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Distribution of Wolbaichia strains in Stegomyia albopicta, Vector of dengue and chikungunya in Kerala, India

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Abstract

Wolbachia are a group of intracellular bacteria known to be prevalent among arthropods. Wolbachia infection cause declines of host populations, and also gives rise to host resistance to a wide range of pathogens. Over the past few decades, Wolbachia is used as a biological tool to control mosquito vectors. The study was aimed to identify Wolbachia strains in field collected St. albopicta from different breeding sites in Kerala. A total of 105 Samples were collected from 3 different areas with different breeding sites, areca nut planation in Kasaragod (North Kerala), rubber and pineapple plantation in Kottayam (Central Kerala) and tyres and containers in Thiruvananthapuram (South Kerala). The positive samples showing clear intensity were selected from general as well as strain specific samples for sequencing. The samples prior to sending for sequencing was extracted and purified using gel extraction kit and lyophilized. The sequences were aligned using ClustalW software. Then further phylogenetic analysis were made using MEGA 5 software. The positive samples were found to have 95% A(wAlbA) and B(wAlbB) super infection with 5% and 3% single infection of wAlbA and wAlbB strains respectively.

Distribution of Wolbaichia strains in *Stegomyia albopicta*, Vector of dengue and chikungunya in Kerala, India

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and lyophilized. The sequences were aligned using ClustalW software. Then further phylogenetic analysis were made using MEGA 5 software.

The positive samples were found to have 95% A(*wAlbA*) and B*(wAlbB)* super infection with 5% and 3% single infection of*wAlbA* and *wAlbB* strains respectively.

Key Words : Wolbaichia strains, *Stegomyia albopicta* , Dengue, Chikungunya, Rubber plantations, Kerala**.**

Introduction

Chikungunya virus (CHIKV), is transmitted to humans through*Stegomyia* mosquitoes. Kerala was the worst affected State in Indian during 2007 with a total of 55.8% of the reported Chikungunya fever cases in the country from the State (NVBDCP, India). Almost all the districts of Kerala were affected with the infection during 2006-2007. The factors attributed to this outbreak were attributed to crucial mutation "A226" acquired by the virus in 2007 and the abundance of the vector species *Stegomyia albopicta* in the region (Kumar,*et al* ., 2008). *Stegomyia aegypti* and*Stegomyia albopicta* , the vectors for CHIKV were widely distributed and abundant during the pre and post monsoon season. The prevailing climate, terrain and agricultural practices in these districts were highly conducive for the breeding of *St. albopicta and St.aegypti* , the vector of Chikungunya and Dengue in Kerala. The worst affected region was the mid-highland regions which harbour the vast rubber plantation sectors in the State. The hilly and semi-forested districts of Kottayam and Pathanamthitta were the most affected districts during 2007 epidemic, which constituted the major portion in the rubber plantation sectors of Kerala. About 63.0% of people living in the rubber plantation areas (Kerala contributes 80.0% of the rubber production in India), was afflicted with this disease (Kumar *et al* ., 2011), *St. albopicta* acted as main vector species (Kumar*et al* ., 2008).

Incidence of Dengue fever, another mosquito borne arbo-viral disease is also on an increasing trend in Kerala (Kumar *et al* ., 2013).*St. albopicta* (Skuse) 1894 (Diptera: Culicidae), the predominant*Stegomyia* species prevalent in Kerala was recorded as the vector species of both these arbo-viral diseases (Kumar *et al* ., 2008; Thenmozhi *et al* ., 2007). Innumerable Discarded Latex Collection containers (DLCCs), unused Fixed Latex Collection Containers (FLCCs), and tree holes, leaf axils of pine-apple plants and fallen leaves of areca-nut tree etc. were recorded the key breeding habitats of this species in rubber plantation sectors in Kerala (Kumar *et al* ., 2011; Sumodan, 2003) .

