Original article

Distribution of *Aedes aegypti, Aedes albopictus* and *Culex sp.* and detection of *Wolbachia* among them in city district Lahore

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ABSTRACT

Background: Dengue is one of the important public health concerns world over is known to cause significant increase in overall disease burden in Pakistan. Since 2011, the disease has penetrated from urban to rural skirts of the most congested cities. A number of control measures have been tested but none of them is exhaustive. *Wolbachia* is most common parasite of arthropods and insects. Due to its heritable endosymbiosis relationship with mosquitoes, it has created interest among scientific community as a potential biological control agent. It infects wild dengue vector species and blocks the transmission of dengue virus through feminization, mutagenesis and parthenogenesis. In Pakistan, limited data is available regarding *Wolbachia* infestation among mosquito species therefore the present cross-sectional study was designed to detect the presence or absence of *Wolbachia* among wild mosquito species *(Aedes aegypti, Aedes albopictus* and *Culex sp.*) in the city district Lahore through Polymerase Chain Reaction (PCR) and to establish PCR model for easy detection of *Wolbachia* in mosquitoes.

Materials and methods: Mosquito traps were set randomly in different areas of Lahore. Total 725 mosquitoes were captured and segregated into 76 pools. Housefly and *Culex sp.* were used as a control. DNA was isolated and subjected to PCR. Data was analyzed using SPSS version 15.

Results: From total 725 mosquitoes, 564 were *Aedes aegypti*, 137 were *Culex sp.* and 24 were *Aedes albopictus*. *Aedes aegypti* and *Aedes albopictus* were all negative for *Wolbachia*. Only 3 out of 20 pools of Culex species were positive for *Wolbachia pipientis*. Housefly also showed none *Wolbachia* infestation.

Keywords:
Wolbachia, Dengue, *Aedes albopictus, Culex sp.*, Polymerase Chain Reaction

INTRODUCTION

Several mosquito species are responsible for transmission of serious diseases like Zika virus, Malaria, Chikungunya, Yellow fever, West Nile virus and Dengue fever. These diseases are among the major causes of fatality and morbidity[1,2] in many parts of the world including Australia, Sub-Saharan Africa, Southern Europe, Latin America Eastern Mediterranean countries and South-East Asia.[2]

At present dengue is the most important and notable disease threatening Pakistani population along with many other parts of the world. A staggering 390 million dengue infections take place annually of which 96 million are clinical cases.[3] In Pakistan, the first ever epidemic was confirmed in Karachi in 1994.[4] There was a dengue fever peak in 2011 particularly in Lahore and its suburban areas.[3,4] Total 21,685 cases inclusive of 17,610 cases from Lahore alone were notified followed by out breaks in Faisalabad, Rawalpindi, Sheikhupura and Peshawar during subsequent seasons.[3,4]

Dengue virus spreads through mosquito bites from infected to non-infected human beings mostly during summer and rainy season. Dengue is transmitted primarily by the mosquitos *Aedes aegypti* (*Ae. aegypti*) and by less efficient vector *Aedes albopictus* (*Ae. albopictus*). *Ae. albopictus* adapts easily to the human environment thus can exist in cooler regions as well.[5] It has caused dengue outbreaks in Japan, Indonesia, Thailand, Malaysia and Hawaii during past many years.[6]

Till the time effective vaccines against all strains of Dengue virus are developed and made available to developing countries like Pakistan, all possible resources are needed to be utilized to curb the disease.[1] Key interventions included removing larval breeding sites, spraying with pesticides including indoor residual spraying to kill or repel mosquitoes and use of personal protective clothing and insecticide treated nets.[5,7-9] Container management including water disposal and hygienic measures have also been applied with.[5,8]

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Table 1. Primers sequence and characteristics

Primers	Sequence	Characteristic
328F	5'CCAGCAGATACTATTGCG3'	Specific for *Wolbachia* AlbA
183F	5'AAGGAACCGAAGTTCATG3'	Specific for *Wolbachia pipientis* AlbB
Wsp81F	5'TGGTCCAATAAGTGATGAAGAAAC3'	Specific *Wolbachia* surface protein
691R	5'AAAAATTAAACGCTACTCCA3'	Reverse primer
12SArRNA	5'AAACTAGGATTAGATACCCTATTAT3'	Negative control against *Aedes albopictus*
12SBrRNA	5'AAGAGCGACGGGCGATGTGT3'	Negative control against *Aedes albopictus*

Wolbachia is a genus of intracellular (endosymbiont) bacteria in the family Rickettsiae.[8] It resides in many species of insects and other arthopodes including mosquitoes. It competes with other parasites for its proliferation. This makes it good candidate for biological control of vectors. The different mechanisms by which *Wolbachia* brings changes in host's reproductive machinery are parthenogenesis, feminization, male-killing and cytoplasmic incompatibility.[6,10-15] Different strains of *Wolbachia* have been detected in many species of mosquitoes. Reports of natural occurrence of *Wolbachia* in *Ae. aegypti* are few and have just began to emerge from Philipines[16], USA[17] and India[18], but non from Pakistan.

