

RESEARCH ARTICLE

Isolation and identification of *Wolbachia* from *Hemiscorpius lepturus* scorpion in Khuzestan province with PCR method

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ABSTRACT

Wolbachia is the most frequent gram negative Endosymbiont bacteria belonging to α -proteobacter group which can effect sexual and somatic tissues of arthropods. Due to manipulate the host's reproductive system can be used as a new method for biological control of insects, bacteria important veterinary disease vectors used. In this study, after collection of scorpion species from field with the help of UV lamp at night, their DNA was isolated and two special primers against 16srDNA and WSP were designed. After setting up PCR conditions with positive control samples, PCR unknown samples were marked. The amplified fragment by agarose gel electrophoresis have shown. Then we have the positive samples sequenced.

During our study on 40 *Hemiscorpius lepturus* scorpions, Twenty-eight of the specimens were positive for *Wolbachia* bacteria, all of which were positive for both primers WSP and 16srDNA primers. The presence of *Wolbachia* bacteria in the *Hemiscorpius Lepturus* scorpion has been proven. This detection was performed by two *16srDNA* and *WSP* genes. The prevalence of the bacterium *Wolbachia* sp. in Khuzestan province can be due to effects of temperature, which is an inhibitory factor for growth of the bacterium in the arthropods. The presence of *Wolbachia* in *H. lepturus* scorpions was approved.

The accessions number of 16Sr DNA sequences is KY554756 and wsp gene is KY554757 that deposit in Gen Bank.

KEYWORDS: *Wolbachia*, *Hemiscorpius lepturus*, PCR

INTRODUCTION

Wolbachia is a gram negative, obligate, intracellular symbiont, that is classified in α -proteobacter (Inaki et al., 2011). *Wolbachia* is a cytoplasmal inherited rickettsia that was found in a wide range of arthropods (Jeyaprakash and Hoy, 2000; Werren and Windsor, 2000; Hilgenboecker et al., 2008). They are widespread symbionts detected in all arthropod classes (infecting between 20 and 70% of insect species) (Bourtzis and Miller, 2003; Zug and Hammerstein, 2012) as well as in a nematode family, the Onchocercidae, commonly known as filarial (Bandi et al., 1998; Sironi et al., 1995). Thus, *Wolbachia* are present in millions of species.

Intracellular bacterium *Wolbachia* first time within the context

of reproductive culex pipiens mosquitoes was reported in 1924 by Wolbach & Hertig. Infections were detected in all of the major insect orders, including Coleoptera, Diptera, Hemiptera/Homoptera, Hymenoptera, Lepidoptera, and Orthoptera (Werren and Windsor, 2000). Depending on both bacterial lineage and host, they may have different effects on host reproduction, such as cytoplasmic incompatibility (Breeuwer and Werren, 1990; O'Neill and Karr, 1990), male killing (Hurst et al., 1999), parthenogenetic reproduction (Stouthamer, 1997; Zchori-Fein et al., 2001) or feminization of genetic males (Bouchon et al., 1998; Juchault et al., 1994). *Wolbachia* cannot be cultured outside the host cells (O'Neill et al., 1992), and its potential application to the bio control of insect pests (Ravikumar et al., 2011) and vector borne diseases

such as dengue fever (Inaki et al., 2011), malaria and filariasis (Chaoyang et al., 2009) have been studied.

Classification of strains of *Wolbachia* is based on the incompatibility of the cytoplasm, the sequence of genes *16srDNA* (Breeuwer et al., 1992; O'Neill et al., 1992; Rousset et al., 1992; Stouthamer et al., 1993) *ftsZ* (the cell cycle belongs) (186), *WSP* (the gene encoding the protein regions vary wildly) (Bourtzis et al., 1994; Braig et al., 1998; Zhou et al., 1998), *coxa*, *glta* and *groEL* done that *16sr DNA* sequence-based classification and *WSP* among them the most important factors are high (Głowska et al., 2015; Vera et al., 2008).

