PCR screening of the *Wolbachia* in some arthropods and nematodes in Khuzestan province

Pourali, P.[1*]; Roayaei Ardakani, M.[2]; Jolodar, A.[3] and Razi Jalali, M. H.[4]

[1]Ph.D. Student in Microbiology, Section of Microbiology, Department of Biology, Fars Science and Research Branch, Islamic Azad University, Shiraz, Iran; [2]Section of Microbiology, Department of Biology, Faculty of Sciences, Shahid Chamran University of Ahvaz, Ahvaz, Iran; [3]Department of Basic Sciences, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran; [4]Department of Pathobiology, Faculty of Veterinary Medicine, Shahid Chamran University of Ahvaz, Ahvaz, Iran

[*]**Correspondence:** P. Pourali, Ph.D. Student in Microbiology, Section of Microbiology, Department of Biology, Fars Science and Research Branch, Islamic Azad University, Shiraz, Iran. E-mail: Parastoo_pourali@yahoo.com

(Received 30 Jan 2008; revised version 7 Feb 2009; accepted 11 Feb 2009)

Summary

Wolbachia is an obligatory, intracellular α-proteobacterium which infect the reproductive and somatic tissues of some arthropod and nematode populations. Because there are not any available data on the presence of this bacterium in Iran, the present study was done to determine the presence of this bacterium among 30 species of arthropods and nematodes. After DNA extraction from samples, we screened *Wolbachia* spp. with specific primers using PCR method. A total of 770 arthropods (of 22 genera) and 41 nematodes (of 6 genera) were screened for *Wolbachia*. Overall 167 arthropod samples (18 colonies) from 7 genera and 1 nematode sample were found positive. Positive PCR products of 16S *rDNA* gene were digested with *RsaI* restriction enzyme and the types of *Wolbachia* were recognized as A supergroup of *Wolbachia*.

Key words: *Wolbachia*, Iran, Arthropod, Nematode

Introduction

Wolbachia is an intracellular bacterium (rickettsia) that is transmitted within the egg cytoplasm and found in reproductive and other tissues of invertebrates. *Wolbachia* is one of the most ubiquitous endosymbiotic bacterium occurring in insects (Werren, 1997; Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000) and is a maternally inherited α-proteobacterium (O'Neill *et al*., 1997; Werren, 1997; Bourtzis and O'Neill, 1998). These remarkable bacteria have been described within a different range of arthropods and filaroid nematodes (Werren *et al*., 1995; Vandekerckove *et al*., 1999; Werren and Windsor, 2000; Lo *et al*., 2002; Bandi *et al*., 2003; Rowley *et al*., 2004; Bordenstein *et al*., 2006) and are extremely common and widespread. They are estimated to occur in 15-20% of insect species (Bandi *et al*., 2001) and have also been found in arachnids, crustaceans, and nematodes (Rousset *et al*., 1992; Breeuwer and Jacobs, 1996). This bacterium has an intracellular lifestyle, and infections occur throughout somatic and germ line tissues of insect species (Dobson *et al*., 1999). The bacteria appear to have an essential role in embryogenesis in nematodes, as antibiotic treatment of infected individuals or experimental animals inhibits production of microfilariae (Hoerauf *et al*., 1999; Hoerauf *et al*., 2000). Although routinely transmitted vertically, *Wolbachia* have also undergone extensive intertaxon transmission, even between different orders of insects and between insects and crustaceans (Werren *et al*., 2008). Within the intracellular environment, they seem to have a wide host tolerance. Equally the phenotypic effects of these bacteria are remarkable. *Wolbachia* are known to cause a number of reproductive alterations in hosts, including cytoplasmic

incompatibility in a broad range of insects, parthenogenesis in hymenopterans, feminization of genetic males in isopods and male killing (O'Neill *et al*., 1992; Rousset *et al*., 1992; Werren *et al*., 2008). *Wolbachia* has evolved several means of altering its reproduction in the host, thereby optimizing its vertical cytoplasmic inheritance.

