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A preliminary survey for *Wolbachia* and bacteriophage *WO* infections in Indian mosquitoes (Diptera: Culicidae)

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Abstract. Maternally inherited *Wolbachia* endosymbiotic bacteria are known to induce various kinds of reproductive alterations in their arthropod hosts. It has been proposed that this bacterium can be used as a tool for gene drive system in mosquitoes and also for the reduction of population size and modulating population age structure in order to reduce disease transmission. In the present study, we carried out a survey to determine the prevalence of *Wolbachia* and its phage *WO* infection in Indian mosquitoes and classified *Wolbachia* infection into groups A and B based on extensive polymerase chain reaction assay using *Wolbachia* specific *wsp* and *orf7* gene primers. Out of 20 field-caught mosquito species, eight species have shown to be infected. Singly infected with *Wolbachia* A was found in two species and B group found in four species, while double infection with AB group were found in two species. All the screened mosquito species with positive *Wolbachia* infection were also infected with phage *WO*. The knowledge of variation in *Wolbachia* and phage *WO* infection rates and inferred susceptibility to infection among different mosquito genera has fundamental implications for designing and successful application of *Wolbachia* based vector-borne disease control strategies.

INTRODUCTION

The vector-borne diseases are a cluster of infectious diseases mainly transmitted by mosquitoes. India's population suffers significantly in the form of morbidity and mortality from these diseases namely, malaria, lymphatic filariasis, Japanese encephalitis, chikungunya and dengue. At present, the application of insecticides as a primary strategy for controlling mosquitoes has led to resistance and environmental problems (Hemingway & Ranson, 2000). As a consequence, molecular biology and genomics tools are being applied to develop genetically engineered mosquitoes that are resistant to pathogen development, as an alternative vector control strategy. Recently, the ability of *Wolbachia* to spread through populations has generated an applied interest in using the symbionts as a mechanism to drive introduced transgenic traits into vector populations to control mosquito-borne diseases (Turelli & Hoffmann 1999; Rasgon *et al.*, 2003)

The genus *Wolbachia* is an alpha-proteobacterium closely related to the genera *Ehrlichia*, *Cowdria* and *Anaplasma*. *Wolbachia* have been found in many arthropods such as insects, mites and isopods and also in filarial nematodes (Stouthamer *et al.*, 1999; Bandi *et al.*, 1998; Weeks & Breeuwer, 2001). In some hosts, *Wolbachia* has ability to manipulate its host's reproduction including cytoplasmic incompatibility, parthenogenesis, feminization and male killing (Werren, 1997;

Stouthamer *et al.*, 1999; Puttaraju & Prakash, 2005a,b,c). Currently, *Wolbachia* is thought to be one of the most prevalent endosymbionts, infecting 15 to 76% of invertebrates (Werren *et al.*, 1995; Jeyaprakash & Hoy, 2000). This alpha-proteobacteria was first described by Hertig & Wolbach (1924) in the mosquito *Culex pipiens*. Later, Hertig (1936) named them *Wolbachia pipientis*. The most common effect of *Wolbachia* infection in mosquitoes is cytoplasmic incompatibility and was first described in *Culex pipiens* (Yen & Barr, 1971). When an infected male mosquito mated with an uninfected female mosquito of the same species, fertilization failed. This phenomenon is called cytoplasmic incompatibility. The *Wolbachia* bacterium comprises of eight super groups, A and B are found in arthropods, whereas C and D are found in filarial nematodes, group E in springtails, group F in termites and scorpions, group G in spiders and Group H in *Dipetalonema gracile* (Vandekerchove *et al.*, 1999; Lo *et al.*, 2002; Baldo *et al.*, 2007).