Dengue a vector – borne and emerging infectious disease is estimated to affect 50-100 million individuals each year in tropical and subtropical areas (Gubler, 2006; Halstead, 2007). Demographic and societal changes, such as population growth, unplanned urbanization, microevolution of the virus, climatic change and modern transportation, have greatly contributed to the increased incidence and geographical spread of Dengue virus infection in recent decades and distributed among about 120 countries globally. Dengue is caused by four serologically distinct types of Dengue virus. DENV-1, DENV-2, DENV-3 and DENV-4, belonging to the family *Flaviviridae* , genus *Flavivirus* . The viruses are transmitted to human beings through the bite of *Stegomyia*mosquitoes such as *St. aegypti* and *St. albopicta* which are considered as primary and secondary vectors of Dengue respectively (WHO, 2015) . The incidence of Dengue fever has increased significantly around the world in recent decades. About half of the global population lives under the risk of infection of this arbo-viral disease(WHO, 2009) .*St. albopicta* is becoming an increasingly important vector of Dengue because of its rapidly changing global distribution (Rezza, 2012) and it is generally believed to be a less efficient vector of arboviruses than *St.aegypti* . *St. albopicta* is highly adaptive and therefore can survive even in cooler temperate regions. Its spread to new areas is due to its tolerance to temperatures below freezing, hibernation and ability to shelter in microhabitats (WHO, 2015).

St. albopicta is (the Asian tiger mosquito), is an aggressive day-biting species known in different parts of the world including South East Asia (Russell *et al.,* 1969) , and Southern China (Qui e*t al.,* 1981) . It belongs to the East and Southeast Asia, where it was originally lived at the edges of forests breeding in tree hole, containers and other natural reservoir (Rezza, 2012) .Worldwide trade in second hand tires transported to various places, which often contain water could be an ideal place for eggs and larvae of these mosquitoes, has been a key factor in the large-scale conquest of *St. albopicta* in new areas which easily adapts to new

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environments, even in a temperate climate. This expansion is creating new opportunities for viruses to circulate in new areas, becoming a common cause of epidemics in *St. aegypti* -free countries from Hawaii to Mauritius. However*St. albopicta* is considered as an inefficient vector of Dengue because not well adapted to urban domestic environments and is less anthropophilic than *St.aegypti* .

The entire population of India lives under the risk of Dengue. In India the first case of Dengue has been reported from Kolkata (previously Calcutta) during 1963. The number of Dengue cases had been an increasing trend since 2001 and the maximum number of suspected cases (28055) has been recorded during 2010. In Kerala Dengue fever was first recorded in Kottayam District in 1997 (Kalra and Prasittisuk, 2004). This district continues to contribute the maximum number of cases of Dengue fever in the State, next to Trivandrum District in the south Kerala every year. Topographically, Kottayam District is semi-forested region with hills and hillocks located at the foothills of the Western Ghats. This district is the abode of rubber plantations in the country and grows rubber in about 60% of its net sown area under agriculture. *St. albopicta* the vector of Dengue fever, is abundant throughout the plantation belt of Kerala, including Kottayam District (Kumar *et al.* , 2011). All the four Dengue serotype are prevalent in the State, however, Dengue 2 and 3 are the major serotypes prevalent in Kerala State (Kumar *et al* ., 2013).

Rubber plantations in the region could be classified as immature ones and mature ones based on the age and productivity of the trees, broadly. Latex collection is carried out by placing hemispherical plastic containers of either 450ml/600ml/900ml capacity, attached to the bark of the mature trees about 7 years old. A small portion of bark of the tree is removed in a horizontal oblique manner by a sharp knife in an interval of 2-5 days and the latex oozing out is collected into the containers fitted to the tree. Also rain guards are fitted to the latex yielding section of the bark to prevent rainwater interfering with the tapping process and collection of latex. The latex oozing out of the scar is removed about 3 hours after making the cuts in the bark. The container is kept in the tree itself after latex collection in upright position to collect the residual latex.

