Research Article

Molecular Evidence of Wolbachia Species in Wild-Caught Aedes albopictus and Aedes aegypti Mosquitoes in Four States of Northeast India

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Wolbachia, a Gram-negative intracellular bacterium, naturally infects many arthropods, including mosquito vectors responsible for the spread of arboviral diseases such as Zika, chikungunya, and dengue fever. Certain Wolbachia strains are involved in inhibiting arbovirus replication in mosquitoes, and this phenomenon is currently being studied to combat disease vectors. A study was conducted in four states in north-eastern India to investigate the presence of natural Wolbachia infection in wild-caught Aedes albopictus and Aedes aegypti mosquitoes, the established vectors of dengue. The detection of a Wolbachia infection was confirmed by nested PCR and sequencing in the two mosquito species Ae. aegypti and Ae. albopictus. Positivity rates observed in Ae. aegypti and Ae. albopictus pools were 38% (44 of 115) and 85% (41 of 48), respectively, and the difference was significant (chi-square = 28.3174, p = 0.00000010). Sequencing revealed that all detected Wolbachia strains belonged to supergroup B. Although Wolbachia infection in Ae. aegypti has been previously reported from India, no such reports are available from north-eastern India. Data on naturally occurring Wolbachia strains are essential for selecting the optimal strain for the development of Wolbachia-based control measures. This information will be helpful for the future application of Wolbachia-based vector control measures in this part of the country.

1. Introduction

Vector-borne diseases cause significant loss of life in terms of morbidity and mortality. In north-eastern (NE) India, dengue fever and malaria are endemic and outbreaks are common in various states in the region [1–4]. Mosquito vectors such as Ae. aegypti and Ae. albopictus cause many dengue outbreaks each year and traditional measures to combat them have not yielded the expected results [5]. Increasing insecticide resistance among the mosquito population further exacerbates this problem. In India, both mosquito species showed different degrees of resistance to dichlorodiphenyltrichloroethane (DDT) in most states [6, 7]. Therefore, more effective and biologically active vector control measures are needed to prevent these vector-borne diseases [8]. Wolbachia, a class of Alphaproteobacteria, is endosymbiotic in several arthropods and filarial nematodes in the biosphere. There are currently 17 supergroups of endosymbiotic Wolbachia (A–S, excluding G and R), most of which infect terrestrial arthropods, particularly insects and arachnids [9–11]. Interestingly, certain Wolbachia species have been shown to possess a natural ability to alter the biology of the infected host mosquito, making them less susceptible to infection by arboviruses such as dengue virus...
(DENV), chikungunya virus (CHIKV), yellow fever virus (YFV), and Zika virus (ZIKV). This property has been exploited by various groups to transfect *Ae. aegypti* with *Wolbachia* strains such as wMel, wMelPop - CLA, wAlbB, and wMel/wAlbB [5]. In Orissa, genetically distinct and unique *Wolbachia* species have been reported in the coastal plains, which show completely different characteristics from the other populations of the country [12]. In addition, both wAlbA and wAlbB *Wolbachia* endosymbionts were observed in *Ae. albopictus* population from the Andaman and Nicobar Islands [13]. A low prevalence of these *Wolbachia* endosymbionts has been observed in Indian wild mosquitoes of *An. culicifacies* and *An. stephensi* species from Tamil Nadu [14]. However, there can be significant overlap in *Wolbachia* strains infecting one host, and different strains can affect the survival of the other [15]. Geographical and ecological factors must also be taken into account. This could have an important impact on the selection of the optimal strain for transfection in *Wolbachia*-based vector control strategies since a detailed assessment of native strains in mosquito populations is first required. The current study was conducted to detect *Wolbachia* infection in adult *Ae. aegypti* and *Ae. albopictus* mosquitoes collected in four different states (Assam, Arunachal Pradesh, Nagaland, and Meghalaya) in NE India by nested PCR using 16S rRNA-specific primers followed by sequencing.

### 2. Materials and Methods

We used archived mosquito samples collected as part of the previously conducted project “Vector Surveillance for ZIKV in Selected High-Risk Areas” [16, 17]. *Ae. albopictus* and *Ae. aegypti* mosquitoes (adult) were sampled from February 2018 to February 2019 in urban areas from four dengue-prone regions in four different states in NE India: Guwahati (Kamrup Metro district, Assam), Tura (West Garo Hills district, Meghalaya), Pasighat (East Siang district, Arunachal Pradesh), and Dimapur (Dimapur district, Nagaland). The location of the study sites is shown in Figure 1. Adult mosquitoes were originally collected using suction tubes from indoor and outdoor resting sites such as open water tanks, garages, tire dumps, and leaf axils. Mosquitoes collected in the field were separated according to species, collection site, date, sex, and blood-fed status of the female mosquitoes. A maximum of 20 mosquitoes were pooled in one tube and transported to the laboratory in 50 μL of TRI Reagent (Molecular Research Center, Inc., USA) at 4°C. Samples were uniformly homogenized, and DNA was extracted using the commercially available DNeasy Blood and Tissue Kit (Qiagen) and stored at −20°C until further processing.