The exact mechanism by which *Wolbachia* induce cytoplasmic incompatibility are still unknown but several factors have been found to modulate cytoplasmic incompatibility strength (i.e egg hatchability) such as bacterial density and host genotypes (Weeks *et al.*, 2002). *Wolbachia* and incompatibility type has led to propose that genes responsible for *Wolbachia* incompatibility are conveyed by extra chromosomal particles such as phages (Guillemaud *et al.*, 1997). Identification of a bacteriophage in the *Wolbachia* of *Culex pipiens* mosquitoes was reported in late 1970's (Wright *et al.*, 1978), but confirmation of a *Wolbachia* phage did not occur until 20 years later, when a prophage region was identified in the genome of *Wolbachia* strain *w*Tai *Teleogryllus taiwanemma* crickets (Masui *et al.*, 2000). Screening of *Wolbachia* infections from a variety of invertebrate hosts indicate that phage *WO* is widespread in the genus (Gavotte *et al.*, 2007) and *WO* might provide some factors of importance to *Wolbachia* contributing to the reproductive alterations they induce in their hosts (Masui *et al.*, 2000).

Recent studies pertaining to *Wolbachia* transfection showed that the introduction of pathogenic *Wolbachia* strains reduce the fitness and shortens the lifespan of their hosts as compared to the uninfected mosquito vectors (Xi *et al.*, 2005a,b; McMeniman *et al.*, 2009). Since most of the screened mosquito species devoid of *Wolbachia* infection and being potential vectors, the present preliminary survey facilitates in identifying the niche to introduce and open new avenues for *Wolbachia* to be used as a potential biocontrol agent.

Although the diversity of *Wolbachia* infection in mosquito genera have been well investigated to certain extent in southeast Asia, Europe and Africa (Kittayapong *et al.*, 2000; Ricci *et al.*, 2002), there is a lack of such investigation in mosquitoes of India. To address this issue, this survey was carried out for the first time in Indian mosquito genera for *Wolbachia* and Bacteriophage *WO* infection. Knowledge of prevalence and variation of *Wolbachia*, *WO* infection rates and inferred susceptibility to infection among different mosquito genera has fundamental implications to design and successful application of *Wolbachia*-based vector borne disease control strategies.

MATERIALS AND METHODS

Collection of mosquitoes

Mosquitoes were collected across 12 various places of Karnataka, Hosur (Tamil Nadu) and Pondicherry in India between June and December 2009 (Table 1). Larvae were collected through dip sampling at appropriate habitats and adults were collected by aspiration from resting boxes in their natural habitats. The larvae collected were brought to the laboratory in plastic bottles and were reared to adults in the insectaria at 26°C ± 1°C temperature and 70-80% relative humidity, with a photo period of 12:12 light: dark conditions. The larvae were reared in round enamel pan by

feeding fish food and yeast tablet in tap water with corresponding label according to their localities until identified using keys (Christophers, 1933; Barraud, 1934; Reuben *et al.*, 1994). The reared adults were sorted to species, and stored at -80°C until processed for DNA extraction.

Extraction of DNA

The genomic DNA of mosquitoes was extracted using ZR Insect/Tissue DNA Kit-5™ (ZYMO RESEARCH, USA www.zymoresearch.com). Individual insect tissues (whole abdomen or ovaries) were ground in ZR BashingBead™ Lysis tube and homogenized in 600 µl Lysis Solution and DNA extracted according to the manufacturer's protocol. DNA was stored at -20°C until further used for polymerase chain reaction (PCR).