In absence of effective vaccines or prophylaxis against most of the arboviruses, control of these vector borne diseases heavily rely on vector control. Community oriented Integrated Vector Management (IVM) strategies involving inter-sectoral collaboration is the best approach proposed for the same. However, during outbreak situations, usage of adulticidal measures using insecticides are being resorted to, towards rapid management of the outbreaks (WHO, 2008). Recently, studies have indicated that *Stegomyia species* when infected by the intracellular proteobacteria called *Wolbachia* limits pathogen replication, in particular the enveloped, positive single-stranded RNA viruses such as dengue (DENV), yellow fever (YFV) and chikungunya (CHIKV) (Moreira *et al* ., 2009; Bian *et al* ., 2010; Walker*et al* ., 2011). Based on this observation, a global campaign to eliminate dengue (http://www.eliminatedengue.com) was initiated to control dengue in countries such as Australia, Vietnam, Brazil, Colombia, Indonesia etc. by introduction of *Wolbachia* into the populations of these vector species. However, the mechanism of pathogen inhibition by *Wolbachia* is still being investigated, but this blocking phenomenon has been linked to priming of the host innate immune system and competition for limited resources between pathogens and*Wolbachia* (Caragata *et al.,* 2013). *Wolbachia* is found in many arthropods such as insects, mites and isopods and also in filarial nematodes. This natural insect symbiont is reported to be most concentrated in reproductive organs, but often have a wide tissue distribution within the host insect, including midgut and salivary glands. *Wolbachia* infection in *St* . *albopicta* was discovered by Wright and Barr in 1980 and is reported to be super infected with *wAlbA* and *wAlbB* strains. These strains are known to play a crucial role in dynamics, evolution and reproductive system of their host. Usually the arboviruses acquired through the blood meal must invade midgut cells and ultimately disseminate to the salivary glands to be transmitted, so that *Wolbachia* and the virus can potentially be present within the same cells. *Wolbachia* itself is not an infectious agent, but is maternally transmitted in the egg cytoplasm and is able to invade host populations by manipulating their reproduction. Hence, considerable scientific attention was directed at better understanding the impact of *Wolbachia* infections on the evolution and ecology of host populations (Charlat *et al* , 2003). Moreover, the self-spreading ability of *Wolbachia* makes it an attractive biocontrol agent to interrupt or reduce arbovirus transmission (Sinkins, 2013). However, the infection prevalence details of Wolbachia in natural populations of *St. albopictus* in endemic regions of the arbo-viral Diseases as Dengue and Chikungunya remains not well documented. Hence, we undertook a study

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to elucidate the natural infection status of this parasite in this vector species in a highly endemic state of Dengue Kerala.

Materials and methods

This investigation was carried out in Kerala, India in 3 areas, Thiruvananthapuram, Kottayam and Kasaragod. The sites were selected according to different nature of breeding habitats of *St. albopicta* in these areas. Also these sites were distributed to South Kerala, central Kerala and North Kerala. All these study sites were affected by arboviral diseases. *St. albopicta* was found to be abundant in all the study sites.

In the present study, field collected *St . albopicta* from different parts of the Kerala with distinct breeding sites were subjected to PCR amplification of the Wolbachia Surface protein(WSP) gene to determine the prevalence of this intracellular bacteria and further amplified using strain specific primers to confirm the strain. Also, selected amplified sequences were then be subjected to phylogenetic analysis.

Sample Collection

For the study, adults as well as larvae from the three study sites were collected. The larval and adult collections were made accordingly to known distinct breeding habitats in the study sites. The collected mosquito larvae and pupae were brought to the laboratory in plastic bottles and were reared to adults, at $28^{o}C \pm 1^{o}C$ temperature and 70-80% relative humidity, with a photo period of 12:12 light: dark conditions. The containers were labeled corresponding to their localities with date of collection.

