Original Article

Detection of a New Strain of *Wolbachia pipientis* in *Phlebotomus perfiliewi transcaucasicus*, a Potential Vector of Visceral Leishmaniasis in North West of Iran, by Targeting the Major Surface Protein Gene

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Abstract

Background: *Wolbachia pipientis* is maternally inherited endoparasitic bacterium belonging to the α-proteobacteria, infecting 20–75% of all insect species including sand flies. The *Wolbachia* surface protein (*wsp*) was employed as an appropriate marker for strain typing. The objective of our research was to find the possibility of detection of *W. pipientis* in *Phlebotomus perfiliewi transcaucasicus.*

Methods: Individual sand flies were screened for the presence of *W. pipientis*. The obtained sequences were edited and aligned with database sequences to identify *W. pipientis* haplotypes.

Results: Two haplotypes of *W. pipientis* were found in *P. perfiliewi transcaucasicus.* The common haplotype of *W. pipientis* was found to be identical to the sequences of those submitted in GenBank. New strain (haplotype) of *W. pipientis* was found novel. The sequence of new strain of *W. pipientis* occurs in *P. perfiliewi transcaucasicus* is very different from those already submitted in GenBank.

Conclusion: Finding one genetically modified new strain of *W. pipientis* in *P. perfiliewi transcaucasicus,* now we can conclude that further documents and studies need to reach the role of cytoplasmic incompatibility of *W. pipientis* through wild sand fly populations to drive a deleterious gene into and to reduce the density of natural populations of sand flies.

Keywords: *Wolbachia pipientis*, *Phlebotomus perfiliewi transcaucasicus*, *Leishmania infantum*, Kala-azar, Iran

Introduction

Phlebotomus perfiliewi transcaucasicus transmits 'infantile visceral leishmaniasis' (IVL) in northwest of Iran to the east of Turkey which has a Mediterranean climate (Nadim et al. 1978, Parvizi et al. 2008, Franco et al. 2011, Mahamdallie et al. 2011). The intracellular *Rickettsia*-like bacterium *Wolbachia pipientis* Hertig has been detected in phlebotomine sand flies (Diptera: Psychodidae, Phlebotominae) using PCR to amplify a fragment of the major *Wolbachia* surface protein (*wsp)* gene (Zhou et al. 1998, Cui et al. 1999, Ono et al. 2001, Benlarbi and Ready 2003, Kassem et al. 2003, Parvizi et al. 2003).

Wolbachia, are gram negative, polymorphic and wide spread bacteria belonging to the family Anaplasmataceae within the order Rickettsiaceae, related to α-proteobacteria that infect reproductive tissues of many arthropods and nematodes. In addition, these bacteria have been found in approximately 80% of insect species all over the world (Zhou et al. 1998, Cui et al. 1999, Benlarbi and Ready 2003, Kassem et al. 2003, Parvizi et al. 2003).

Wolbachia are unique endosymbionts that is maternally inherited, intracellular *Rickettsia* like bacteria which spread themselves to next generation, by transovarian transmission

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(Weeks et al. 2002, Benlarbi and Ready 2003). This transmission is known as vertical transmission. *Wolbachia* has been implicated in causing reproductive manipulations on its hosts. They induce a number of reproductive abnormalities that appear in their host phenotypes including cytoplasmic incompatibility (CI), parthenogenesis, feminization, male killing (Curtis and Sinkins 1998, Cui et al. 1999, Ono et al. 2001, Weeks et al. 2002).

Cytoplasmic incompatibility is a common observed phenotype of *Wolbachia*, when infected populations of same species cross to each other, results may appear to be signs of incompatibility, unidirectional incompatibility or bidirectional incompatibility that can be completed or partial (Curtis and Sinkins 1998, Kassem et al. 2003).

In case of an infected male mates with an uninfected female (or infected with another strain of *Wolbachia*, this cross is incompatible and no offspring will be produced (Unidirectional CI). In case of male and female populations are infected with more than one strain of *Wolbachia*, Bidirectional incompatibility will be observed (Braig et al. 1998, Werren 1998, Cui et al. 1999, Weeks et al. 2002, Kassem et al. 2003).