The initial report for the presence of *Wolbachia* in scorpion is related to southern African genus, *Opisthophthalmus* (Laura et al., 2007). Lincoln Suesdek-Rocha et al in 2007 reported the *Wolbachia* bacteria isolated from scorpion *Tityus serrulatus* (Lincoln Suesdek-Rocha et al., 2007).

The main purpose of this study was to search for *Wolbachia 16S rDNA* and *WSP* gene in the genomic DNA preparation made from the dangerous scorpions *H. lepturus* (*Hemiscorpiidae*) as the most lethal and stinging a great number of people annually one with major concern (Moghadam et al., 2011) Among different types of available scorpions in Khuzestan.

MATERIALS AND METHODS

Scorpion's samples

40 Iranian scorpions *H. lepturus* were collected from Khuzestan province Cities include: Masjed Soleiman

(10 sample), Izeh

(10 sample), Ramhormoz

(10 sample), Ahvaz

(10 sample). All scorpions were identified by trained health-caring personnel of the laboratory reference of Razi Institute in Ahvaz. Scorpions are individual and have maintained separately. Samples of both sexes were used.

Scorpions were washed with 70% alcohol (Roche, Germany). After catching the scorpion tail end zone (stinger), tail (mentasoma), pendipalp, the head, the chest (shoulder sex), abdomen and legs separated and extracted DNA and kept at -80°C.

DNA extraction

All DNA extraction using High Pure PCR Product Purification Kit (Roche, Germany) with the instructions of kit. The extracted DNA was dissolved in DEPCddH₂O (Roche, Germany) solution and frozen at -20°C and kept until it was used for rDNA and *wsp* amplification by PCR.

16S rDNA and *wsp* amplification by PCR

The PCR amplification reactions were carried out by Bio-Rad thermocycler (Bio-Rad Laboratories, Inc. USA). In 25 µl reaction mixtures consisting of 0.6 µl of dNTP, 0.6 µl of each primer, 5 µl of the crude DNA extract, 2 µl MgCl₂, 2.5 µl of 10X PCR buffer, 0.4 µl of Taq polymerase DNA and 13.3 µl of D.D.W for primer 16S rDNA. In 25 µl reaction mixtures consisting of 0.6 µl of dNTP, 0.5 µl of each primer, 5 µl of the crude DNA extract, 2 µl MgCl₂, 2.5 µl of 10X PCR buffer, 0.5 µl of Taq polymerase DNA and 13.4 µl of D.D.W for primer *wsp*.

Specific primers used in this work were *Wsp-R* (5'AAA AAT TAA ACG CTA CTC CA) and *Wsp-F* (5'TGG TCC AAT AAG TGA TGA AGA AAC)(Zhou et al., 1998) and rDNA-R (5'GAA TAG GTA TGA TTT TCA TGT) and rDNA- F (5'TTG TAG CCT GCT TAT GGT ATA ACT)(O'Neill et al., 1992). 16S rDNA amplifies a 890 bp fragment and *wsp* amplify a 620 bp fragment.

16S rDNA sequence are designed from the moderately variable regions of 16S rDNA and are relatively conserved among *Wolbachia*. *WSP* primers are the gene encoding a surface protein.

The PCR *Wsp* primer conditions for initial denaturation were carried out for 5 min at 95°C, followed by 35 cycles consisting of 94°C for 45 s, 49°C for 1 min and 72°C for 45 s, and a final extension for 7 min at 72°C.

The PCR rDNA primer conditions for initial denaturation were carried out for 5 min at 95°C, followed by 35 cycles consisting of 94°C for 45 s, 50°C for 1 min and 72°C for 1 min, and a final extension for 7 min at 72°C as described.

10 µl of the amplified product with a 100 bp DNA ladder were separated by 1% agarose gel (Roche, Germany) electrophoresis including ethidium bromide and visualized by UV transilluminator (UVitec Limited, United Kingdom) to determine the presence and size of the amplified DNA.