Screening for *Wolbachia* and Bacteriophage *WO* by polymerase chain reaction

Screening for *Wolbachia* was carried out through PCR using the group specific *wsp* primers for A group; (136A F5'-TGAAATTTTACCTCTTTTC-3 and 691R 5-'AAAAATTAAACGC TACTCCA-3') and for B group (81F 5'-TGG TCCAATAAGTGA TGAAGAAAC-3' 522 R 5'-ACCAGCTTTT GCTTGATA-3'), which were designed from the conserved regions of the outer surface protein gene to determine the type of infection of individual field collected mosquitoes (Zhou *et al.*, 1998). Amplification was done with PCR thermocycler (Eppendorf AG, Hamburg, Germany) using Hot Start *Taq* polymerase (5 Prime Eppendorf) and 1 or 2 µl of DNA sample in a reaction volume of 25 µl. The PCR condition followed for each step include 3 min at 95°C for the initial denaturation step followed by 30 cycles of 1 min at 95°C (denaturation), 1 min at 50°C (annealing), 1min at 72°C (extension) and 10 min at 72°C for the final extension. The PCR conditions for phage *WO* using *WO orf7* gene: (*WO orf7*F - 5'-CCC ACA TGA GCC AAT GAC GTC TG-3' and *WO orf7*R - 5'-CGT TCG CTC TGC AAG TAA CTC CAT TAA AAC-3') (Masui *et al.*, 2000) were 1 min at 95°C for the initial denaturation step followed by 35 cycles of 30 sec at 94°C (denaturation), 40 sec at 57°C (annealing), 1min 15 sec at 72°C (extension) and 10 min at 72°C for the final extension. The reaction mixture contained 10 µl 10X buffer (5 Prime Eppendorf), 3 µl 25 mM $MgCl_2$, 1.25 µl dNTPs (10 mM each), 1 µl 10 pmoles of both forward and reverse primers and 1 unit of *Taq* DNA polymerase (5 Prime Eppendorf). A negative control for the PCR assay (sterile distilled water instead of DNA in the reaction mixture) and positive specimens from a colony of *Wolbachia*-infected Uzifly, *Exorista sorbillans* (Prakash & Puttaraju, 2006, 2007) were used as a positive control to confirm the PCR amplification. Analysis of the PCR products was conducted by gel electrophoresis. Ten microliters of the PCR product was loaded onto 1.2% agarose gel containing ethidium bromide (0.5 µg/ml, GeNei™, Bangalore) and the amplicons were documented by gel documentation unit (Alpha Imager(R) EP, Canada). The size of the PCR product was determined using a 3-kb ladder (GeNei™, Bangalore).

RESULTS AND DISCUSSION

In total, 20 species belonging to 6 genera and 9 subgenera of field-caught mosquitoes were screened for *Wolbachia* infection by PCR assay using *Wolbachia* specific *wsp* gene primers. The infection status of each species is shown in Figure 1 and Table 1. Total, eight species (40%) showed positive for *Wolbachia* infection. In the genus *Aedes* 20% of five species, *Culex* 50% of eight species, *Armigeres* 100% of two species and *Toxorhynchites* 100% of one species screened for *Wolbachia* showed positive infections status, whereas genera *Anopheles* and *Lutiza* were not positive for *Wolbachia* infection. The *Wolbachia* positive members of *Aedes* and *Culex* included several medically important species involved in the transmission of chikungunya, dengue, Japanese encephalitis and human filariasis in India. Broad classification of *Wolbachia* into major group A and B showed that two of the

eight mosquito species were infected with A super group, four with B super group and two were doubly infected with AB super group in the genus *Aedes* and *Toxorhynchites* as shown in the Figures 2 and 3. Within infected mosquito genera, A, B and AB super group appear to be unequally distributed. There is no earlier report about *Wolbachia* infection in the eight mosquito species of Indian populations. However, 19% in Europe (5/26, discounting filarial *Wolbachia* infections) (Ricci *et al.*, 2002) and 28% (25/89) in Southeast Asian (Kittayapong *et al.*, 2000) mosquito species were tested positive for infection. In addition, 48% (12/25) of infected Southeast Asian species harboured multiple *Wolbachia* strains (Kittayapong *et al.*, 2000). The present survey revealed 40% of 20 Indian mosquito species harbour *Wolbachia* infection and of which *Wolbachia* infection is reported for the first time in *Toxorhynchites* species which was infected with both AB super group *Wolbachia*.

