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Molecular typing and phylogeny of Wolbachia: A study from Assam, North-Eastern part of India

Monika Soni[a], Chandrakanta Bhattacharya[a], Jitendra Sharma[b], Siraj Ahmed Khan[c], Prafulla Dutta[c,*]

[a] Regional Medical Research Centre (ICMR), NER, Dibrugarh, Assam, India
[b] District Surveillance Unit, Office of the Joint Director of Health Services, Lakhimpur, Assam, India
[c] Entomology & Filariasis Division, Regional Medical Research Centre (ICMR), NER, Dibrugarh, Assam, India

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ABSTRACT

Background: Wolbachia are maternally inherited endosymbiotic alphaproteobacteria, infecting 40–75% of arthropod species. Knowledge on distribution of native strains infecting mosquito vectors from endemic regions is essential for successful implementation of vector control interventions utilizing potential strains of *Wolbachia*. Study identified various native strains of *Wolbachia* inhabiting different mosquito species from field and colonised conditions of Assam. The fly *Drosophila melanogaster* was also included in our study.

Methods: Different mosquito species collected from field viz; *Aedes albopictus*, *Aedes aegypti*, *Anopheles hyrcanus, Anopheles annularis, Culex vishnui, Toxorhynchites splendens, Armegeries obturbans and* fly *Drosophila melanogaster* were included in the study. *Anopheles stephensi and Culex quinquefasciatus* were obtained from RMRC, Dibrugarh mosquito colony y for *Wolbachia* screening. DNA was extracted from these species, amplified using group specific *wsp* primers followed by sequencing and phylogenetic analysis.

Results: Aedes albopictus from Dibrugarh, Tinsukia and Sivasagar district showed superinfection with A and B group of *Wolbachia* but, *Aedes albopictus* from Tezpur district presented infection with A group only. Our study reports for the first time natural infection of *Wolbachia* A and B group from colonised *Anopheles stephensi* mosquito but reported no infection from field collected *Anopheles hyrcanus or Anopheles annularis.* Similarly *Armigeres obturbans* and *Culex vishnui* presented infection with only B group of Wolbachia. *Drosophila melanogaster* showed superinfection with A and B group. *Toxorhynchites splendens, Aedes aegypti and Culex quinquefasciatus* reported no infection with *Wolbachia.*

Conclusion: To the best of our knowledge this is the first study on *Wolbachia* screening from Northeast part of India and also first report of natural *Wolbachia* infection from colonised *Anopheles stephensi species.* The current understanding on distribution of *Wolbachia* strains naturally present within insect species from this geographical region should aid future *Wolbachia* mediated vector control strategies.

1. Background

Communicable diseases are still regarded as leading disease burden in low and middle income countries. (Boutayeb, 2010; Gupta and Guin, 2010) More than 10% of all communicable diseases are due to vector-borne infections (Golding et al., 2015) out of which dengue alone document 50–200 million infections every year (Murray et al., 2013; WHO, 2016). Vector borne diseases are greatest peril to human population in terms of health and economic burden. Rapid urbanization, globalization and climate change has enabled expansion and transmission of such diseases to newer geographical regions.

The vector species responsible for spreading life-threatening diseases such as dengue, Chikungunya, Japanese Encephalitis, Malaria, West Nile and Lymphatic Filariasis are widely distributed throughout India and across major part of globe (Dev et al., 2015; Dutta et al., 1998; Kalluri et al., 2007; Lemon et al., 2008; Organisation, 2014). Lack of effective therapeutics, vaccines and increasing emergence of insecticide resistance, necessitates finding alternate and effective tools for management of mosquito associated diseases (Karunamoorthi and Sabesan, 2013; Mnzava et al., 2015). In our ongoing efforts to find effective vector control strategies, *Wolbachia* due to its bizarre qualities has emerged as potential candidate and gaining more importance to address vector controls strategies. *Wolbachia*, are maternally inherited, obligate, endosymbiotic *alphaproteobacteria* estimated to infect 40–75%

* Corresponding author.
E-mail addresses: jitendra.du.biotech@gmail.com (J. Sharma), duttaprafulla@yahoo.com (P. Dutta).

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of insect species [6]. They cause various types of reproductive manipulations in the host like feminization, parthenogenesis, male killing and cytoplasmic incompatibility (CI) to enhance their own expansion by conferring fitness benefit to their host (Stouthamer et al., 1999; Werren et al., 2008). The antiviral protection capability of *Wolbachia* was first identified in *Drosophila melanogaster* and anticipated to perform similarly in other medically important vectors (Hedges et al., 2008). These characteristics of *Wolbachia* gained scientific attention towards curbing vector population by utilizing life shortening strain of *Wolbachia* or those which directly interfere with the virus infection (Alam et al., 2011; Zhou and Li, 2016).

There is considerable variations within *Wolbachia* strains where single host can be infected with multiple strain and multiple host infected with same strains (Osei-Poku et al., 2012; Valette et al., 2013). This may affect the overall results as native strains may complement or interfere with the trans-infection studies. Incidence of horizontal transfer in closely related species are common among arthropods (Ahmed et al., 2016; Russell et al., 2009). Therefore, precise combination of strains and host are required for successful implementation of *Wolbachia* based bio control strategy to curb the mosquito population. It is imperative to conduct systematic screening of mosquito species for native infections.