Wolbachia are of wide interest as a potential mechanism for rapid speciation (Werren, 1997) and genetic modification. Because of the phenotypes induced by these infections, it has been suggested that the manipulation of endosymbiotic bacteria can be used as a novel method for the biocontrol of pest arthropods of medical, veterinary and agricultural importance (Werren, 1997; Turelli and Hoffmann, 1999; Zabalou *et al*., 2004; Xi *et al*., 2005; Bordenstein *et al*., 2006). Eight major *Wolbachia* "supergroups" (A to H) exist based on phylogenetic clustering of *ftsZ* gene sequences (Lo *et al*., 2002; Werren *et al*., 2008). A, B and E infect the arthropods; C and D infect nematodes; G infects spiders; H infects termites and F infects both arthropods and nematodes (O'Neill *et al*., 1992; Vandekerckove *et al*., 1999; Bandi *et al*., 2003; Rowley *et al*., 2004; Bordenstein and Rosengaus, 2005; Werren *et al*., 2008).

The phylogenetic data also show extensive horizontal transmission of *Wolbachia* between insect taxa although the mechanisms are still unclear (Werren *et al*., 1995). Polymerase chain reaction (PCR) technologies have opened new avenues for research on this organism and have facilitated significant insights into relationships between host and endosymbiont.

Wolbachia are widespread and common in insects. In a PCR-based screening study, Werren *et al*. (1995) found that over 16% of insect species in their sample were infected with *Wolbachia*. In a study using similar methods, West *et al*. (1998) revealed that 22% of British insects were infected (West *et al*., 1998). The latter screening study has indicated that the prevalence may even be underestimated and that *Wolbachia* infection levels are as high as 76% of all insect species (Jeyaprakash and Hoy, 2000).

The objectives of this study were to determine the distribution and type of *Wolbachia* in a sample of arthropods collected from Khuzestan province, Iran. Until now, there is no report on the existence of *Wolbachia* in arthropods in Iran. In this study, a PCR screening method was used to determine the prevalence of *Wolbachia* among 30 species of arthropods and nematodes.

Materials and Methods

Sample collection and DNA extraction

A total of 770 arthropods from 22 genera and 41 nematodes of 6 genera were collected from April to August 2007 from Khuzestan province, Iran. Nematode samples were obtained from the digestive tracts of both genders of mice, gallinaceans, sheep, buffaloes and cows. Their digestive systems were washed by parasitological standard methods: *Haemonchus contortus* samples were obtained from abomasums of sheep and buffaloes, *Ascaridia galli* from small intestine of gallinaceans, *Trichosomoides* from digestive system of mice and *Setaria* species from abdominal cavity of cows. The samples were stored in 95% ethanol at -20°C before analysis. Before DNA extraction, the tissues were dissected in sterile, double-distilled, deionized water on a sterile petri dish and then serially rinsed in droplets of sterile H_2O and air-dried for 15 min. Positive control DNA samples were prepared using pupae or adults of known infected strains of *Drosophila melanogaster* (Turelli and Hoffmann, 1995). PCR reactions were performed on blanks and water was used as a negative control for the PCR because uninfected standard flies as negative controls were not available. DNA was extracted from either single, whole individuals (for arthropods equal to or less than 0.5 mm in size) or single, whole abdomens (arthropods greater than 0.5 mm in size) or colony of very small insects. Predatory arthropods were starved for 24 h before DNA extraction to limit gut content contamination. DNA was extracted using a phenol-chloroform extraction method (Werren *et al*., 1995).

Screening using 16S *rDNA* and *wsp* primers

Polymerase chain reaction was

performed using 16S *rDNA* to test for the presence of *Wolbachia*. Arthropods yielding a product of the expected size (438 bp) were tentatively scored as positive for *Wolbachia*. To confirm the presence of *Wolbachia*, we used second specific primers for *wsp* gene of *Wolbachia* that amplified a 632 bp fragment and the samples were tested again with these primers.