The incidence of phage *WO* infections among *Wolbachia* in natural populations of different mosquito species was determined based on PCR detection of *WO* putative minor capsid protein (*orf7*). The present study detected 100% infection of Phage *WO* in *Wolbachia* positive mosquito species as shown in Figure 4. The observations suggest that the phage *WO* might have transmitted vertically and was wide spread in infected mosquito species. The wide spread association of *Wolbachia* and phage *WO* means that the phage *WO* may be beneficially auxiliary to mosquito *Wolbachia*, as is found in various other phages/bacteria couples (Miao & Miller, 1999). Furthermore, according to Chauvatcharin *et al.* (2006) *Wolbachia*-associated bacteriophages may have played a key role in the evolution of these diverse bacterial genera of arthropods. *Wolbachia* are detected all over the world and spreading rapidly, by causing various reproductive alterations to their hosts, through which they can efficiently spread in the host populations (Werren *et al.*, 1995). It is probable that phage *WO* has associated with *Wolbachia* for a very long time, enabling *Wolbachia* to remain in insect hosts by producing some virulence factors. However, due to the lack of a genetic system to manipulate this bacterium, very little is known about molecular mechanisms that underlie the interaction of this agent with its host and phage *WO* can hopefully be used as a vector to transform these fastidious bacteria without any culture step as stated by Masui *et al.* (2000).

Wolbachia have been receiving much attention in recent years for its role as a tool for controlling economically important insect pests and disease vectors. *Wolbachia* induced cytoplasmic incompatibility mechanism is an useful tool for implementing population suppression and replacement strategies used in biocontrol (Brelsfoard & Dobson, 2009). In this direction, introduction of *Wolbachia* infection in previously uninfected vectors such as *Aedes aegypti* with *wMelPop* strains have shown potential implication in reducing the fitness of their hosts. It is encouraging to note that such transfection studies have high rate of maternal inheritance and this can be easily exploited in controlling mosquitoes through cytoplasmic incompatibility (McMeniman *et al.*, 2009)

Information about the presence/absence of *Wolbachia* infection in their host also encourages identifying their interaction with viral infections. Recent studies in this direction by Teixerira *et al.* (2008) suggest that *Wolbachia* infection may confer resistance to virus infection in their host, which gives a ray of hope in combating the vectors effectively. Previous investigations are also helpful in identification of *Wolbachia* infection and the reproductive phenotype they induce in their hosts, which are not previously studied or described.

The phenomenon of cytoplasmic incompatibility has made *Wolbachia* attractive as a potential gene driving system for the modification of insect vectors so that they cannot transmit disease (Sinkins & Gould, 2006). Since

Table 1. *Wolbachia* and Phage *WO* infection status in screened mosquito species collected from various places in India

Genus	Sub genus	Species	*Place of Collection*	*No. Tested*	*Wolbachia* Infection status	*WO* Phage Infection status
Aedes	*Stegomyia*	*Ae. aegypti*	Hosur (12°43¹N 77°49¹E)	24	–	–
		Ae. albopictus	Bangalore (12°58¹N 77°38¹E)	27	AB	+
	Fredwardius	*Ae. vittatus*	Chamarajanagar (11°56¹N 77°00¹E)	18	–	–
	Finlaya	*Ae. chrysolineatus*	Jog Falls (14°18¹N 74°55¹E)	10	–	–
		Ae. pseudotaeniatus	Shimoga (13°56¹N 75°38¹E)	13	–	–
Anopheles	*Cellia*	*An. stephensi*	Bangalore (12°58¹N 77°38¹E)	15	–	–
		An. culicifacies	Pondicherry (11°55¹N 79°49¹E)	12	–	–
		An. subpictus	Pondicherry (11°55¹N 79°49¹E)	11	–	–
Culex	*Culex*	*Cx. pseudovishnui*	Mandya (12°31¹N 76°53¹E)	19	–	–
		Cx. gelidus	Chickballapur (13°26¹N 77°46¹E)	09	A	+
		Cx. vishnui	Mandya (12°31¹N 76°53¹E)	13	B	+
		Cx. tritaeniorhynchus	Ramanagara (12°54¹N 78°02¹E)	16	–	–
		Cx. bitaeniorhynchus	Srirangapattana (12°41¹N 76°70¹E)	21	–	–
		Cx. sitiens	Hosur (12°43¹N 77°49¹E)	19	B	+
		Cx. quinquefasciatus	Mysore (12°18¹N 76°42¹E)	30	B	+
	Culiciomyia	*Cx. pallidothorax*	Chickmagalur (13°18¹N 75°49¹E)	15	_	_
Armigeres	*Armigeres*	*Ar. subalbatus*	Bangalore (12°58¹N 77°38¹E)	24	A	+
		Ar. kesseli	Sringeri (13°25¹N 75°15¹E)	21	B	+
Lutzia	*Metalutzia*	*Lu. fuscana*	Pondicherry (11°55¹N 79°49¹E)	16	_	_
Toxorhynchites	*Toxorhynchites*	*Tx. splendens*	Udupi (13°20¹N 74°45¹E)	12	AB	+