There are four known forms of parthenogenesis that have been observed in different types of insect species (Thelytoky, Pseudogamy, Automixis, Apomixis). They have different aspects but there was the same one in which female individual produces offspring without participation of male partner in mating. Parthenogenesis in insects can cover a wide range of mechanisms. W*olbachia* induces a particular type of parthenogenesis in some species, called Thelytoky Parthenogenesis. By this action, *Wolbachia* causes duplication in gametes in some insects therefore the resulting offspring would be all *female*, they also carry the *Wolbachia* infection (Anonymous 1997).

In addition, *Wolbachia* causes embryonic mortality of male zygotes in some arthropods. In addition, *Wolbachia* has horizontal transmission between different species of arthropods (O' Neill et al. 1992, Breeuwer and Jacobs 1996, Braig et al. 1998). This transmission has been observed in groups of parasitic wasps that they were not infected with *Wolbachia* naturally, due to predating and feeding from a species of *Drosophila*, after a short period of time within the host cells of mentioned wasps, they have become infected with the same strain that was present in *Drosophila* species (Rousset and Solignac 1995).

In addition, *Wolbachia* was found in Isopods, mites and nematodes. The following reasons can describe research on *Wolbachia*: the wide spread of bacteria, manipulations on its hosts and its role to make speciation, the affect of bacteria of host's fertility and being a potential natural enemy or a vector. Useful genes can be synthesized by genetic engineering and then transferring both into *Wolbachia* and insect populations for biological control which might cause decreasing arthropod transmitted diseases in human as a secondary reservoir (Curtis and Sinkins 1998, Turelli and Hoffmann 1999).

The selection of *Wolbachia* surface protein (*wsp*) gene has been due to free availability of this protein at the surface of the bacteria, ease of its identification, diversity of this gene in various genus and the possibility of usage of this gene for the purpose of studying the evolutional relationship and phylogenetic proximity of these groups of bacteria (Werren 1997, Bandi et al. 1998, Werren 1998, Sinkins and O'Neill 2000).

The *Wolbachia* surface protein (*wsp*) gene is useful marker for strain typing (Stouthamer et al. 1999, Baldo et al. 2006). There are no reports of the bacterial *wsp* gene being isolated and being sequenced from the same individual specimens of *Phlebotomus perfiliewi transcaucasicus*, in order to investigate the number of strains of *W. pipientis* infecting wild populations of this insect. We have

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reached to beneficial consequences. This is now reported of *P. perfiliewi transcaucasicus* from an endemic of (IVL) in Iran, and it is an essential piece of information for detecting *W. pipientis* through the wild populations of this sand fly (BenIsmail et al. 1987, Seccombe et al. 1993, Benlarbi and Ready 2003, Parvizi et al. 2009).

Materials and Methods

Sand flies were collected from three locations of Sarab and Kaleybar in the northwest of Iran in Azerbaijan Province as well as Meshkin Shahr in Ardabil Province using CDC traps and sticky papers. Sand flies were dissected, head and genital termination were kept for identifying of species based on morphological characters, thorax and abdomen were stored at -80 °C until further operations for DNA extraction followed PCR assays (Parvizi et al. 2003, Parvizi and Ready 2008).

About 550 base-pairs (bp) (minus primers) of the *wsp* fragment of *Wolbachia* Surface Protein were amplified by PCR using the primer pair *wsp* 81F (Forward) (5´TGGTCCAATAAGTGATGAAGAAAC 3´) and *wsp* 691R (Reverse) (5´AAAAA TTAAACGCTACTCCA3´), PCR amplification was carried out according to the protocol of Benlarbi and Ready (2003).

A 20µl PCR reaction mixture consisted of 2µl 1x Promega buffer, 2µl MgCl2, 0.5µl of each dNTP, 1µl of each primer, 0.2µl Taq DNA polymerase (Promega) and 2µl of sand fly genomic DNA. The PCR amplification was carried out with the following thermal profile using a GeneAmp® PCR System 9700 thermal cycler (PE Applied Biosystems): 2 min. denaturation at 94 °C, 35 cycles of denaturation at 94 °C for 30 sec., annealing at 50 °C for 30 sec, extension at 72 °C for 1.5 min, and a final extension at 72 °C for 10 min (Benlarbi and Ready 2003, Parvizi et al. 2003).

