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Evaluation of prevalence and phylogenetic analysis of *Wolbachia pipientis* isolated from *Dirofilaria immitis* of canids in northern Iran

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ABSTRACT

Dirofilaria immitis and *Dirofilaria repens*, two mosquito-borne filarial nematodes, are the primary causes of subcutaneous and cardiopulmonary dirofilariasis in humans and canines. *Wolbachia pipientis* infection is widespread among arthropods and nematodes globally. One safe, cost-effective, and efficient way to avoid dirofilariasis infections is to eradicate *Wolbachia*. So, herein we determined the prevalence and phylogenetic analysis of *W. pipientis* strains isolated from *D. immitis* of canids in northern Iran. A descriptive-analytical cross-sectional study from March 2019 to April 2020 in Guilan province, north of Iran, was conducted. Sampling consisted of 32 road-killed carnivores (12 *Canis familiaris* and 20 *Canis aureus*) were gathered and necropsied for this study. The 16S ribosomal RNA (rRNA) gene was the basis for the molecular analysis. *D. immitis* infection was discovered in 7/20 (35%) of the jackals and 9/12 (75%) of the dogs. The *W. pipientis* infection was present in all 16 of the infected dogs and jackals. In this study, 16S rRNA sequences had 100% similarity with previously submitted *W. pipientis* sequences from the USA, Russia, Myanmar, and Italy. This paper hopes to provide a new vision for further studies on the symbiotic relationship between *D. immitis* and *Wolbachia,* providing an advance in the therapeutic and diagnostic approach. There is no report of molecular identification for *Wolbachia spp.* isolated from *D. immitis* in northern Iran. So, to fill this study gap, herein we determined the prevalence and phylogenetic analysis of *W. pipientis* strains isolated from *D. immitis* of canids in northern Iran.

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1. Introduction

Filarial nematodes are the main causative agents of zoonotic vector-borne diseases such as lymphatic filariasis. Lymphatic filariasis is a neglected tropical disease that is an important human infection [1]. Many cases of zoonotic filariae infections are reported globally.

These cases have been reported everywhere, especially in temperate regions. With the exception of the eyes, these worms usually reside in human tissue locations that resemble those of their native animal host. Humans have been reported as final hosts to a variety of filariae, including species of *Brugia*, *Onchocerca*, *Dipetalonema*, *Loaina*, and *Dirofilaria*. Filariae worms have been extracted from the central nervous system, lymphatics, heart, and lungs, as well as subcutaneous tissues. It goes without saying that, in the right circumstances, nearly every animal that can spread filarial disease may infect humans and cause some degree of recovery. Future research is anticipated to reveal more filariae species that have been isolated from humans [2].

Mosquitoes of the genera, *Anopheles*, *Aedes*, *Mansonia*, or *Culex* are known as the intermediate hosts and vectors of species that cause lymphatic filariasis. *Aedes aegypti* and *Culex quinquefasciatus* are prevalent worldwide in tropical climates. Zika, dengue, yellow fever, and chikungunya are among the viruses known to be spread by *Aedes aegypti*. Lymphatic filariasis, West Nile virus, and St. Louis encephalitis are among the diseases that are known to be transmitted by *Culex quinquefasciatus*. Both *Culex* and *Aedes* mosquitoes are intermediate hosts and vectors for dirofilariasis diseases [3,4].

Canine cardiopulmonary and subcutaneous dirofilariasis are thought to be mostly caused by zoonotic mosquito-borne filarial worms, *Dirofilaria immitis*, and *Dirofilaria repens*, respectively [5]. Canine heartworm, or *D. immitis*, is a parasite that affects dogs and other canids all over the world. Numerous cases of this infection have also come from other animals, including horses, seals, beavers, muskrats, bears, nutrias, and domestic and wild cats. Adult worms mostly reside on the right side of the heart, where they create microfilariae that move through the peripheral circulation. The parasite moves to the right side of the heart after developing in the subcutaneous tissues for around three months. Most parts of the world have reported cases of *D. immitis* infections in humans, indicating that the parasite is enzootic. Most dogs with *D. repens* infection are asymptomatic, despite the fact that it is recognized as a cause of canine subcutaneous dirofilariasis.

Furthermore, because of the skin and subcutaneous symptoms, which might include uncomfortable or painful nodules or swellings that are temporary, this illness is thought to be a possible zoonotic disease for humans in endemic places. Moreover, live nematodes may produce acute symptoms if they penetrate the conjunctiva [2,6]. *Wolbachia* is an endosymbiotic bacterium that is allegedly present in more than 20% of all insects, mosquitoes, arthropods, and nematodes globally. *Wolbachia* transmits through the cytoplasm of insect egg cells.