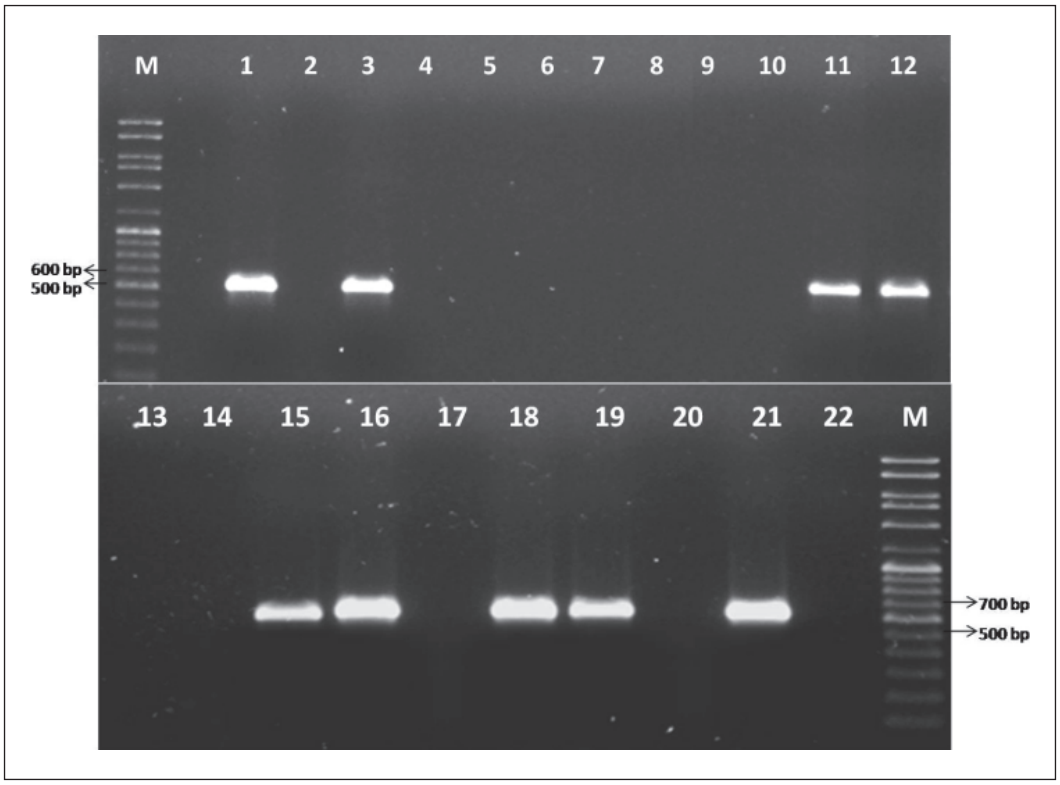

Figure 1. *Wolbachia* specific *wsp* general primer that amplifies at around 600 bp from mosquito species of India. Lane M – molecular weight marker, Lane 1 & Lane 22 – positive and negative controls respectively. Lane 2 to 21, mosquito species as shown in Table 1