PCR methods

The PCR amplification reactions were carried out by Bio-Rad thermocycler, in 25 µl reaction mixtures consisting of 0.5 mM of each primer, 0.6 mM of dNTP, 1 mM $MgCl_2$, 1 µl of the crude DNA extract, 0.2 U of Taq polymerase, 2.5 µl of 10 x PCR buffer and 1 µl of DMSO. The 16S *rDNA* primers used in the assay were W-Specf (5′-CATACCTATTCGAAGGGATAG) and W-Specr (5′-AGCTTCGAGTGAAACCAAT TC) (Werren and Windsor, 2000) and the *wsp* primers were (5′-TGGTCCAATAAG TGATGAAGAAAC) and (5′-AAAAAT TAAACGCTACTCCA) (Zhou *et al*., 1998). Each reaction mixture was overlaid with about 30 µl of mineral oil. PCR was performed with initial denaturation at 94°C for 2 min, followed by 30 cycles consisting of 94°C for 30 s, 50.7°C for 1 min and 72°C for 1 min, and a final extension for 4 min at 72°C. Then, a sample of 8 µl of this reaction mixture was electrophoresed with a 100 bp DNA ladder on 1% agarose gel to determine the presence and size of the amplified DNA. DNA bands were visualized by ethidium bromide staining. Blank sample did not have any template DNA. For the confirmation of the obtained results, some of the PCR products of both primers were chosen randomly and their nucleotide sequences were determined. Therefore, PCR products from at least three different samples were purified by a commercial PCR purification kit (Qiagen) according to the manufacturer's instructions. Then TAG Copenhagen A/S manufacture sequenced purified products by the F primers. Finally, the results were compared with the sequence database at the National Center for Biotechnology Information using BLAST program.

Restriction enzyme digestion

The W-Spec primers were designed from the 3′ half of the 16S *rDNA* gene in order to amplify a 438 bp fragment. This region was chosen because it contains restriction sites which were different between A and B group *Wolbachia*, providing the second confirmation of bacterial group. After amplification of a 438 bp fragment using 16S *rDNA* primers, the type of *Wolbachia* was distinguished by *RsaI* restriction enzyme (Fermentas) digestion (A or B). *RsaI* restriction enzyme digestion of group B *Wolbachia* results in five fragments with length of 146, 165, 16, 67 and 46, only the 146 and 165 fragments were visible as overlapping bands at those positions. Digestion of group A results in 311, 83 and 46 base pair fragments, only the 311 fragment was visible. The digestion was carried out in a total volume of 25 µl containing 18 µl of each PCR product, 2.5 µl of 10 x buffer and 1.5 µl of *RsaI*. The samples were incubated for 16 h before digestion. After the digestion, a sample of 8 µl of this reaction mixture was electrophoresed with a 1 kbp DNA ladder on 1% agarose gel to determine the type of *Wolbachia*.

Precautions were taken to prevent false positives by (i) washing samples with ethanol before DNA extraction; and (ii) including blank controls in all DNA extractions and PCR reactions. In addition, predatory arthropods were starved (when possible) for 24 h before DNA extraction. Furthermore, precautions were taken to prevent false negatives by (i) repeating PCR of negative DNA samples and (ii) using positive controls in all PCR reactions.

Results

Prevalence of the *Wolbachia* among arthropods

The PCR amplification reactions were carried out by *Wolbachia* 16S *rDNA* specific and *wsp* primers, separately. 16S *rDNA* gene amplified a 438 bp and *wsp* gene amplified 632 bp fragments (Figs. 1 and 2). The negative-control samples included in each PCR run did not yield amplification products.

A total of 770 arthropods (of 22 genera) and 41 nematodes (of 6 genera) were screened for *Wolbachia*. According to the

results, 167 arthropod samples from 7 genera (18 colonies) and 1 nematode sample were found positive. By digestion of the positive PCR products with *RsaI* restriction enzyme, the type of *Wolbachia* was determined. The result showed that 14.06% ($^{18}/_{128} \times 100$) of arthropod colonies and 7.14% ($^{1}/_{14} \times 100$) of nematode colonies were positive (Table 1). *Wolbachia* in all 7 *Wolbachia*-positive arthropod species belonged to A supergroup (Fig. 3).

DNA sequencing results

The amplified fragments were sequenced by 16S *rDNA* and *wsp* F primers and then compared with the sequence of *Wolbachia* in *Drosophila melanogaster* that exists in the gene bank. As a result, the amplified sequences of 16S *rDNA* and *wsp* showed 100 and 99% nucleotide identity, respectively.