Sample Identification

The adults collected were identified using keys Christophers, 1933; Barraud, 1934; Reuben *et al.,* 1994 and transferred to 1.5ml Eppendorf tubes and 50ul of 70% ethyl alcohol was added. The tubes were stored at -40degC until molecular analyses.

Molecular analyses

DNA Extraction and Polymerase Chain Reaction (PCR). Total DNA from individual mosquitoes was extracted following a modified method proposed by Collins et al. (1987). The DNA were pelleted, dissolved in water, and subjected to a phenol chloroform isoamyl extraction followed by chloroform. DNA were precipitated using NaAc/ethanol and dissolved in 50 µl of deionized water (Sigma-Aldrich, St. Louis, MO). The extracted and purified DNA were screened initially for Wolbachia infection by PCR using wsp gene, forward primer (81F, 5'-TGGTCCAATAAGTGATGAAGAAAC-3' and reverse primer 691R, 5'-AAAATTAAACGCTACTCCA-3'), which amplified about 600-700 bp product. This served as positive control for Wolbachia genotyping.

PCR conditions were as follows: An initial denaturation of 5 min ($95^{0}C$) was followed by ve cycles of $94^{0}C$ for 40 s (denaturation), $45^{0}C$ for 1 min (annealing), and 72 ^{0}C for 1 min (extension) and 35 cycles of $94^{0}C$ for 40 s (denaturation), $51^{0}C$ for 1 min (annealing), $72^{0}C$ for 1 min (extension), and a final extension at $72^{0}C$ for 10 min following a modified protocol of Hasebe et al. (2002). The $50^{0}C$ reaction included 1.5 U of *Thermus aquaticus* polymerase, 5 µl of 10 × PCR buffer, 2.5m Mmagnesium chloride, 2.5 µl of Q solution (QIAGEN GmbH, Hilden, Germany), and 0.5 µl of 10 pmol each of forward and reverse primers, along with the DNA of the mosquito species.

Wolbachia genotyping among the positive samples was performed by a multiplex PCR using Wolbachia strain specific primers; 328F/691R for wAlb A and 183F/691R for wAlb B. The PCR products obtained using general primers were diluted; 1µL in 99µL of sterilized sigma water prior to the PCR reaction. Similar to general PCR reaction, the volume/ reaction was calculated according to the number of samples to be processed, and once the MM is prepared, 48µL of it is added to tubes followed by 2µL of template DNA. A negative control was placed to determine whether the amplification. PCR cycling conditions Wolbachia genotyping are 95°C for 2 min , 35 cycles at 94°C for 1min, 55°C for 1min 30 sec, 72°C for 2mins, 1 cycle at, 72°C for 7 min, 4°C hold.

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DNA Sequencing and Analysis. The amplified fragments were run on a 1% agarose gel to check the integrity of the fragments and the PCR product was purified by QIAGEN GmbH PCR purification kit. The purified products were eluted to 20 µl of deionized water, and a portion of it was lyophilized in a Speed Vac concentrator (Thermo Electron Corporation, Waltham, MA) and was shipped to MWG (Lederberg, Germany/Microsynth, Balgach, Switzerland) for custom sequencing. Both reads (from forward primer as well as reverse primer) were done, and the sequences were analyzed as follows. The DNA sequences were subjected to alignment using ClustalW. Sequence divergences among individuals were quantified by using the Kimura two-parameter distance model (Kimura 1980). The phytogenic tree was constructed and genetic distance was calculated using MEGA 5.0. The neighbour-joining (NJ) method was followed using Kimura two-parameter (K2P) genetic distances (Kimura, 1980) to deduce the phylogenetic tree.

Results

All DNA extractions were done using adults 105, of which 69 were males and 36 were female. Among *Wolbachia* infection positive samples, 12 of them including 6 samples with general *WSP* primers and 6 samples with strain specific primers were then sequenced for confirmation of the strains amplified.