After amplification, the samples were fractionated by horizontal submerged gel electrophoresis, using 1.5% agarose gels and DNA size markers (Promega PCR markers G316A, or Bioline Hyper ladder IV). DNA fragments were visualized by ethidium bromide staining, then excised and purified using a Geneclean II Kit (BIO 101 Inc) before cycle sequencing each strand. The sequences obtained were edited and aligned with database sequences using Sequencher TM v. 3.1 software (Gene Codes Corp.) to identify unique sequences (=haplotypes), which were analyzed phylogenetically using PAUP* software (Swofford 2002).

Results

Five species of *Larrossius* subgenus and six species of *Adlerius* subgenus identified from three locations of Sarab, Kaleybar in north eastern of Azerbaijan province and Meshkin Shahr in Ardabil Province in northwest of Iran. Majority of sand fly species identified in these locations belonged to *Phlebotomus* and *Paraphlebotomus* subgenera and *Sergentomyia* genus. Abundance of some species of *Larrossius* subgenus and *Adlerius* subgenus were very low but due to their importance in IVL transmission we tried to detect *wsp* gene of *Wolbachia* to all sand fly species (Table 1).

In total 12 out of 183 sand flies were found infected with *Wolbachia* including 11 out of 41 *P. perfiliewi transcaucasicus and* one *P. kandelakii.*

Two out of seven *P. perfiliewi transcaucasicus* were found infected with *wsp* gene in Sarab region in north east of Azerbaijan Province. Seventy three sandflies species belonged to other *Larrossius* and *Adlerius* species were examined for *Wolbachia* but no positive specimen was found for *wsp* gene in this focus.

Nine out of 24 *P. perfiliewi transcaucasicus* were found infected with *wsp* gene in Kaleybar region in north east of Azerbaijan Province.

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Twenty two sandflies species were belonged to other *Larrossius* and *Adlerius* species which were tried to detect *Wolbachia* but no positive *wsp* gene was found in this region.

Three of *P. perfiliewi transcaucasicus* were examined but no infection found with *wsp* gene in Meshkin Shahr in Ardabil Province in North West of Iran. One out 26 *P. kandelakii* was found infected with *wsp* gene in Meshkin Shahr in Ardabil Province but the band of PCR product in agarose gel was too weak to sequence.

In total (12 /183) 6.5% of all screened sand flies were positive with *Wolbachia wsp* gene that (2/81) 2.5% of these specimens were male and (10/102) 9.8% were female (Table 2).

Only 9/11 (80%) positive PCR products of *Wolbachia wsp* gene in *P. perfiliewi transcaucasicus* contained enough DNA for direct sequencing. One haplotype of the *wsp* gene were recognized by aligning of new sequences comparison with homologous ones from GenBank. The common haplotype of *W. pipientis* was found to be identical to the sequences of those submitted in GenBank (GenBank accession number EU780684), and it predominated in Iranian sand flies infected with this species (7/11 infections). New strain of *W. pipientis* (GenBank accession number (JX 488735) was found novel (2/11 infections). This new strain differs pairwise by 36 to 120 bp nucleotide positions from those haplotypes of *W. pipientis* submitted in GenBank (GenBank Accession Numbers AF237882, AY 288297, HM563686, HM775090) (Fig. 2).