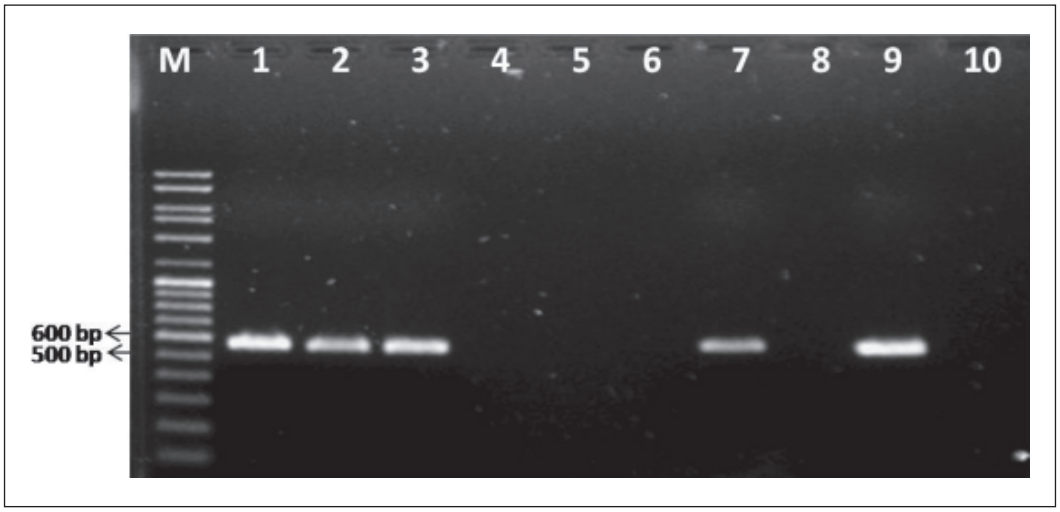

Figure 2. *wsp* A supergroup of *Wolbachia* specific primer that amplifies around 550 bp. Lane M – molecular weight marker; 1 - positive control; 2 – *Ae. albopictus;* 3 – *Cx. gelidus;* 4 – *Cx. vishnui;* 5 – *Cx. sitiens;* 6 – *Cx. quinquefasciatus;* 7 – *Ar. subalbatus;* 8 – *Ar. kesseli;* 9 – *Tx. splendens;* 10 – negative control

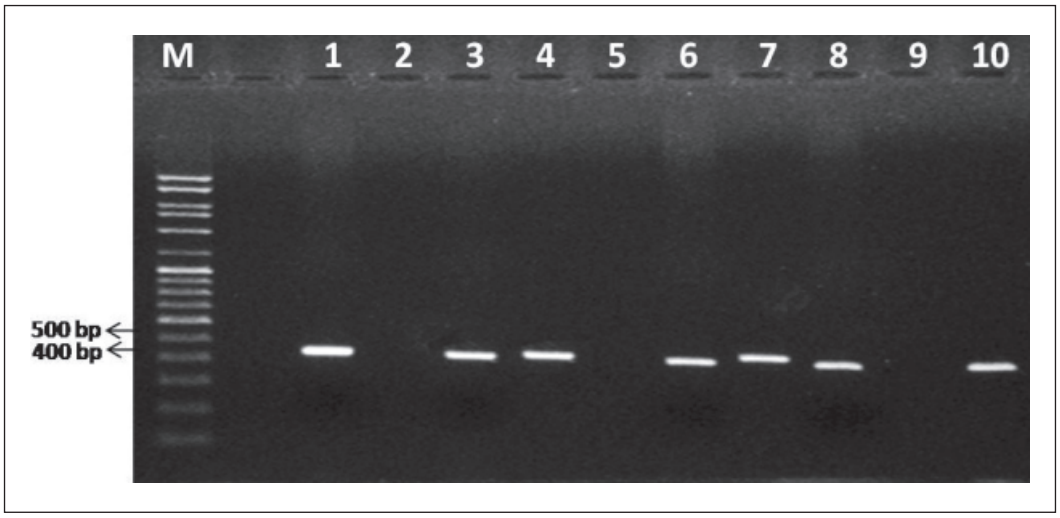

Figure 3. *wsp* B supergroup of *Wolbachia* specific primer that amplifies around 450 bp. Lane M – molecular weight marker; 1 – *Ae. albopictus;* 2 – *Cx. gelidus;* 3 – *Cx. vishnui;* 4 – *Cx. sitiens;* 5 – *Ar. subalbatus;* 6 – *Cx. quinquefasciatus;* 7 – *Ar. kesseli;* 8 – *Tx. splendens;* 9 – negative control; 10 – positive control

