firstly, quantifying Wolbachia density without accounting for mosquito body size and/or DNA extraction efficiency is problematic. Secondly, the wMel is a strain that naturally infects Drosophila melanogaster so a better comparison would be to natural infections in mosquito species (such as wPip in Culex quinquefasciatus or even the wAlbA/wAlbB strains in Aedes albopictus). Therefore, I would question the inclusion of this density data given wMel in Ae. aegypti is an artificial infection.

Discussion
The sentence ‘It was not possible to study more in detail the phylogeny of Wolbachia strain detected in this study by multi locus sequence typing because of the lack of DNA extracts after the screening’ needs clarification. Do the authors mean that they were unable to amplify any of the Wolbachia MLST genes? Did they try using degenerate primer protocols or nested PCR given the qPCR data would indicate low density infections?

The statement “This is consistent with previous attempts to quantify Wolbachia in naturally infected malaria vectors” is incorrect and refers to some (An. gambiae complex) but not all species analysed in Sub-Saharan Africa. The authors should expand this discussion as the low density infections presented in this study are comparable to those strains detected in An. gambiae mosquitoes from Sub-Saharan Africa. Some of these studies have only resulted in 16S gene amplification and sequencing resulting in conflicting phylogenetics which appear incompatible with the traditional criteria for vertically transmitted endosymbionts (reviewed in reference 9). The authors should provide some further discussion points on whether their results only amplifying 16S could have resulted from either 1) integration into the mosquito genome or 2) some form of contamination. However, additional Wolbachia gene analysis would allow more confidence in these detected strains given the high 16S qPCR Cp values are at the limit of detection.

References
4. Wiwatpanaratanutabutr I: Geographic distribution of wobachial infections in mosquitoes from Thailand.


Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
No

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: medical entomology, Wolbachia, Anopheles

I have read this submission. I believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.