With some minor modifications, ITS2-PCR (Internal Transcribed Spacer-2) was performed to validate *Ae. aegypti* and *Ae. albopictus* species in the collected mosquito pools [18]. The PCR contained 1.0 mM MgCl₂ and the primers ITS2-F and ITS2-R (5′-ATCACCTCGGTCTGTGATC-3′, 5′-ATGCTTTAAATTTAGGGGTATG-3′) at a concentration of 1 μM each. The PCR settings were as follows: 95°C for 5 minutes (initial denaturation), then 35 cycles of 95°C for 30 seconds (denaturation), 56°C for 30 seconds (annealing), 72°C for 45 seconds (extension), and 72°C for ten minutes the last extension. Positive controls for *Ae. albopictus* were provided by the Indian Council of Medical Research-Vector Control Research Center (ICMR-VCRC, Puducherry) and internal controls were used for *Ae. aegypti*. *Wolbachia* detection in *Aedes* mosquitoes was performed using nested PCR (nPCR) as described by Shaw et al. [19]. From the extracted individual pools of *Ae. aegypti* and *Ae. albopictus* gDNA, 16S rRNA *Wolbachia* gene was targeted by nested PCR. The initial PCR was performed with *Wolbachia* 16S rRNA-specific primer pairs (W-Specf: 5′-CATACCTATTCGAGGGATAGT-3′ and W-Specr: 5′-AGCTTCAAGTGAACCAATTCC-3′) in a 25 μL reaction volume with 5 μL of gDNA. Then, 5 μL of the primary PCR amplicon was used as a target in the second round of PCR with the following internal primers: 16SNF (5′-GAAAGGGATAGTGCTGTGGTGC-3′) and 16SNR (5′-CAATTCCATGCGGTGTAGC-3′) in a reaction volume of 50 μL. The PCR protocol for nested 16S rRNA PCR was as follows: initial denaturation at 95°C for 15 minutes, followed by 35 cycles of 15 seconds at 95°C, 25 seconds at 66°C, and 30 seconds at 72°C; followed by a final extension step at 72°C for 5 minutes [19]. *Wolbachia* control DNA provided by ICMR-VCRC, Puducherry, was used as a PCR positive control, and double distilled water (ddH₂O) was used as a negative control. The secondary PCR product, 412 bp in size, was considered specific for *Wolbachia* and sequenced using Sanger’s technique. The *Wolbachia* 16S and *Aedes* ITS2 sequences obtained were checked for sequence quality and compared using the Bioedit Version 7.2 software [20]. The aligned nucleotide sequences were checked for matches and compared to pre-existing high-similarity sequences downloaded from the NCBI GenBank database. All sequences were aligned with Clustal W and exported to MEGA X software for further genetic analysis [21].

### 3. Results

The project collected a total of 6,229 adult *Aedes* mosquitoes from dengue-endemic areas in four different Northeast states. Details on the distribution of these mosquitoes can be found elsewhere [17]. In short, it was found that *Ae. aegypti* was the predominant *Aedes* species (63.3%) among all mosquitoes collected in the study. In Guwahati, Dimapur, and Tura, which are predominantly urban areas, *Ae. aegypti* was dominant, while in Pasighat, which is surrounded by forested areas, *Ae. albopictus* was already established in this region, a larger number of *Ae. aegypti* pools were selected for analysis (115 vs. 48). Of the 163 pools, a total of 85 pools were found positive for *Wolbachia* by nPCR (Table 1, Figure 2). Positivity rates observed in *Ae. aegypti* and *Ae. albopictus* were 38% (44 of 115) and 85% (41 of 48), respectively, and the difference was significant (chi-square = 28.3174, p = 0.00000010). A total of 17 *Wolbachia* 16S PCR amplicons (8 from *Ae. aegypti* and 9 from *Ae. aegypti*).
Figure 1: District-level map of NE India (7 states) showing the four study areas (GPS coordinates marked with black ‘*’ sign) with land use/land cover details. (Data source: IRS P6 LISS 111 satellite data from National Remote Sensing Centre, Indian Space Research Organization, Department of Space, Govt. of India, Balanagar, Hyderabad-500037; image created with Bhuvan (https://bhuvan.nrsc.gov.in/) and QGIS version. 3.8.3-Zanzibar).