This bacterium causes cytoplasmic incompatibility between infected males and uninfected females, which leads to phenotypic alterations in host eggs and increases the transmission of the infection in females by eradicating male embryos. Consequently, the percentage of female-infected individuals rises in the following generation. *Wolbachia* can decrease the capacity for various pathogens in the infected vectors; for example, mosquitoes infected with transgenic bacteria harboring a *Wolbachia* protein have shown lower vectorial capacity for *D. immitis* [7]. Consequently, *Wolbachia*-infected mosquitoes are suggested to be used in biological control for vector-borne diseases. In the infected nematodes, *Wolbachia* acts as a compulsory mutualistic bacterium. These bacteria live inside the cells of nematodes, and they are necessary for the survival, reproduction, and development of the parasite by providing vital metabolites to the host [8-10].

Wolbachia provides flavin adenine dinucleotide, riboflavin, nucleotides, and heme for infected nematodes, whereas the parasite provides crucial amino acids for the growth of *Wolbachia* [11]. *Wolbachia* improves the development of the infected nematodes by promoting the progression of the L1 larva to the L3 larva and accelerating the transmission of the L3 larva to the adult worms [12].

Wolbachia has been employed as an anti-filarial treatment approach for infections caused by dirofilariasis in recent years. The symbiotic relationship between *Wolbachia* and nematodes represents an advance in eradicating and controlling filarial infection by targeting *Wolbachia* with antibiotics [8]. The administration of antimicrobial medications like doxycycline and ivermectin can prevent *D. immitis* from undergoing embryogenesis, which will lead to the sterility of the female worms. Thus, this component can be utilized to kill nematodes in the management and treatment of dirofilariasis [12].

Wolbachia spp. are endosymbionts of filarial nematodes, including *D. immitis*, and can be considered a target for the treatment of dirofilariasis [1,8,9,13]. This paper hopes to provide a new vision for further studies on the symbiotic relationship between *D. immitis* and *Wolbachia* providing an advance in the therapeutic and diagnostic approaches. Previous studies have confirmed that northern Iran is an endemic region for Dirofilariasis. However, there is no report of molecular identification for *Wolbachia* spp. isolated from *D. immitis* in northern Iran. So, in order to fill this study gap, herein we determined the prevalence and phylogenetic analysis of *W. pipientis* strains isolated from *D. immitis* of canids in northern Iran.

2. Materials and Methods

2.1 Study area

A descriptive-analytical cross-sectional study from March 2019 to April 2020 in Guilan province (37. 2774 N, 49. 5890 E), north of Iran, was conducted. Sampling consisted of carnivores that were involved in car accidents on the main roads and highways of Guilan province in four main regions: north, south, east, and west. This province is located on the southern shores of the Caspian Sea, which is surrounded by the Alborz Mountains in the south and covers an area of 14,042 km2 with a mild and subtropical climate. Guilan province receives about 104.23 millimeters of mean annual rainfall and comprises 33.73% of rainy days. The humidity in this region is about 71.02%, which experiences a considerable rise in autumn and winter, while in summer and spring, it decreases moderately [14].

2.2 Sample collecting

Totally, 32 carnivores (12 *Canis familiaris* and 20 *Canis aureus*) that were involved in car accidents on the main roads of Guilan province were collected. Samples were gathered from 13 cities in different locations in Guilan and transferred to the parasitology and microbiology laboratories of Guilan Medical School. Demographic data, including carnivore species, sex, and location of carcasses, were reported. After the abdominal cavity was opened, the diaphragm muscles were cut. The thoracic area was opened with the help of rib scissors before the heart and pulmonary arteries were carefully inspected for *D. immitis* infection. After the *D. immitis* worms were identified based on their morphological characteristics, they were kept in 70% ethanol for molecular and phylogenetic studies of *Wolbachia* infection [14].

2.3 Molecular analysis

In order to extract DNA, nematodes extracted from infected jackals and dogs were triple-washed in distilled water. Following the manufacturer's recommendations, the extracted total genomic DNA was kept at −20 °C after being extracted using Yekta Tajhiz Azma DNA extraction kit. 30 µL quantities of 2X red PCR premix (Ampliqon, Odense, Denmark), 20 pmol of each primer, and 1 µL of extracted DNA were used for the PCR. A 967 bp fragment of the 16S rRNA gene was amplified using the forward primer (5′-GAAGATAATGACGGTACTCAC-3′) and the reverse primer (5′-AGCTTCGAGTGAAACCAATTC-3′) for the 16S ribosomal RNA gene. The PCR conditions for the 16S rRNA gene were as follows: an initial denaturing step of 95 °C for 5 min and 35 cycles, a subsequent denaturing step of 95 °C for 1 min, an annealing step of 52 °C for 45 s, and 50 s of extension at 72 °C, with a final extension at 72 °C for 5 min. After loading the PCR products in 1.5% agarose gel, electrophoresis was conducted, and by using a UV document (UVITEC, Cambridge, UK), specific bands were finally observed. Subsequently, the pcr products were transferred to a genetic company (Pishgam Biotech Company, Tehran, Iran) for Sanger DNA sequencing.