Discussion

Wolbachia has mutualistic relationship with nematodes therefore, if we clear the bacteria from nematodes we can cure the

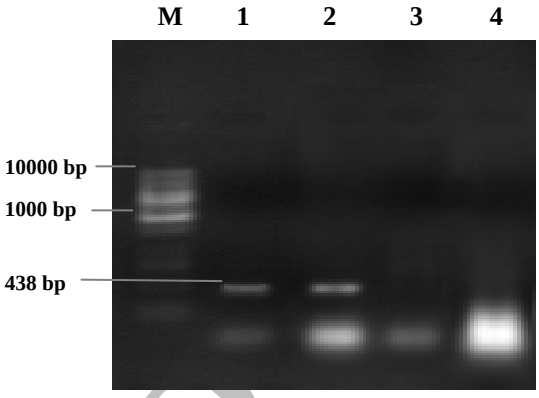

Fig. 1: Detection of *Wolbachia* via amplification of specific gene fragment from different arthropods. *Wolbachia* detection with primers of R and F for a fragment (438 bp) of the 16S *rDNA* gene. Lane M: DNA marker, Lane 1: Amplification of the 16S *rDNA* gene fragment for *Boophilus annulatus*, Lane 2: Amplification of the 16S *rDNA* gene fragment for *Drosophila melanogaster* (positive control), Lane 3: Blank sample (negative control) and Lane 4: Amplification of the 16S *rDNA* gene fragment for *Haemaphysalis* sp.

Table 1: Arthropods tested for infection with *Wolbachia* using PCR

Organisms	Number of insects in each colony	Number of colonies	Positive colonies (16S *rDNA*)	Type (A or B)	Positive colonies (*wsp*)
Arthropods					
Drosophila melanogaster	113	5	5	A	5
Musca domestica	25	25	2	A	2
Sarcophaga haemorrhoidalis	17	17	-	-	-
Goniodes meleagridis	27	1	-	-	-
Goniocotes gallinae	23	1	-	-	-
Microcertermes	18	1	-	-	-
Amitermes	52	5	-	-	-
Coccinella trifasciata	6	6	2	A	-
Coccinella septempunctata	4	4	-	-	-
parasitoid wasp	39	3	1	A	1
Aphis_fabae	89	4	-	-	-
Braconidae	32	2	-	-	-
Apis mellifera	6	6	2	A	2
Agrotis segetum	6	6	-	-	-
Achaearanea tepidariorum	5	5	-	-	-
Cimex lectularius	6	1	-	-	-
Boophilus annulatus	5	5	3	A	3
Hyalomma anatolicum	154	13	-	-	-
Rhipicephalus sanguincus	76	7	3	A	3
Haemaphysalis sp.	53	4	-	-	-
Dermanyssus gallinae	8	1	-	-	-
Linguatula serrata	6	6	-	-	-
Nematodes					
Ostertagia circumcincta	22	4	-	-	-
Haemonchus contortus	7	1	-	-	-
Dirofilaria immitis	2	2	1	-	1
Ascaridia galli	5	5	-	-	-
Setaria sp.	1	1	-	-	-
Trichosomoides	4	1	-	-	-

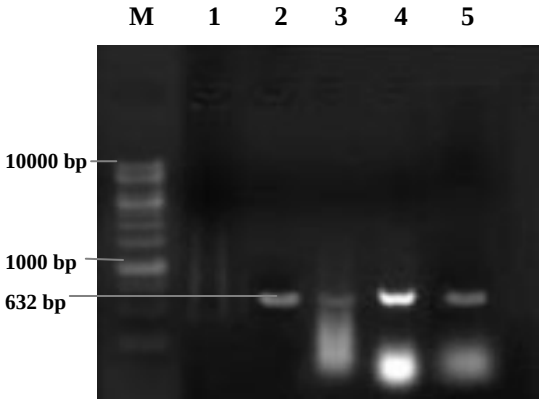

Fig. 2: Detection of *Wolbachia* via amplification of specific gene fragment from different arthropods. *Wolbachia* detection with primers of R and F for a fragment (632 bp) of the *wsp* gene. Lane M: DNA marker, Lane 1: Blank sample (negative control), Lane 2: Amplification of the *wsp* gene fragment for *Drosophila melanogaster* (positive control), Lane 3: Amplification of the *wsp* gene fragment for *Boophilus annulatus*, Lane 4: Amplification of the *wsp* gene fragment for *Rhipicephalus sanguincus* and Lane 5: Amplification of the *wsp* gene fragment for *Musca domestica*