Table 1: Distribution and characteristics of *Wolbachia* positive pools by nPCR.

<table>
<thead>
<tr>
<th>Locations</th>
<th>Mosquitoes species</th>
<th>Gender</th>
<th>No. of pools processed</th>
<th><em>Wolbachia</em> positive pools by nPCR</th>
<th>Pool positivity rate (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nagaland (Dimapur)</td>
<td><em>Aedes aegypti</em></td>
<td>Male</td>
<td>16</td>
<td>4</td>
<td>25.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>22</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td><em>Aedes albopictus</em></td>
<td>Male</td>
<td>1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>3</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>42</td>
<td>4</td>
<td>9.52</td>
</tr>
<tr>
<td>Assam (Guwahati)</td>
<td><em>Aedes aegypti</em></td>
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<td>19</td>
<td>10</td>
<td>52.60</td>
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<tr>
<td></td>
<td></td>
<td>Female</td>
<td>22</td>
<td>4</td>
<td>18.10</td>
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<tr>
<td></td>
<td><em>Aedes albopictus</em></td>
<td>Male</td>
<td>3</td>
<td>1</td>
<td>33.30</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>3</td>
<td>2</td>
<td>66.60</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Total</td>
<td>47</td>
<td>17</td>
<td>36.17</td>
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<tr>
<td>Arunachal Pradesh (Pasighat)</td>
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<td>2</td>
<td>66.60</td>
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<tr>
<td></td>
<td></td>
<td>Female</td>
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<td>2</td>
<td>66.60</td>
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<tr>
<td></td>
<td><em>Aedes albopictus</em></td>
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<tr>
<td></td>
<td></td>
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<td>44</td>
<td>42</td>
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<td>Meghalaya (Tura)</td>
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<td>11</td>
<td>10</td>
<td>90</td>
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<td>12</td>
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<tr>
<td></td>
<td><em>Aedes albopictus</em></td>
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<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Female</td>
<td>0</td>
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</tr>
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<td></td>
<td></td>
<td>Total</td>
<td>30</td>
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<td>73.33</td>
</tr>
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</table>
albopictus) pools) were sequenced in the current study using Sanger’s technique. The resulting sequences have been deposited in the NCBI GenBank database (accession numbers: OL477363.1-OL477379.1). The phylogenetic maximum likelihood tree was constructed using the Tamura–Nei model [22]. The tree with the highest log likelihood (−663.46) comprised 27 nucleotide sequences (295 positions each) (Figure 3). MEGA X was used to perform evolutionary analysis [21]. When compared to other known sequences from groups A and B, it was found that all isolates in the current study belonged to Wolbachia supergroup B. For the confirmation of the field-collected Aedes samples as Ae. aegypti and Ae. albopictus, an ITS2-PCR was performed with corresponding positive controls (Figure 4). A total of 8 ITS2-PCR amplicons (4 Ae. aegypti and 4 Ae. albopictus) were sequenced and submitted to the NCBI GenBank (ITS2 sequence accession numbers: OP327745 – OP327752). A total of 25 nucleotide sequences were used to construct the phylogenetic tree using the neighbour joining method and the Kimura-2 parameter in MEGA 11 [23, 24]. Analysis of the phylogenetic tree (Figure 5) showed good agreement of the study samples with Ae. aegypti and Ae. albopictus [18, 25].

4. Discussion

The current study has shown the prevalence of Wolbachia species in two important dengue vectors viz. Ae. aegypti and Ae. albopictus from four dengue endemic areas spread over four states in northeastern India. In both mosquito species, Wolbachia supergroup B was detected. The positivity rate was higher in Ae. albopictus compared to that of Ae. aegypti (85% vs. 38%), and the difference was significant. Although previous studies from Northeast India have reported Wolbachia in various mosquito vectors, there are no previous reports of Wolbachia infection in Ae. aegypti [15]. Traditionally, it was assumed that natural infection of Ae. aegypti with Wolbachia was not common [15, 26]. Previously, researchers used different sets of primers to detect natural infection of Wolbachia in mosquito vectors. Wolbachia surface protein (wsp)-based primers have been widely used to detect Wolbachia superinfections in many arthropods. A comparison between wsp primers and 16S rRNA-based primers in Ae. aegypti mosquitoes has shown that the highest detection rate was achieved with 16S rRNA primers in the US [27]. Malaria vectors such as Anopheles mosquitoes were also assumed not to be naturally infected with Wolbachia until a unique 16S rRNA-based PCR demonstrated that An. gambiae carry low-level natural Wolbachia infection [19].