2.4 Phylogenetic analysis

Chromas version 2.6.6 (Technelysium Pty Ltd., Brisbane, Queensland, Australia) was used to trim and modify the sequence findings before they were compared to the reference sequences in the GenBank using the BLAST tools (http://www.ncbi.nlm.nih.gov/). The 16S rRNA gene's sequences were also added to the GenBank database (Accession Numbers: OR939250 and OR947699 for the 16S rRNA gene). Along with the reference sequences in the GenBank, phylogenetic analysis was also carried out on the sequences acquired in this study. The MEGA 6.0 program was used to execute a Tamura-3-parameter model and the Maximum-Likelihood method. Based on a thousand replications, the phylogenetic trees with bootstrap values were deemed reliable.

3. Results

In this study, 9/12 dogs (75%) and 7/20 jackals (35%) were reported with *D. immitis* infection. All 16 infected cases (100%) harbored a *Wolbachia* infection. We confirmed the presence of about 967 bp *Wolbachia*-specific sequences in all 16 DNA extracts. One sample from infected dogs and one sample from infected jackals were molecularly analyzed in the phylogenetic tree.

In this study, no genetic divergence within the specimens of *W. pipientis* was achieved based on the partial 16S rRNA gene. The inter-species distance rate within sequences of *Wolbachia* in this study and those submitted in the GenBank was 0 to 2.1% for the 16s rRNA gene. The BLAST analysis based on the 16S rRNA gene indicated that sequences of *W. pipientis* obtained from canids (OR939250 and OR947699) in this study had 100% homology with *Wolbachia* collected from *D. immitis* in the USA (AF088187), Russia (MN200331), Myanmar (ON259766), and Italy (Z49261). Furthermore, our sequences presented 97.9% similarity with *Wolbachia* isolated from *D. repens* in Croatia, Lithuania, and Russia (KY114937, MK050782, and MN200313, respectively). Moreover, pairwise distances based on the partial 16S rRNA gene between our sequences of *W. pipientis* and *Wolbachia* isolated from *Onchocerca* in Italy (FR827937), *Wolbachia* isolated from *Onchocerca* in the UK (CU062464), and *Wolbachia* isolated from *Onchocerca* in France (KX853433) were 1.6%, 1.6%, and 1.2%, respectively.

The 16S rRNA gene phylogenetic tree (Figure 1) revealed that all *W. pipientis* isolates obtained from *D. immitis* of infected canids in this study were placed

along with other *Wolbachia* isolates obtained from *D. immitis* in the USA, Russia, Myanmar, and Italy in one clade with high statistical support of 99% (AF088187, MN200331, ON259766, and Z49261, respectively). This clade was classified as a sister taxon of *Wolbachia* isolated from *D. repens* in Croatia, Lithuania, and Russia (KY114937, MK050782, and MN200313, respectively).

4. Discussion

Many classes of arthropods and nematodes can potentially harbor *W. pipientis*, and approximately 20–70% of them may be infected with this bacterium [15-18], as it is frequently reported in mosquitoes, butterflies, sand flies, fruit flies, fleas, ticks, bugs, wasps, silkworms, filarial nematodes, and canine heartworm [19-23].

Wolbachia has a vast distribution worldwide in filarial nematodes [24]. This endosymbiont was previously reported in 30.6% of dogs with filariasis infections in the Mediterranean region. In this study, the prevalence of *W. pipientis* collected from *D. immitis* of canids was in accordance with previous studies conducted in Brazil with a 100% infection rate. *Wolbachia* spp. DNA was detected in all blood tests obtained from dogs with dirofilarial infections in Brazil [25,26]. Moreover, *Wolbachia* has also previously been reported from *W. bancrofti*, *O. volvulus*, *Brugia malayi*, and *Setaria tundra* [16,27-29]. Dirofilariasis in canids has been reported from various regions of Iran with different rates of infection, from 0.9% in Esfahan to 78.6% in Guilan [30-32], but no previous studies were conducted aiming for the prevalence and phylogenetic analysis of *W. pipientis* isolated from *D. immitis* of canids in northern Iran. Our results shed new light on the molecular characterization of *W. pipientis* isolated from *D. immitis* of canids in northern Iran, where, zoonotic dirofilariasis is endemic. In our study, the results of phylogenic analysis of the 16S rRNA gene obtained from *W. pipientis* of *D. immitis* infected canids demonstrated no generic differences. Moreover, comparing sequences obtained from *W. pipientis* in this study and those submitted in the GenBank revealed an interspecies variation of 0-2.1%. Based on the 16S rRNA gene phylogenetic tree, sequences obtained in this study clustered with the major *W. pipientis* group, including isolates obtained from various hosts and regions in the Americas, Europe, and Asia continents. In this study, 16S rRNA sequences had 100% similarity with previously submitted *W. pipientis* sequences from the USA, Russia, Myanmar, and Italy. Our findings, along with those of other genes submitted to the GenBank, indicate low genetic differentially based on the 16S rRNA gene among *W. pipientis* isolates in different countries.