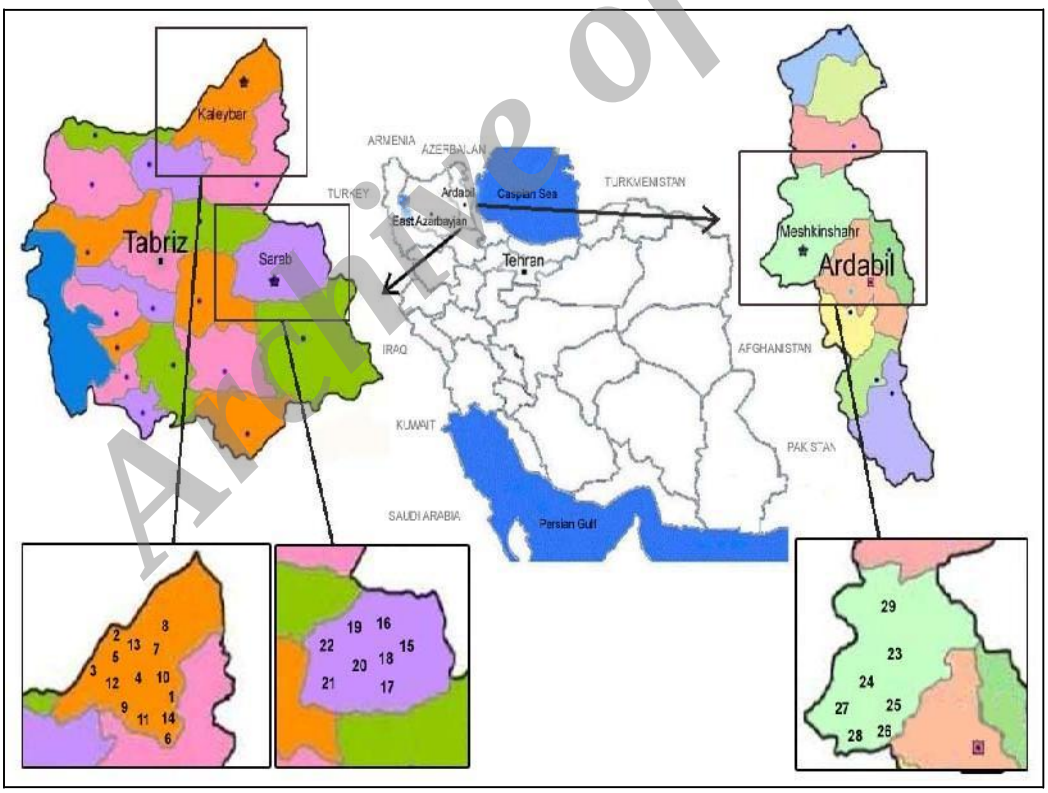

Fig. 1. Locations of Iranian provinces, cities and villages where *Larrossius* and *Adlerius* group sand fly species were sampled. (Villages: 1- Sheghlan, 2- Bastam lou, 3- Safar lou, 4- Aslanbagh lou, 5- Sarma lou, 6- Aylily, 7- Shekhm lou, 8- Olou gheshlagh, 9- Oliurdy, 10- Abdolrazagh, 11- Aghamir lou, 12- Molan, 13- Jou aghaj, 14- Ajoudan abad, 15- Agh miun, 16- Sahzab, 17- Hasan jan, 18- Sanzigh, 19- Razligh, 20- Ghalajough, 21- Dowlat abad, 22- Arzanagh, 23- Alni, 24- Ourkandi, 25- Mizan, 26- Mouyil, 27- Ghassabeh, 28- Aghbolagh, 29- Ghourt tappeh)

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Table 1. *Wolbachia* infections in two subgenus species using *wsp* gene in three endemic visceral leishmaniasis locations in North West of Iran

Province / Region		Subgenus	*Larrossius*							*Adlerius*					
		Region Species	*P. kandelakii*	*P. tobbi*	*P.perfiliewi transcaucasicus*	*P. keshishiani*	*P. major*	*P. simici*	*P. brevis*	*P. halepensis*	*P. longidoctus*	*P. balcanicus*	*Chinensis Group*	Total
East Azarbayejan Province	Sarab	Ghalajough	5		2			2	1				3	13
		Razligh			2			2		6				10
		Sahzab	1		6 (2+ve)									7 (2+ve)
		Sanzigh					2			3			1	6
		Agh miun	1					3						4
		Arzanagh	1	5						5			6	17
		Hasan jan	2		2								4	8
		Dowlat abad	4		2					7		1	1	15
		Total	14	5	14 (2+ve)	0	2	7	1	21	0	1	15	80 (2+ve)
	Kaleybar	Sheghlan	2		1						1		1	5
		Bastam lou			1									1
		Safar lou			1									1
		Aslanbagh lou	3		4									7
		Sarma lou	1		1									2
		Aylily			6 (4+ve)									6 (4+ve)
		Shekhm lou			1 (1+ve)								3	4 (1+ve)
		Olou gheshlagh											4 (2+ve)	4 (2+ve)
		Oliurdy	2										1	3
		Abdolrazagh			4 (2+ve)								2	6 (2+ve)
		Aghamir lou	1											1
		Molan	1		2								1	4
		Jou aghaj											1	1
		Ajoudan abad											1	1
		Total	10	0	24 (7+ve)	0	0	0	0	0	1	0	11 (2+ve)	46 (9+ve)
Ardabil Province	Meshkin shahr	Alni								1	3		2	6
		Ourkandi	2								3		5	10
		Mizan	3				5			3				11
		Mouyil	9											9
		Ghassabeh	2		1									3
		Aghbolagh	4 (1+ve)		2									6 (1+ve)
		Ghourt tappeh	6							3			3	12
		Total	26 (1+ve)	0	3	0	5	0	0	7	6	0	10	57 (1+ve)
Total		29 Village	50 (1+ve)	5	41 (9+ve)	0	7	7	1	28	7	1	36 (2+ve)	183 (12+ve)