Subsequently, researchers from Malaysia (25%), the Philippines (16.8%), and the USA (44.8%) have reported natural Wolbachia infection in Ae. aegypti using Wolbachia 16S rRNA primers [27–29]. In 2019, the natural infection of Ae. aegypti with Wolbachia supergroup B was detected using 16S rRNA-based primers from Coimbatore, Tamil Nadu, India [5]. The reported strain showed 99% homology with the wAlbB strain in Ae. albopictus [5]. This is similar to the homology levels observed in our study (98%–99%) compared to published sequences in the NCBI database. The abovementioned studies from Malaysia, Philippines, USA, and India observed Wolbachia infection in Ae. aegypti mosquitoes by screening individual mosquitoes. However, Wolbachia infection in screened pools of Ae. aegypti has also been described previously [30, 31]. Coon et al. reported two Wolbachia 16S rDNA OTUs (operational taxonomic units) in a pool of 30 Ae. aegypti larvae collected in Florida in 2014 [30].
Similarly, *Wolbachia* 16S OTUs have been detected in pooled *Ae. aegypti* mosquitoes in Thailand [31].

In most countries, including India, *Ae. albopictus* mosquitoes have traditionally been subjected to *Wolbachia* detection using primers based on *Wolbachia* surface protein (*wsp*). In a study conducted in Orissa, India, 1291 male and female *Ae. albopictus* mosquitoes collected from 15 districts across the state were tested for *Wolbachia* infection using *wsp* primer-based PCR. Among these, 1281 (99.2%) mosquitoes tested positive for *Wolbachia* infection; most were individually infected with supergroups B and A, and some had mixed infection with A and B [12]. Another study from
Orissa found that 5% of *Ae. albopictus* were monoinfected with *wAlbA*, 10% with *wAlbB* and 80% with both *wAlbA* and *wAlbB* [32]. Similarly, Ravikumar H et al. also reported *Wolbachia* infection in eight of twenty mosquito species using *wsp* primer sets, with *Wolbachia A* and *B* observed in *Ae.albopictus* mosquitoes [33]. From Assam, NE India, Soni et al. showed superinfection with *Wolbachia A* and *B* from Dibrugarh, Tinsukia, and Sibsagar districts; whereas from Tezpur, in *Ae. albopictus*, only the *Wolbachia supergroup A* was detected. [15]. In the Andaman and Nicobar Islands, a total of 57 *Ae. albopictus* mosquitoes (100%) were found to be infected with *Wolbachia* supergroups A and B by *wsp* primer-based PCR [13].

The *wAlbB* strain has been shown to confer protection against arboviruses in mosquito vectors. However, the route of infection seems to play a crucial role. Whether a particular mosquito species is infected with this strain naturally, through transient or stable transfection, has a major impact on resistance to arboviruses such as dengue and chikungunya [34, 35]. While natural infection of *Ae. albopictus* with *wAlb A* and *wAlbB* showed no antiviral activity, stable transinfection with the *wMel* strain has been reported to reduce transmission of DENV and CHIKV [34, 35]. Likewise in the case of *Ae. aegypti*, stable transinfection with *wMelPop*, *wAlbB*, and *wMel* strains all showed reduced infection rate, viral load, and transmission rates for DENV and CHIKV compared to *Wolbachia*-free mosquitoes [34, 36]. However, such studies on natural populations are scarce and large-scale studies across different geographic locations are needed to obtain conclusive evidence for the potential antiviral role of wild-caught, naturally infected *Ae. aegypti* and *Ae. albopictus*. Different types of *Wolbachia* strains provide different survival benefits to host mosquitoes and may also entail fitness costs. One variety can also complement or compete with the other. Experimenting with *Ae. albopictus* triple infections with *wMel*, *wAlbA*, and *wAlbB* has shown that the introduction of new *Wolbachia* strains can sometimes lead to unexpected complications in uninfected or naturally infected mosquito vectors [36–38].

Our observation on the molecular detection of *Wolbachia* using 16SrRNA primer-based PCR and sequencing in *Ae. aegypti* and *Ae. albopictus* could have important implications for future intervention strategies based on the transfection of *Wolbachia*-infected mosquitoes. It may or may not play an important role in reducing arbovirus transmission under natural conditions. However, further validation of the definitive presence of natural infection in these mosquito hosts requires additional molecular tests such as the detection of bacteria in the host tissue and their removal after antibiotic treatment and whole genome sequencing [39]. We propose that natural infection of *Wolbachia* in mosquito vectors needs to be delineated, preferably based on multiple lines of evidence in different geographic regions, before initiating vector control measures based on *Wolbachia*-infected mosquitoes.
Data Availability

All the sequences generated in this study have been submitted to the NCBI GenBank and can be obtained from the database. The relevant Accession Numbers are mentioned in the text.

Conflicts of Interest

The authors declare that there are no conflicts of interest.

Authors’ Contributions


Acknowledgments

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