Among the positive samples 22 amplified products were sequenced (**Table 1**). The sequences were then aligned and subjected to phylogenetic analysis and further analyzed between and among the groups formed within the phylogenetic trees. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model **(** Tamura and Nei, 1993). The two trees with the highest log likelihood were constructed using Bootstrap method with 1000 replicates. The trees are drawn to scale, with branch lengths measured in the number of substitutions per site (next to the branches). The analysis involved 7 nucleotide sequences. Evolutionary analyses were conducted in MEGA 5.

The phylogenetic tree based on general amplified products as apparent, shown in **figure 1** , showed two major division designated A and B. The samples sequenced after general amplification from Thiruvananthapuram when analyzed with strain specific primers showed single infection with *walbA* strain as similar to the phylogenetic tree observed. As well as the sample from Kottayam showed single infection with B strains.

The strain specific samples showed two distinct groups showing*wAlbA* and *wAlbB* strain shown in **figure 2** . The genetic difference between the groups was found to be 0.301 (Kimura 2-parameter model. Kimura's two parameter model (1980) corrects for multiple hits, taking into account transitional and transversional substitution rates, while assuming that the four nucleotide frequencies are the same and that rates of substitution do not vary among sites. There was no gene flow between group A and group B (Nm = 0.00. (P value= 0.0011) A variable site contains at least two types of nucleotides or amino acids. Some variable sites can be singleton or parsimony-informative. The sequence analysis showed 4 singleton variable sites within the groups, with no parsimony informative sites. Also 1 segregating site was observed causing to have 2 haplotypes within each group. The nucleotide diversity was found to be similar in both A and B (0.00099).

The *Wolbachia* infection was detected in *St.albopicta* in all the 3 study sites having homology of superinfection with A and B groups. Then the selected samples representing the study sites were subjected to phylogenetic analysis. However the second and third study areas showed infection with single strains; Thiruvananthapuram, showed 5 samples with single infection of*wAlbA* strain and 3 samples in Kottayam with *wAlbB* strains. A total of 7 samples were found to be uninfected. The **table 2**shows the details of the *Wolbachia* infection status**, table 3** shows infection status among males and females.

Discussion

In the present study, field collected *St . albopicta* from different parts of the Kerala with distinct breeding sites were subjected to PCR amplification of *WSP* gene to determine the prevalence of this intracellular bacteria and further amplified using strain specific primers to confirm the strain. Also, selected amplified sequences will then subjected to phylogenetic analysis.

The study revealed higher percentage of *Wolbachia* infection; 93% of the 105 studied samples emphasizing the natural infection of*Wolbachia* in *St . albopicta* in Kerala, South India. The two *Wolbachia* groups found

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were genetically distant and there was no gene flow between *wAlbA* and *wAlbB* . Rather *Wolbachia* in higher densities would cover the way for exploiting this association for controlling vector mosquitoes of important arboviral diseases. The study was aimed at identification of *Wolbachia* strains in field collected *St. albopicta* from different breeding sites in Kerala. A total of 105 Samples were collected from 3 different areas with different breeding sites, areca nut planation in Kasaragod (North Kerala), rubber and pineapple plantation in Kottayam (Central Kerala) and tyres and containers in Thiruvananthapuram (South Kerala). The purified products were subjected to PCR amplification (**shown in Fig 3, 4, 5, 6, 7 and 8**), using *WSP* general primers (81F/ 691R). High prevalence and distribution of *Wolbachia* strains are currently being reported from different countries in Asian region including Korea and Malaysia (Park *etal.,* 2016; Joanne *et al.,* 2015). The importance of such baseline studies play a vital role, mainly due to the initiative on global campaign on eliminate dengue using *Wolbachia* as a biological control measure (Eliminatedengue.com, 2016). Similarly in India, studies in different states showed *Wolbachia* infection and more recently a focus on natural infection with genotyping of its strains in *St. albopicta* are reported (Park *et al.,* 2016; Joanne *et al.,* 2015). However, an earlier report by Pidiyar (2003) in Pune showed single infection with super group A forming a separate group within *wAlbA* strain.