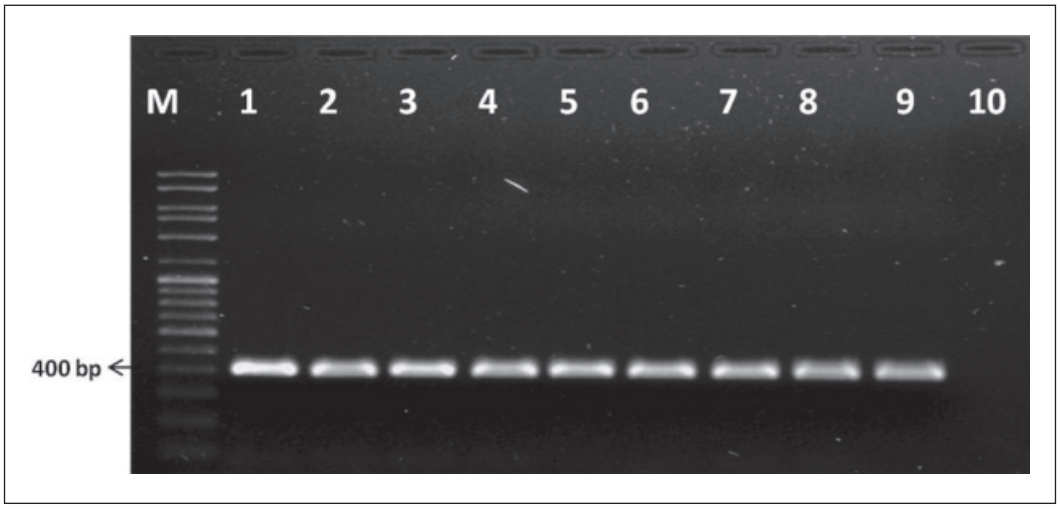

Figure 4. *WO* phage of *orf7* gene that amplifies at around 400 bp. Lane M – Molecular Weight Marker; 1 – positive control; 2 – *Ae. albopictus;* 3 – *Cx. gelidus;* 4 – *Cx. vishnui;* 5 – *Cx. sitiens;* 6 – *Cx. quinquefasciatus;* 7 – *Ar. subalbatus;* 8 – *Ar. kesseli;* 9 – *Tx. splendens;* 10 – negative control

Wolbachia infections cause cytoplasmic incompatibility leading to few or no offspring in mosquitoes, there is considerable interest in using *Wolbachia* for biological control of mosquitoes to reduce the reproductive potential of mosquito species. An implementation programme of this magnitude requires such preliminary survey for identifying the infection status of vectors. So the strategies could be devised to use both naturally occurring infection and genetically modified *Wolbachia* strains for biocontrol programmes.

The present study reveals that species of *Aedes albopictus*, *Culex vishnui*, *Culex quinquefasciatus*, *Armigeres*

subalbatus were infected with *Wolbachia*, whereas, *Aedes aegypti*, *Anopheles stephensi*, *Anopheles culicifacies*, *Culex pseudovishnui*, *Culex tritaeniorhynchus* and *Culex bitaeniorhynchus* are free from *Wolbachia* infection. All the mosquitoes which are free from *Wolbachia* are also of primary importance as vectors in India and this offers a potential advantage for the application of *Wolbachia* for the genetic control of these disease vectors because these species represent an empty niche into which *Wolbachia* could be experimentally introduced. Through this preliminary survey naturally occurring *Wolbachia* infection types in Indian mosquitoes have been identified. However, *Wolbachia* infection in other mosquito species needs to be investigated to understand the variation in *Wolbachia* infection. Such studies will provide basic descriptive information to devise experimental strategies by exploiting a *Wolbachia*-cytoplasmic incompatibility based mechanism to control vectors.

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