In Pakistan, limited data is available regarding *Wolbachia* infestation in local population of mosquitoes and other insects. This study was designed to see distribution of mosquito species and search for *Wolbachia* species in local population of mosquitoes. Detection of *Wolbachia* among local isolates is a first step to understand its transmission to the *Ae. aegypti*, a major vector of the dengue virus.

MATERIALS AND METHODS

Mosquitoes were collected from different union councils of Lahore city during August to October. The collected mosquito samples were brought to the laboratory of Medical Entomology and Parasitology Department of Institute of Public Health Lahore and were segregated species wise. Mosquitoes were exposed to -20°C to kill them and were later stored at the same temperature to halt the metabolism and hence prevention of lysis. Six samples of houseflies were also taken as a control and kept along with mosquitoes.

Mosquitoes were tested for *Wolbachia* strain A (*AlbA*) and *pipientis (AlbB)* through PCR. For this purpose, already described primers 328F[11] (*Wolbachia AlbA),* 183F[11] (for *Wolbachia pipientis/AlbB*), Wsp81F[11] (*Wolbachia* surface protein) and 691R[11] were used. Two primers against ribosomal RNA of *Ae. albopictus* were also used to act as negative control.[15] These were synthesized by Gene Link, Inc. 190 Saw Mill River Rd. Hawthorne, NY 10532, USA. The details of the primers are given in Table 1.

DNA extraction was done using FavorPrep Tissue Genomic DNA Extraction Mini Kit (catalogue # FATGK001, Favorgen® Taiwan). Three to five mosquitoes (approximately 25 mg) were used for each extraction. The extracted DNA was stored at -20°C. All the extracted DNA samples were amplified on GeneAmp (Applied Biosystems) at Center of Excellence in Molecular Biology (CEMB). The extracted DNA samples were amplified using 2X PCR Mix (Thermo Scientific, K1081). The PCR conditions for multiplex wAlb A and wAlb B genes were 95°C for 5 min, 95°C for 3 min, 50°C for 30 sec, 72°C for 1 min and 72°C for 2 min. Statistical analysis was carried out using SPSS version 15.0.

RESULTS

Total 725 mosquitoes were collected and divided into 76 pools according to union councils from where these were captured. Samples were collected from indoor, outdoors including different parks of Lahore city (Shalimar Garden, Race Course Park and Jallo Park). Each pool consisted of total 3-9 mosquitoes.

The collected mosquitoes were identified and segregated according to different species. Three species *Aedes aegypti, Aedes albopictus* and *Culex sp.* were identified. Of the mosquitoes collected 564/725 (77.7%) were *Ae. aegypti*, 37/725 (18.8%) were *Culex sp.* and only 24/725 (3.3%) were *Ae. albopictus.* The highest numbers of *Ae. aegypti* and *Culex sp.* were collected from Data Ganj Baksh Town, i.e., 129/564 (22.8%) and 42/137 (30.6%), respectively. The highest number (37.5%) of *Ae. albopictus* were collected from Allama Iqbal Town (Table 2). Two samples were collected from the Parks; Shalamar park, Shalamar town, Race Course Park in Data Ganj Baksh Town and Jallo Park.

The extracted DNA samples were subjected to species specific PCR. There were total 51 pools of *Ae. aegypti* and five pools of *Ae. albopictus* tested and none found positive for *Wolbachia* infestation. However,

Table 2. Distribution of mosquito species in each town of city district Lahore

Location	*Ae. aegypti*	*Ae. albopictus*	*Culex sp.*
Aziz Bhatti Town	36	0	13
Lahore Cantonment	12	0	16
Data Ganj Baksh Town	129	4	42
Gulberg Town	17	4	9
Iqbal Town	84	9	9
Nishtar Town	25	0	6
Parks Town	19	5	21
Ravi Town	34	1	11
Samanabad Town	51	0	1
Shalamar Town	81	0	5
Wahga Town	5	1	4
Total	564	24	137

three pools of *Culex sp.* out of 20 were found positive for *Wolbachia*. From this it was inferred that 15% of *Culex sp.* population is infected with *Wolbachia* in the Lahore region. The *Wolbachia* strain isolated from *Culex* species was wAlbB or *Wolbachia pipientis* (results were positive with 183F/691R primers) (Table 3; Figure 1). Three pools of *Culex sp.* which were positive for *Wolbachia* infestation were from Aziz Bhatti Town, cantonment area and one sample from the Parks.