The present study focused on identification of *Wolbachia* strains in *St . albopicta* from different breeding sites in Kerala. The findings of this study concur with other published results showing 93% positive *Wolbachia* infection. The A and B superinfection rate among positives sample was found to be 95%, and single strain infection of *wAlbA* and *wAlbB was* 5% and 3% respectively. Moreover, the negative or uninfected samples were recorded from Thiruvananthapuram (4 samples) where major breeding sources, mostly man made habitats. The samples may have been feeding from natural or synthetic bactericide containing food source causing the *Wolbachia* in them to perish in the wild. Besides, negative samples may indicate maternal transmission leakage of *Wolbachia*(Park *et al.,* 2016). Future studies should take this into consideration specifically studies focused on prevalence of *Wolbachia* in an area. In addition five samples among 31 positive samples from Thiruvananthapuram showed single infection with *wAlbA* strain; 16% within the study site samples. Similarly in Kottayam 9% of the samples were singly infected with *wAlbB*strain. Generally single infection points to incomplete cytoplasmic incompatibility, Armbruster *et al.,* 2003 conducted a study focused on 18 different populations and found 100% positive infection of *Wolbachia* , hence more extensive sampling has to be conducted within study sites before conclusions can be deciphered.

Phylogenetic analysis of *Wolbachia* strains based on the fast evolving *WSP* gene showed that the prevalent *Wolbachia*isolate was closely related with *Wolbachia* strains reported from different geographical locations of India, such as Pune and Odisha (Das*et al.,* 2014). Further analysis suggested that there was no difference between the strains observed from the field collected mosquitoes from different breeding sites, except for minor difference from two sites having single infection; in Thiruvananthapuram and Kottayam. The two strains belonging to group A and B, in *St. albopicta* ; *wAlbA* and *wAlbB* respectively, were found to be genetically distant (K2P=0.301) with no gene flow. There can be further evolutionary strain within the groups.

Furthermore, evolutionary theory predicts that, due to maternal inheritance, *Wolbachia* should evolve in a mutualistic fashion to increase the fitness of infected females (Turelli, 1994). Overall the effect of *Wolbachia* infection on insect fitness can be mutualistic, parasitic or neutral, or even a combination. As for *St. albopicta* , a laboratory studies of the mosquito *Wolbachia* -infected females lived longer and had more offspring than their uninfected counterparts (Dobson *et al.,* 2004, Dobson*et al.* , 2002) but these benefits are offset by an increased rate of death in infected larvae (Islam and Dobson, 2006). In addition intraspecific transfer of *Wolbachia* strains into *St. albopicta* was demonstrated by Xi *et al.,* (2006) and these strains as well as native strains in *St. albopicta* is known to modulate arboviral infection specifically CHIKV (Mousson*et al.,* 2012, 2010; Xi *et al.,* 2006). Further, few*St. aegypti* samples; 20 were subjected to PCR amplification

using *WSP* general primers (81 forward and 691 reverse), to determine if *Wolbachia* infection is present. But all the 20 samples including 8 males and 12 females were found to be uninfected with *Wolbachia.*

Conclusion

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The study demonstrated the detection of *Wolbachia* from field-collected *Ae. St . albopicta* in kerala, India. Totals of positive samples were found to have 95% A(*wAlbA*) and B*(wAlbB)* super infection with 5% and 3% single infection of*wAlbA* and *wAlbB* strains respectively, samples were determined to be infected with *Wolbachia* using the *WSP* and*16S* rDNA markers, respectively. Overall, 97 (93%) individual mosquito samples showed positive amplifications in both markers, indicating a low infection rate. Our study supports previous studies that the potential *Wolbachia* strain in *St .albopicta* belongs to supergroup B. In addition, other*Wolbachia* strains (e.g. supergroup A) could potentially infect this mosquito vector.

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Declaring of competing interest:

The authors declare that they have no known competing financial interests or personal relationships that they could have appeared to influence the work reported in this paper.

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