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P. papatasi (EU780683)   AGCTACTACGTTCGTTTGCAATACAACGGTGAATTTTTACCTCTTT-TCACAAAAGTTGA  59
P. perfiliewi            AGCTACTATGTTCGTTTGCAATACAACGGTGAAATTTTACCTCTTTAT-ACAAAAGTTGA
                         ********.************************.************.*.***********

P. papatasi (EU780683)   TGGTGCTACAGGTGCTAAGAAGACTGCAGATACTGCTACAACTACT-GACCTTTATA-AA  117
P. perfiliewi            TGGTATTACAAATG-TAA-CAG---GTA-A-A--G--AAAAGGA-TAGTCCCTTA-ACAA
                         ****..****..**.***..**...*.*.*.*..*..*.**..*.*.*.**.***.*.**

P. papatasi (EU780683)   GCTTCTTTTATGGCTGGTGGTGGTGCATTTGGTTATAAAATGGACGACATCAGGGTTGAC  177
P. perfiliewi            G-ATCTTTTATAGCTGGTGGTGGTGCATTTGGTTATAAAATGGACGACATTAGAGTTGAT
                         *..********.************************************.**.*****.

P. papatasi (EU780683)   GTTGAAGGGCTTTATTCGCAGCTAAGC-AAGGATGCA-CTTGCT-GTAGCTCCTACTCCA  234
P. perfiliewi            GTTGAAGGGCTTTACTCACAATTG-GCTAAAGATACAGCT-G-TAGTAAATACTTCTGAA
                         **************.**.**..*..**.**.***.**.**.*.*.***..*.**.**..*

P. papatasi (EU780683)   GCAA-T-T-GCAGACAGTTTAACAGCAATTTCAGGGCTAGTTAACGTTTATTACGATATA  291
P. perfiliewi            ACAAATGTTGCAGACAGTTTAACAGCATTTTCAGGATTGGTTAACGTTTATTACGATATA
                         .***.*.*.*****************.*******..*.*********************

P. papatasi (EU780683)   GCAATTGAAGATATGCCTATCACTCCATACATTGGTGTTGGTGTTGGTGCAGCATATATT  351
P. perfiliewi            GCGATTGAAGATATGCCTATCACTCCATACGTTGGTGTTGGTGTTGGTGCAGCATATATC
                         **.*************************.*****************************.

P. papatasi (EU780683)   AGCACA-CCTTTGGCAACTGCTG-TG-AGT-A--G-TCAAAATGGTAAATTTGCTTTTGC  404
P. perfiliewi            AGCA-ATCCTT---CAAAAGCTGATGCAGTTAAAGATCAAAAAGG-A--TTTGGTTTTGC
                         ****.*.****.:.***.*.****.**.***.*..*.******.**.*..****.******

P. papatasi (EU780683)   TGGTCAAGCAAGAGCTGGTGTT        426
P. perfiliewi            TTATCAAGCAAAAGCTGGTGTT
                         *..********.**********
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Fig. 2. Alignment of the single *wsp* gene sequence of *W. pipientis* isolated from Iranian *P. papatasi* with the GenBank sequence EU780683 reported by Parvizi et al. (2009). Nucleotide differences are marked by a point, not a star

Table 2. *Wolbachia* detection in sand flies screened by PCR using *wsp* gene in North West of Iran (+ve = *Wolbachia* positive)

Location	No. female sand flies screened by PCR	No. +ve female sand flies	+ve female sand flies%	No. male sand flies screened	+ve male sand flies	+ve male sand flies%	Total sand flies	Total +ve sand flies	Total +ve sand flies %
Kaleybar	39	9	23.1	7	0	0	46	9	19.6
Sarab	29	0	0	51	2	3.9	80	2	2.5
Meshkin Shahr	34	1	2.9	23	0	0	57	1	1.7
Total	102	10	9.8	81	2	2.5	183	12	6.5