DNA sequence analysis

Wolbachia positive samples then had their *16S rDNA* and *WSP* gene fragment amplified and sequenced with the forward (5'TTG TAG CCT GCT TAT GGT ATA ACT) and reverse (5'GAA TAG GTA TGA TTT TCA TGT) PCR primers, and *WSP* gene fragment amplified and sequenced with the forward (5'TGG TCC AAT AAG TGA TGA AGA AAC) and reverse (5'AAA AAT TAA ACG CTA CTC CA) PCR primers. Amplicons were subjected to electrophoresis to confirm that they consisted of a single product and then purified using GF-1 gel DNA recovery Kit (vivantis ind.). After purification, sequencing was performed sent to Bioneer Company (Munpyeongseo-ro, Daedeok-gu, Daejeon 306-220, Republic of Korea) for nucleotide sequencing in both directions.

Phylogenetic and sequence alignment analyses

Alignment and Phylogenetic analysis of these sequences were performed using BioEdit 7.2 (<http://www.mbio.ncsu.edu/bioedit/bioedit.html>) and MEGA7 (<http://www.megasoftware.net/>) software respectively. The phylogenetic analysis was based on the Neighbor-Joining method with bootstrap test (1000 replicates) method. In addition to the isolate obtained in the present study, Sequence was compared with Gen Bank database using the BLAST software from NCBI site (<http://www.ncbi.nlm.nih.gov>) and the sequences of isolates from GenBank were also added to the analysis.

RESULTS

All scorpions were morphologically *Hemiscorpius lepturus*. Total of 40 scorpions *H. lepturus* were screened for *Wolbachia* by PCR method. *Wolbachia* positive by both *16S rDNA* and *WSP* gene PCR were found in 28 of them. 8 from Masjed Soleiman, 9 from Izeh, 7 from Ramhormoz, 4 from Ahvaz. PCR reaction amplified fragments in the range of about 620 bp for *wsp* of *Wolbachia* endosymbiont (Figure 1). PCR reaction

amplified fragments in the range of about 890 bp for 16S rDNA of *Wolbachia* endosymbiont (Figure 2). Therefore, the presence of *Wolbachia* in *H. lepturus* scorpions was approved.

The accessions number of *16Sr DNA* sequences is KY554756 and *WSP* gene is KY554757 that deposit in Gen Bank.

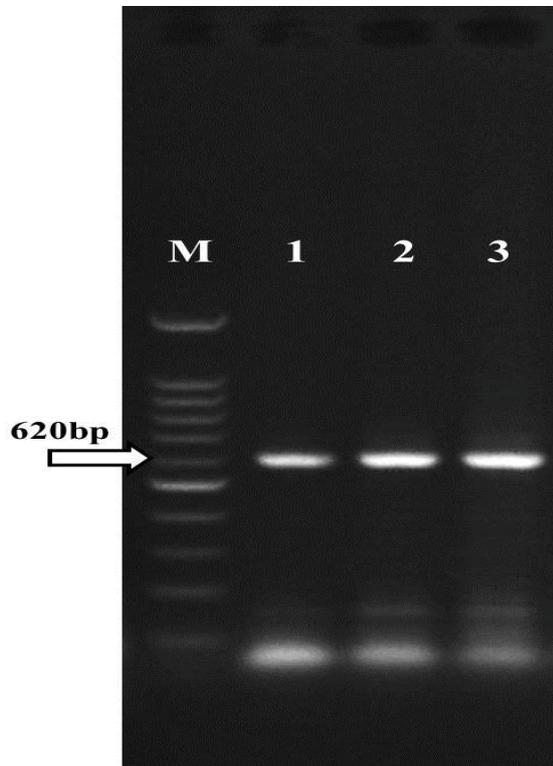


Figure 1. PCR amplification of *WSP* gene from *Wolbachia* endosymbiont of scorpion *H. lepturus* using specific primers. Lane M: DNA ladder, Lane 1, 2 and 3: production of amplification of *WSP* gene.