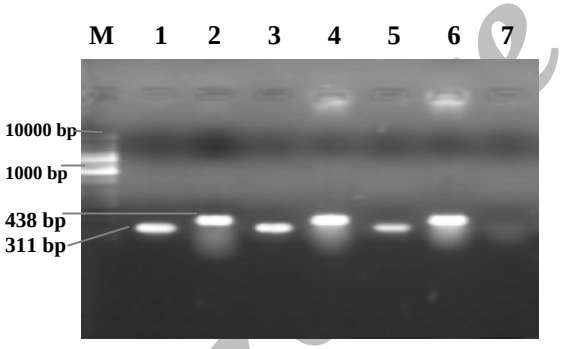

Fig. 3: Detection of *Wolbachia* supergroups via digestion of specific gene fragments (16S *rDNA* gene) with *RsaI* enzyme. Lane M: DNA marker, Lanes 1, 3 and 5: Digestion of the 16S *rDNA* gene fragment for *Boophilus annulatus*, *Rhipicephalus sanguincus* and *Drosophila melanogaster* (positive control) with *RsaI* enzyme, respectively. This fragments belong to the A supergroup of *Wolbachia* (311 bp). Lanes 2, 4 and 6: Amplification of the 16S *rDNA* gene fragment for *Boophilus annulatus*, *Rhipicephalus sanguincus* and *Drosophila melanogaster* (positive control), respectively and Lane 7: Blank sample (negative control)

humans who are infested by nematodes. Because *Wolbachia* induce parthenogenesis in female arthropods, treatment of *Wolbachia* can decline the population of the pests. The present study shows that overall, 14.08% of the colonies of arthropods were infected with *Wolbachia* (Table 1). However, the status of most species in these samples was based on a single or few individuals. As a result, infected species were less likely to be positive and the actual frequencies of the infected species may be underestimated in this study.

In the present study, one sample (*Coccinella trifasciata*) that was negative in the initial *wsp* screen, proved to be positive for the A *Wolbachia* with 16S *rDNA* primers (Table 1). In this particular sample, the 16S *rDNA* primers may be more sensitive in detecting *Wolbachia* infection than the *wsp* primers. This may be attributed to the relative storage ages of the samples when they were tested. We conducted a study showing that the detection of *Wolbachia* by *wsp* declines with the time of storage (95% ethanol and freezing at -20°C); whereas the detection levels with the 16S *rDNA* primers remained high (Werren and Windsor, 2000). This is most probably due to some degradation of the DNA within the samples which affects the amplification of the *wsp* product.

In the previous studies, Werren and Windsor (2000), using similar polymerase chain reaction methods, reported that 19.3% of temperate North American insects are infected with *Wolbachia*. They showed that there were clear differences between insect orders, in their relative frequencies of infection with A versus B *Wolbachia*. In particular, Hymenoptera showed higher infection levels with A *Wolbachia* and Lepidoptera showed higher infection levels with B *Wolbachia*. These results may indicate differences in the ability of A and B *Wolbachia* to infect different taxa. They showed that only 0.025% of the samples were infected with B *Wolbachia.*

In the present study, by *RsaI* restriction enzyme digestion of the positive PCR products, the type of *Wolbachia* was determined. All of the positive samples were infected with A *Wolbachia* and there was not any infection with B type of *Wolbachia*, and we can relate it to taxa-specific presence of *Wolbachia* supergroups (Table 1) (Fig. 2).

Weeks *et al*. (2003), used a sensitive hemi-nested polymerase chain reaction

method to screen 223 species from 20 arthropod orders for infection with *Wolbachia*. This bacterium was found to infect 49 species (22%) and their results were similar to our results.

Duron and Gavotte (2007) showed that *Wolbachia* infection was never detected by PCR using experimental conditions that are able to detect *Wolbachia* in arthropods and filarial nematodes. Only bacteria of the *Xenorhabdus* and *Photorhabdus* genus have been actually detected in some nematodes. All suggest that *Wolbachia* do not infect non-filaroid worms despite sharing a common arthropod-parasitic lifestyle with filarial nematodes. Why *Wolbachia* are absent in non-filaroid worms parasitizing infected arthropods remains intriguing. In the Steinernematidae and Heterorhabditae worms, infection with the symbiotic bacteria *Xenorhabdus* and *Photorhabdus* are well known to produce antibiotic (Duron and Gavotte, 2007) and could be an explanation for the absence of *Wolbachia* in these species. Non-filarial nematodes and arthropods have desirable cellular environments for *Wolbachia*, but filarial nematodes have not. Indeed, *Wolbachia* has limited metabolic capacity, resulting from the loss of genetic material following adaptation to the intracellular environment (Duron and Gavotte, 2007).