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Discussion

Recently, *wsp* gene has been used to improve phylogenetic resolution within the species clade of *W. pipientis*, which was divided into four groups (A–D) and 12 subgroups (Zhou et al. 1998, Ono et al. 2001). The groups A and B are concordant with those identified by 16S rDNA for the strains of *W. pipientis* from insects, mites and crustaceans, whereas groups C and D harbor the strains from filarial nematodes. Populations of *P. perfiliewi transcaucasicus* from Iran were screened and for the first time, *W. pipientis* were found in this sand fly. Two haplotypes including, one new haplotype were obtained from *wsp* gene (Fig. 2).

The common widespread strain (GenBank ID: EU780683, AY288297) was the A-group strain of *W. pipientis* (wPap) but the new haplotype was indistinguishable from that of the A-group strain of *W. pipientis* (wPap) previously isolated from *P. papatasi* originating from Israel/West Bank (AF237883 (Ono et al. 2001) and India (GenBank accession number AF237882 (Ono et al. 2001), as well as from Spain and Iran (Benlarbi and Ready 2003, Parvizi et al. 2003).

From the three regions under study, 183 sand flies were selected in order of their genus and diversities of types and regions. *Wolbachia* infection was detected in 1 case out of 57 samples in Meshkin Shahr (1.7%), 9 cases out of 46 samples in Kaleybar (19.6%), and 2 cases out of 80 samples in Sarab (2.5%) out of subgenus of *Larroussius* "*Wolbachia* Surface Protein genes".

Larroussius and *Adlerius* subgenus species were first identified as male because only males have good morphological characters of the head and abdominal terminalia for differentiation of species but females do not have this advantage. So it is obvious that this females are mates of that detected male geneses that were taken from this area (the males of these three species were taken from the given three regions, same as females) (Parvizi et al. 2003, Akhondi et al. 2012).

Out of *Adlerius* subgenus, only two cases in female sand flies were infected by *Wolbachia* which had no morphological characters for identification of genus (as mentioned above), but the males had good morphological characteristics for identification. As in these regions, male sand flies of *Adlerius* subgenus "*P. longiductus, P. halepensis, P. brevis, P. balcanicus, P. simici*" have been found, the two cases of detected *Wolbachia* in female sand flies of *Adlerius* subgenus in Kaleybar region, could be of any of the above five mentioned types. We would like to mention that all collected sand flies of above 5 mentioned species from Kaleybar were not examined because it was not the purpose of this paper.

The selection of *Wolbachia* surface protein gene has been due to free availability of this protein at the surface of the bacteria, ease of its identification, diversity of this gene in various genus and the possibility of usage of this gene for the purpose of studying the evolutional relationship and phylogenetic proximity of these groups of bacteria (Werren 1997, Bandi et al. 1998, Werren 1998, Sinkins and O'Neill 2000).

There is a natural *Wolbachia* infection in sand flies. Cytoplasmic incompatibility is the recognized phenotype in sand flies which have been shown to be infected with *Wolbachia* (McGraw et al. 2002, Rasgon 2003). Moreover, it has been suggested that *Wolbachia* can prevent the carriage and transferring of parasites and viruses via infected insects. In addition, it is a unique unknown manner which allows *Wolbachia* to be a good selection and used as a transgene of the target genes and controlling the leishmaniasis which has been recently adapted among the population of sand flies (Breeuwer and Jacobs 1996, Werren 1998,

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Sinkins and O'Neill 2000, Brownstein et al. 2003).

As the existence of *Wolbachia* in many sand flies which carry cutaneous leishmaniasis agents of urban and rural types is not known, it is suggested that the existence of this bacteria in other types of carriers of leishmaniasis is surveyed (Dobson et al. 2002).

Wolbachia can be used as a transferring gene or transgene in insects. This can be done by designing and synthesizing of target genes by genetic engineering techniques and then to transfer them into *Wolbachia* genome the point at which proper genes of *Wolbachia* can be released to the mass of insects. It appears that the use of this technology would be very useful for the purpose of biologically control and combat against varieties of parasites and viruses of the region by employing arthropods (Werren 1998, Dobson et al. 2002, Mitsuhashi et al. 2002, Rasgon 2003).

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