We speculate that *Wolbachia* spp. ancestors had an association with the nematodes of the phylum filariae, and this relationship was highly likely generated over time. Because of the vast distribution of *Wolbachia* spp. in nematodes of the phylum filariae, it is unknown whether the absence of *W. pipientis* in uninfected nematodes is because of an ancestral behavior of this bacterium or is regarding the removal of the specific sequence over time as a result of low transpositional activity in some filarial species [27]. Several questions remain unanswered in these criteria, and there is abundant room for further progress in determining host-parasite relationships. Phylogenetic studies conducted on *Wolbachia* spp. illustrate that this endosymbiont bacterium may have evolved along with ancestors of the nematodes of the phylum filaria.

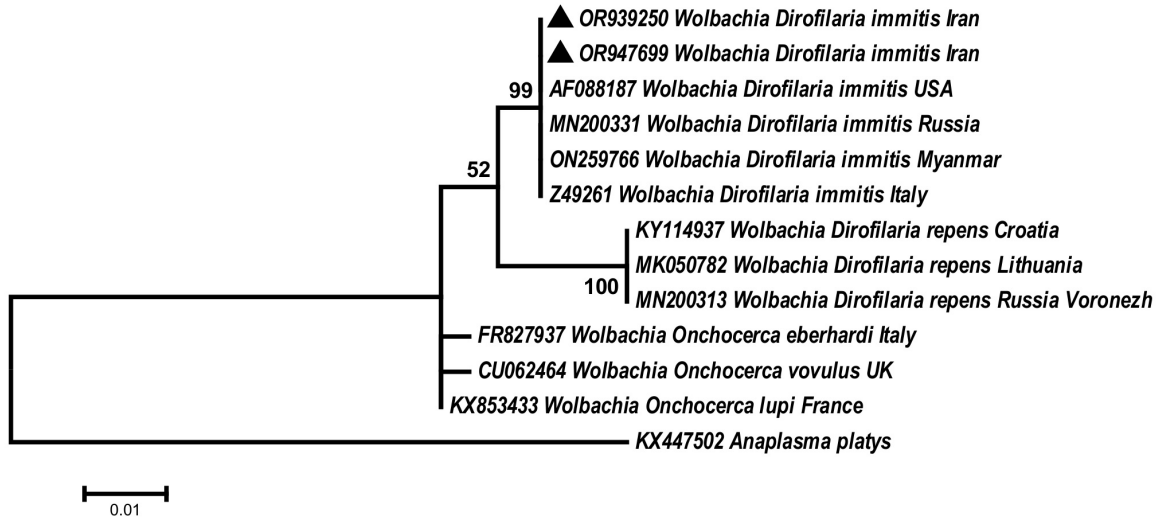

Figure 1. Phylogenetic tree of 16S rRNA gene sequences of the W. pipientis isolated from D. immitis in this study (▲) and reference sequences obtained from GenBank. The tree was constructed based on the maximum likelihood method and the Tamura three-parameter model in MEGA6 software.

The results of this study confirmed a high percentage of *Wolbachia* spp. isolated from *D. immitis* in carnivores in northern Iran, so it is highly recommended that the transmission risk of the zoonotic filarial nematodes be communicated to doctors and veterans in the region. Our study provided the molecular characterization of *W. pipientis* based on the 16S rRNA gene, without any polymorphism among the two nematodes in the different hosts.

Providing further molecular data as well as gathering more isolates from different hosts in other regions would be useful for achieving a better comprehending of molecular differentiation between *Wolbachia* species. Another implication of this study is the possibility of *Wolbachia* being a target for treatment and biological control for dirofilariasis and other mosquito-borne diseases since this method of prevention is harmless, economical, and more effective.

Authors' contributions

Methodology, Conceptualization, Supervision and Funding Acquisition: MS, MH, HS. Data Curation and Molecular Analysis: FA. Visualization, Validation and Study Design: MH, EM. Investigation, and Phylogenetic Analysis: AM. All authors were involved in Writing original draft, Review, and Editing. All authors read and approved the final manuscript.

Conflict of interest

No potential conflict of interest was reported by the authors.

Ethical declarations

The study design was approved by the Ethics Committee of Guilan University of Medical Sciences, Iran (IR.GUMS.REC.1399.335). The authors confirm that they have adhered to ARRIVE Guidelines to protect animals used for scientific purposes.

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