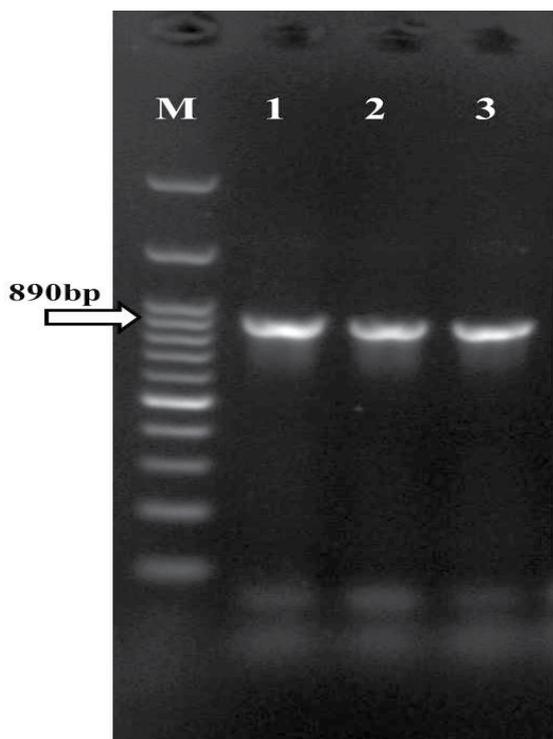


Figure 2. PCR amplification of *16S rDNA* gene from *Wolbachia* endosymbiont of scorpion *H. lepturus* using specific primers. Lane M: DNA ladder, Lane 1, 2 and 3: production of amplification of *16S rDNA* gene.