DISCUSSION

Most of the preventive methods are directed towards the control of the dengue vector. Scientists are continuously trying to devise methods that can control both the vector as well as dengue virus.[10,11] *Wolbachia* transmission in dengue vector is introduced as a biological control method in Australia. Many studies have been conducted to detect the prevalence of *Wolbachia* in dengue vector species. This study aimed at detection of naturally occurring species of *Wolbachia* among local isolates of mosquito population of city district Lahore.

Wolbachia infections are more commonly reported in *Ae. albopictus*, however, only few reports exist for primary dengue vector *Ae. aegypti*.[11,16] Similarly, in the present study, total 564 *Ae. aegypti* were collected from different areas in city district Lahore and none was found infected with *Wolbachia*. Many studies have shown that *Ae. albopictus* population is heavily infected with *Wolbachia*. In Sri Lanka, 48.8% *Ae. albopictus* population had *Wolbachia* infection which is contradictory to this study.[11] Out of 24 *Ae. albopictus* samples, none were found to be PCR positive for *Wolbachia*. There might be a possibility that some other strain of *Wolbachia* is present in *Ae. albopictus* population for which different set of primers are

Table 3. Distribution of PCR positive *Wolbachia* pools from different towns of city district Lahore

Location	PCR Results		Total pools
	Positive	Negative	
Aziz Bhatti Town	1	4	5
Lahore Cantonment	1	6	7
Data Ganj Baksh Town	0	15	15
Gulberg Town	0	8	8
Iqbal Town	0	8	8
Nishtar Town	0	6	6
Parks Town	1	1	2
Ravi Town	0	7	7
Samanabad Town	0	7	7
Shalamar Town	0	6	6
Wahga Town	0	5	5
Total	3	73	76

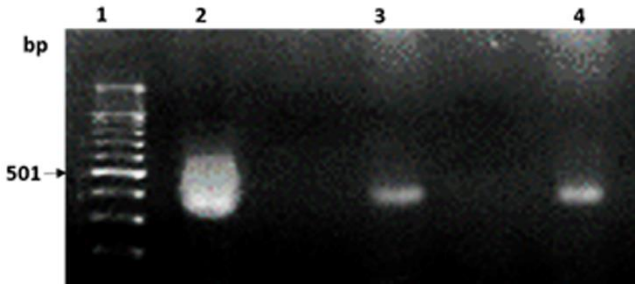

Figure 1. Agarose gel showing PCR positive results. Lane-1 shows DNA ladder, lane 2 to 4 shows amplified products

required to isolate the strain. There could be a possibility that the native *Ae. albopictus* population was negative for *Wolbachia*. One other reason could be that there is an anticipation of *Wolbachia* leakage high temperatures (wMel and wMelPop-CLA strains cannot pass from mother to progeny at temperature above 26°C) and most of the sampling was done during hot weather.[15] Many PCR tests were tried at different melting temperatures and different reagent concentrations but all the samples were found to be negative.

Total 6 housefly samples collected were also negative for *Wolbachia*. A study was conducted in Denmark in 2017 in which the scientists assessed the bacterial communities associated with housefly. The results showed a 42% prevalence of Campylobacter in houseflies whereas no *Wolbachia* or Rickettsiae family was reported positive in that study.[19] An another study indicated the presence of a small proportion of *Wolbachia* in housefly population.[20]

Culex sp. mosquitoes showed 15% prevalence of *Wolbachia* infection among the population in Lahore district. Out of 20 pools, 3 pools were positive for *wAlbB/ W. pipientis* strain.[11,21] Sri Lankan population showed 30% infection with *Wolbachia* which can be due to different climatic conditions as Sri Lanka has a humid weather.

Another possibility of none or low prevalence of *Wolbachia* in *Culex sp.* and other mosquitos could be attributed to the fact that there was a lot of indoor and outdoor spray activities performed in Lahore district since the dengue epidemic in 2011.[2,8,22] These vector killing sprays could have undesirable effects on untargeted *Wolbachia* population.

According to WHO, 23,019 lab confirmed cases of dengue were reported in Pakistan in 2011 which dropped to 3,668 in 2013 but after that a rising trend was seen again reaching the total number of 18,892 lab confirmed cases in the year 2017.[23] The early reduction could be either due to the active surveillance of the disease or could be the result of rigorous control measures and population awareness. But the increase in number of cases after 2013 evokes the point that there should be some promising method to control the disease. The results of this study mark first step towards understanding the biological control of dengue vector as characterization and search for *Wolbachia* present in *Culex sp.* or other mosquito species can be transmitted in *Ae. aegypti* population. Further research will help to characterize the local strains of *Wolbachia* and their efficacy toward eradication of dengue virus in Pakistan.

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