Tsai *et al*. (2007) based on alignment of the sequences from the *wsp*, *ftsZ*, and 16S *rRNA* genes, demonstrated that *Wolbachia* exist in *Angiostrongylus cantonensis*, a non-filaroid nematode.

According to our results, a total of 41 nematode samples (from 6 genera) were screened for *Wolbachia* and this bacterial infection was never detected in non-filaroid nematode samples.

Bandi *et al*. (2001), found that *Dirofilaria immitis* (filaroid nematode) was positive for *Wolbachia* infection. As we described before, the type of *Wolbachia* in filaroid nematode is C or D. So in our results, the 438 bp fragment that belong to the *Wolbachia* in filaroid nematodes, did not digest with *RsaI* restriction enzyme. They showed that all colonies of *D. immitis* were infected by *Wolbachia* but our results showed that only 50% of the colonies were infected. This difference may be related to preservation of our samples in lacto phenol in a period of 80 days, before DNA extraction.

In conclusion, our result shows that *Wolbachia* is not a very common bacterium in some arthropod and nematode samples in Khuzestan province. In spite of this result, it seems that further studies should be performed in this field to determine the existence of this bacterium in other parts of Iran.

Acknowledgements

We wish to acknowledge the support of Department of Biology officials, and great thanks to Mr. Papizadeh in microbiological research laboratory.

References

Bandi, C; Bourtzis, K; Braig, H; Lo, N; O'Neill, S; Stouthamer, R; Vandekerckove, TTM and Werren, JH (2003). *Wolbachia* nomenclatural system: a discussion paper. News and Forums. Available on the website: www.wolbachia.sols.uq.edu.au/news.cfm?action=thread&id=25.

Bandi, C; Trees, AJ and Brattig, NW (2001). *Wolbachia* in filarial nematodes: evolutionary aspects and implications for the pathogenesis and treatment of filarial diseases. Vet. Parasitol., 98: 215-238.

Bordenstein, SR; Marshall, ML; Fry, AJ; Kim, U and Wernegreen, JJ (2006). The tripartite associations between bacteriophage, *Wolbachia*, and arthropods. PLoS Pathogens. 2: 43.

Bordenstein, S and Rosengaus, RB (2005). Discovery of a novel *Wolbachia* super group in Isoptera. Curr. Microbiol., 51: 393-398.

Bourtzis, K and O'Neill, SL (1998). *Wolbachia* infections and their influence on arthropod reproduction. Bioscience. 48: 287-293.

Breeuwer, JAJ and Jacobs, G (1996). *Wolbachia*: intracellular manipulators of mite reproduction. Exp. Appl. Acarol., 20: 421-434.

Dobson, S; Bourtzis, K; Braig, HR; Jones, BF; Zhou, W; Rousset, F and O'Neill, SL (1999). *Wolbachia* infections are distributed throughout insect somatic and germ line tissues. Insect Biochem. Mol. Biol., 29: 153-160.

Duron, O and Gavotte, L (2007). Absence of *Wolbachia* in nonfilariid worms parasitizing arthropods. Curr. Microbiol., 55: 193-197.

Hoerauf, A; Nissen-Pähle, K; Schmetz, C; Henkle-Dührsen, K; Blaxter, ML; Büttner, DW; Gallin, MY; Al-Qaoud, KM; Lucius, R and Fleischer, B (1999). Tetracycline therapy targets intracellular bacteria in the filarial nematode *Litomosoides sigmodontis* and results in filarial infertility. J. Clin. Invest., 103: 11-18. Quoted by: Fenollar, F; Maurin, M and Raoult, D (2003). *Wolbachia pipientis* growth kinetics and susceptibilities to 13 antibiotics determined by immunofluorescence staining and real-time PCR. Antimicrob. Agents Chemother., 47: 1665-1671.

Hoerauf, A; Volkmann, L; Nissen-Pähle, K; Schmetz, C; Autenrieth, I; Büttner, DW and Fleischer, B (2000). Targeting of *Wolbachia* endobacteria in *Litomosoides sigmodontis*: comparison of tetracyclines with chloramphenicol, macrolides and ciproflaxcin. Trop. Med. Inter. Health. 5: 275-279.

Jeyaprakash, A and Hoy, MA (2000). Long PCR improves *Wolbachia* DNA amplification: *wsp* sequences found in 76% of sixty three arthropod species. Insect Mol. Biol., 9: 393-405.