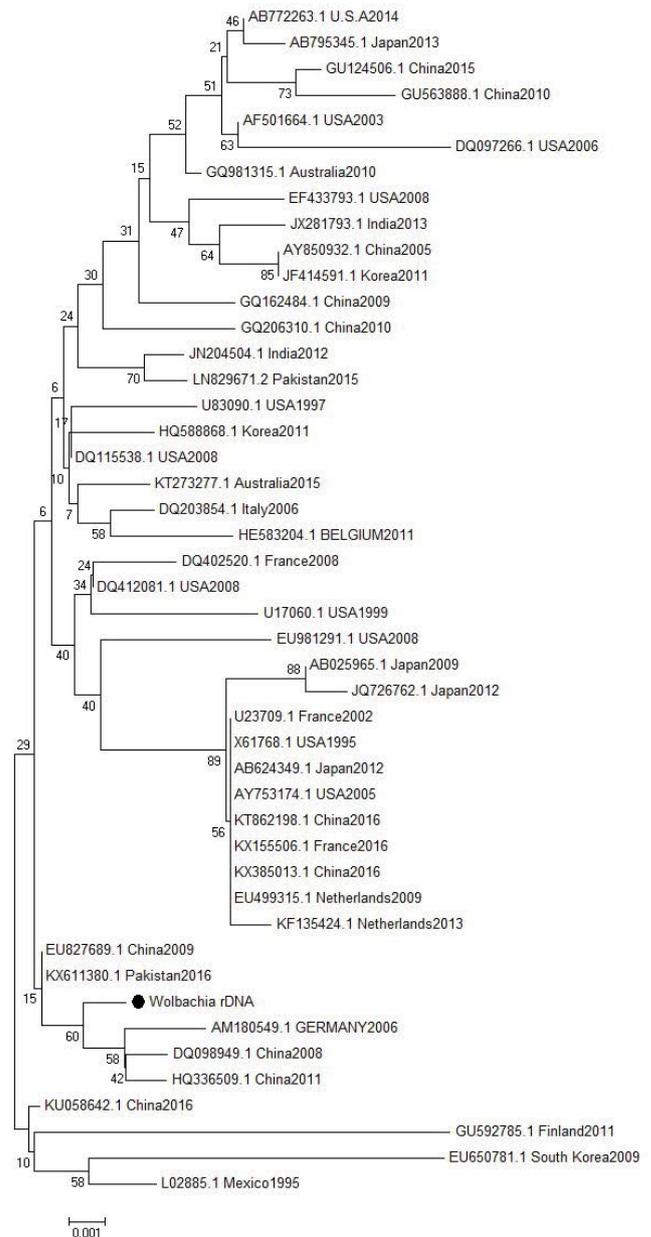


Figure 3. Dendrogram constructed by the neighbour-joining method based on the partial sequence of the *16sr DNA* gene of *Hemiscorpius lepturus*. The sequences isolates in this study (●). Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. Accession numbers are shown for GenBank sequences.

According to the phylogenetic tree, sequences derived from the sequence of the *Wolbachia 16srDNA* sequences isolated from China, Pakistan and Germany have similar (accession numbers are shown in the dendrogram), but the sequences isolated from Netherland, America, Australia and India is different (Figure 3). According to the phylogenetic tree, sequences derived from the sequence of the *Wolbachia WSP* gene sequences isolated from China and America and have very similar (accession numbers are shown in the dendrogram), but the sequences isolated from America, France, china and japan is very different (Figure 4).

The most similar rDNA sequences with sequences (Pakistan, 2016) and (China, 2009) 99.6% and Differences with (Korea, 2011) and (Netherlands, 2013) was 97%. The most similar wsp sequences with sequences (japan, 2016) 99.3% and (Canada, 2009) 98% and Differences with (China, 2015) 79% and