This study aims to gauge our understanding on the aboriginal diversity of *Wolbachia* strains present within the mosquito species. Samples of mosquitoes are collected from different locations of Assam, India along with selected species maintained at institute's colony. *Aedes albopictus*, *Aedes aegypti*, *Anopheles stephensi, Anopheles hyrcanus, Anopheles annularis, Culex vishnui, Culex quinquefasciatus, Toxorhynchites splendens, Armigeres obturbans and* fly *Drosophila melanogaster* are the species incorporated to identify *Wolbachia* strains inhabit within them based on Wolbachia Surface Protein (*wsp*) gene.

2. Methods

2.1. Sample collection sites

Mosquitoes were collected from various locations of Dibrugarh, Tinsukia, Sivasagar and Tezpur districts of Assam, India. The collection areas with sampling GPRS coordinates are shown in the map. (Fig. 1)

2.2. Sample collection and identification

Adult mosquito species were included in the study. Mosquito species *Aedes albopictus* and *Aedes aegypti* were collected from Sivasagar, Dibrugarh, Tinsukia and Tezpur districts of Assam with BG-Sentinal Trap (Bio Gents GmbH (Germany)) using commercial human bait during day time where *Armigeres obturbans* was found mixed with Aedes species collection. *Culex quinquefasciatus* and *Anopheles stephensi* were obtained from RMRC, Dibrugarh mosquito colony. Species of Culex and Anopheles were obtained from dusk collection carried out in cattle shades from Tinsukia district using suction tube method. *Toxorhynchites splendens* were found in close proximity to Aedes habitat. They were collected during day time from tree trunks using suction tube methods. *Drosophila melanogaster* were attracted using sliced citrus fruits for collection purpose. Collected species were identified by applying standard morphological keys (Barraud, 1934; Christophers, 1933; Harbach, 2007) separated, pooled and stored accordingly.

2.3. DNA extraction, PCR amplification and sequencing

Total genomic DNA was extracted from whole mosquito using QIAGEN DNeasy Blood and Tissue Kit, Catalog.no: 69506 (Qiagen, Inc., Hilden, Germany) following manufacturer's instructions. Mosquito were grinded using Microtube Homogenizer from Sigma individually. DNA was eluted in 100 ul of elution buffer (buffer AE) as suggested in protocol.

2.4. PCR reaction

Insects were screened for Wolbachia strains based on *wsp* gene (Fig. 2A) sequence by PCR using group specific primers as described by Zhou (Zhou et al., 1998). The list of primers used for Wolbachia identification is provided in Table 1. Amplification was performed in 50 µl PCR reactions with 5 µl of extracted DNA, 25 µl of 2 X Master mix (Promega, USA), and 0.5 µM of each primer. Cycling conditions followed were: 95 °C for 5 min as initial denaturation followed by 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s with final extension at 72 °C for 10 min. Product were visualized on 1.5% Agarose gel for bands of expected size. Amplified, desired PCR products were purified using Qiagen gel extraction kit and sequenced bidirectional at SciGenom Labs Pvt. Ltd, Kerala, India using same set of primers. Sequences were analysed by bioinformatics tools such as BioEdit version 6, chromatograms were analysed for single and double peaks. Final processed and trimmed sequences were deposited in the GenBank (http://www.ncbi.nlm.nih.gov) database.

3. Results

3.1. Group specific identification of Wolbachia infections in mosquito samples and fly

Adult mosquito species were included in our study. Number of individual female mosquito processed for each species and the prevalence of *Wolbachia* is listed in Table 2. Study found *Aedes albopictus* mosquitoes collected from Sivasagar, Dibrugarh and Tinsukia districts are superinfected with *Wolbachia* A and B group with reported strains *Mel + AlbA* and *wAlbB* (Table 3). However, *Aedes albopictus* samples collected from Tezpur district are only infected with *wAlbA* strain (Fig. 2B). Our study reported for the first time natural *Wolbachia* infection in colonised *Anopheles stephensi* which has been maintained for years at this research institute. It was found to be superinfected with both A and B group whereas, *Anopheles hyrcanus and Anopheles annularis* collected from field areas did not show any possibility of Wolbachia infection (Table 3). Both *Armegeries obturbans* and *Culex vishnui* collected from Dibrugarh district presents infection with *wPip* strain of Wolbachia B group (Fig. 2C). The *Drosophila melanogaster* is found to be infected with Mel clade of supergroup and *Wolbachia pipientis* (Table 3). Sequence generated in this study were submitted to GenBank sequence database.

3.2. Phylogenetic analysis

Wolbachia wsp gene sequences obtained from the study were aligned using program package ClustalW. It includes fifteen number of sequence from this study and twelve number of *Wolbachia wsp* reference sequence from public repository NCBI (Fig. 3). Phylogeny revealed two clusters A and B representing A and B supergroup of *Wolbachia* (Fig. 3). Sequence obtained in the study showed 100% identity among themselves in both the clusters. Evolutionary analyses were conducted in MEGA6 (Tamura et al., 2013).

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model with a bootstrap value of 1000 (Tamura and Nei, 1993). Round symbols indicate sequences with accession numbers generated from this study. A discrete Gamma distribution was used to model evolutionary rate differences among site. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site.

4. Discussion

The results confirm the presence of *Wolbachia* infection in the following species; *Aedes albopictus*, *Culex vishnui*, *Anopheles stephensi, Armegeries obturbans, and Drosophila melanogaster*. It is interesting that