Lo, N; Casiraghi, M; Salati, E; Bazzocchi, C and Bandi, C (2002). How many *Wolbachia* supergroups exist? Mol. Biol. Evol., 19: 341-346.

O'Neill, SL; Giordano, R; Colbert, AME; Karr, TL and Robertson, HM (1992). 16S *ribosomal-RNA* phylogenetic analysis of the bacterial endosymbionts associated with cytoplasmic incompatibility in insects. Proc. Natl. Acad. Sci. USA, 89: 2699-2702.

O'Neill, SL; Pettigrew, MM; Sinkins, SP; Braig, HR; Andreadis, TG and Tesh, RB (1997). *In vitro* cultivation of *Wolbachia pipientis* in an *Aedes albopictus* cell line. Insect Mol. Biol., 6: 33-39. Quoted by: Kozek, WJ (2005). What is new in the *Wolbachia*/*Dirofilaria* interaction? Vet. Parasitol., 133: 127-132.

Rousset, F; Bouchon, D; Pintureau, B; Juchault, P and Solignac, M (1992). *Wolbachia* endosymbionts responsible for various alterations of sexuality in arthropods. Proc. Biol. Sci., 250: 91-98.

Rowley, SM; Raven, RJ and McGraw, EA (2004). *Wolbachia pipientis* in Australian spiders. Curr. Microbiol., 49: 208-214.

Tsai, KH; Huang, CG; Wang, LC; Yu, YW; Wu, WJ and Chen, WJ (2007). Molecular evidence for the endosymbiont *Wolbachia* in a non-filaroid nematode, *Angiostrongylus cantonensis*. J. Biomed. Sci., 14: 607-615.

Turelli, M and Hoffmann, AA (1995). Cytoplasmic incompatibility in *Drosophila simulans*: dynamics and parameter estimates from natural populations. Genetics. 140: 1319-1338.

Turelli, M and Hoffmann, AA (1999). Microbe-induced cytoplasmic incompatibility as a mechanism for introducing transgenes into arthropod populations. Insect Mol. Biol., 8: 243-255.

Vandekerckove, TTM; Watteyne, S; Willems, A; Swing, JG; Mertens, J and Gillis, M (1999). Phylogenetic analysis of the 16S *rDNA* of the cytoplasmic bacterium *Wolbachia* from the novel host *Folsomia candida* (Hexapoda, Collembola) and its implications for wolbachial taxonomy. FEMS Microbiol. Lett., 180: 279-286.

Weeks, AR; Velten, R and Stouthamer, R (2003). Incidence of a new sex-ratio-distorting endosymbiotic bacterium among arthropods. Proc. Biol. Sci., 270: 1857-1865.

Werren, JH (1997). Biology of *Wolbachia*. Annu. Rev. Entomol., 42: 587-609.

Werren, JH; Baldo, L and Clark, ME (2008). *Wolbachia*: master manipulators of invertebrate biology. Nat. Rev. Microbiol., 6: 741-751.

Werren, JH and Windsor, DM (2000). *Wolbachia* infection frequency in insects: evidence of a global equilibrium? Proc. R. Soc. Lond. B. Biol. Sci., 267: 1277-1285.

Werren, JH; Zhang, W and Guo, LR (1995). Evolution and phylogeny of *Wolbachia*: reproductive parasites of arthropods. Proc. R. Soc. Lond. B., 161: 55-63. Quoted by: Brownlie, JC and O'Neill, SL (2005). *Wolbachia* genomes: insights into an intracellular lifestyle. Curr. Biol., 15: 507-509.

West, SA; Cook, JM; Werren, JH and Godfray, HCJ (1998). *Wolbachia* in two insect host-parasitoid communities. Mol. Ecol., 7: 1457-1465.

Xi, Z; Khoo, CC and Dobson, SL (2005). *Wolbachia* establishment and invasion in an *Aedes aegypti* laboratory population. Science. 310: 326-328.

Zabalou, S; Riegler, M; Theodorakopoulou, M; Stauffer, C; Savakis, C and Bourtzis, K (2004). *Wolbachia*-induced cytoplasmic incompatibility as a means for insect pest population control. *Proceedings of national academy of sciences USA*., 101: 15042-15045.

Zhou, W; Rousset, F and O'Neill, S (1998). Phylogeny and PCR-based classification of *Wolbachia* strains using *wsp* gene sequences. Proc. R. Soc. Lond. B., 265: 509-515.