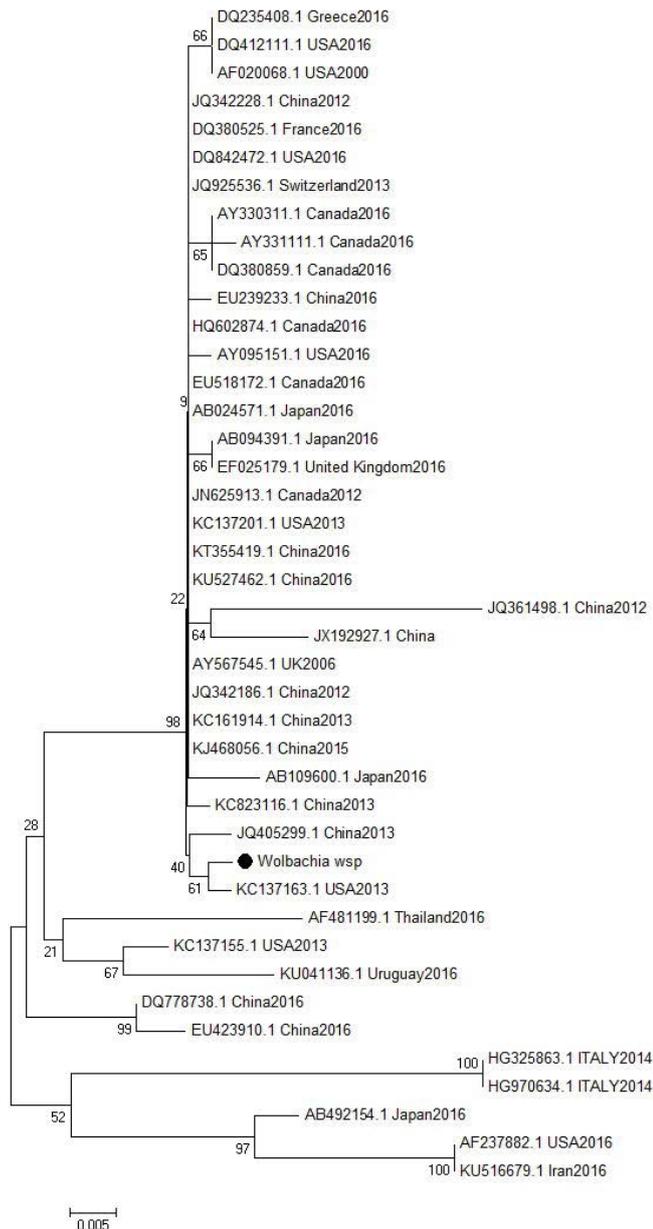


Figure 4. Dendrogram constructed by the neighbour-joining method based on the partial sequence of the *WSP* gene of *Hemiscorpius lepturus*. The sequences isolates in this study (●). Bootstrap values (expressed as percentages of 1000 replications) are shown at the branch points. Accession numbers are shown for GenBank sequences.

(France, 2016) was 72%.

DISCUSSION

In this study, *Hemiscorpius lepturus* scorpion in Khuzestan, Iran could test based on PCR for *16S rDNA* and *WSP* genes was isolated in the bacterium *Wolbachia*. Scorpions coming from Izeh were collected, have more infections than scorpions were collected from more temperate regions (Ramhormoz and Ahvaz). According to this study, *Wolbachia* infection in scorpion *Hemiscorpius lepturus* was detected by PCR method.

Sylvia Joanne et al. (2014), in Malaysia studied on *Aedes albopictus* been, a total of 286 samples based on the sequence *wsp* tested (67 male and 219 female), which (91.60%) for both *Wolbachia* (A, B) were infected, (3.15%) only had type A, (1.05) percent were infected with only one of two types or (2.05%) of

the unknown and (5.25) percent were without infection. (Joanne et al., 2015).

Didier Bouchon et al. (1998) in a study carried out on the 689 number of insects that includes (231 male and 458 female) of arthropod species reported 22 cases of insects *Wolbachia* bacteria were extracted.

O'Neill et al. in 1992 studied on different genes and *16S rDNA* sequences on *Culex pipiens* and several other species of insects showed, Their DNA sequences and insect colony PCR and investment in different species, different sequences of these genes from *Wolbachia* were found. (O'Neill et al., 1992).

According to our findings, *Wolbachia* bacteria isolated from scorpion *Hemiscorpius lepturus* by PCR method in both *WSP* gene and *16S rDNA* gene. The method used in this study is similar to the studies mentioned.

Bradaran et al. (2011), examined the 20 scorpion *Hemiscorpius lepturus* were collected from Khuzestan (Iran). *Wolbachia* bacteria are present in 10 samples patented Hemi scorpion. This study was conducted by PCR method and 16Sr DNA primers. (Baradaran et al., 2011).

Comparison of the 16S rDNA fragment and *wsp* fragment with the GenBank database revealed that the nucleotide sequence of 16S rDNA of *Wolbachia* endosymbiont of *H. lepturus* was highly similar to that of *Drosophila melanogaster*.

Some horizontal transmission has nevertheless occurred between scorpions, *Wolbachia 16S rRNA* and *WSP* gene sequences are identical in infected species of the *H. lepturus*. Similarly, the *16S rRNA* gene sequence of *Wolbachia* from *Drosophila melanogaster*, an insect (Arthropoda), is identical to that of scorpion *H. lepturus* were examined.

The present study is the first report of the presence of *Wolbachia* in *H. lepturus*. Since the sampling were performed through Khuzestan, the prevalence of the bacterium *Wolbachia* sp. in these area can be due to effects of temperature, which is an inhibitory factor for growth of the bacterium in the arthropods.

In this study, 40 samples from scorpion *H. lepturus* were examined to detect presence of bacteria *Wolbachia*; we were able to isolate the bacteria from scorpion *H. lepturus*. The survey has correlation with the similar studies. In 40 samples, 28 samples were positive for the *WSP* gene and the *16S rDNA* gene.

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