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Morphological and molecular identification of *Dirofilaria immitis* from Jackal (*Canis aureus*) in North Khorasan, northeast Iran

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ABSTRACT

Background & objectives: The heartworm *Dirofilaria immitis* is an important mosquito-borne zoonotic nematode of domestic and wild mammals throughout the world, causing cardiopulmonary dirofilariasis. This parasite has been reported from carnivores in some provinces of Iran. However, in the present study, the occurrence of this filarial nematode is reported for the first time in wild canids of the North Khorasan Province, located in northeast Iran, based on morphological and molecular characteristics.

Methods: The carcasses of 45 golden jackals (*Canis aureus*), 16 foxes (*Vulpes vulpes*), 15 dogs (*Canis familiaris*), and one wolf (*Canis lupus*) were necropsied between 2013 and 2014.

Results: By gross examination, adult filarial nematodes were found in the cardiovascular system of four jackals (8.9%). The morphological characteristics of the recovered heartworms were compatible with *D. immitis*. DNA sequencing of the cytochrome c oxidase subunit I (cox1) gene of all four isolates was identical, showing 100% homology with several sequences registered in GenBank from other countries. No adult *D. immitis* was found in any of the other animals examined.

Interpretation & conclusion: D. immitis is circulating in wildlife of the study area, suggesting the relevance of developing control programmes to prevent transmission of the disease to humans and domestic animals.

Key words Cox1; *Dirofilaria immitis;* homology; Iran; Jackal

INTRODUCTION

The heartworm *Dirofilaria immitis* is an important mosquito-borne zoonotic nematode, affecting wild and domestic canids, felids, and humans in tropical and temperate regions throughout the world[1]. In humans, immature worms cause pulmonary dirofilariasis in the absence of circulating microfilariae[2]; however, in canids and felids, heartworms reach sexual maturity, usually resulting in cardiopulmonary dirofilariasis[1]. The vectors are females of various mosquito species of the Culicidae family. During a blood meal, infectious larvae penetrate the host's skin, and following sexual maturity in the pulmonary artery and right ventricle of canine hearts, female adults release microfilariae into the blood stream. The lifecycle of *D. immitis* in any given area depends on the presence of suitable vector species, which makes transmission and distribution of dirofilariasis susceptible to global climate change[1].

In Iran, *D. immitis* has been reported in canids from different parts of the country[3-5], and in humans as case reports[3, 6]. Nevertheless, no information is available from the northeastern part of the country, which has a favourable climate for sustaining filaroid nematodes. Therefore, the aim of the present study was to investigate the occurrence of *Dirofilaria* species in wild canids of North Khorasan Province and differentiate isolates by molecular characterization.

MATERIAL & METHODS

Study area

This study was conducted in North Khorasan Province (37.47 °N, 57.33 °E), a temperate region with cold winters, comprising area of 28,434 km^2 and situated in northeast Iran (Fig. 1). It is one of the three provinces created after the division of Great Khorasan in 2004[7]. Bojnurd is the center of the province.

Sampling

Sampling included 77 carcasses of canids either collected from the roads following car accidents or provided

Fig. 1: Map of the study area: (a) Map of Iran; and (b) Map of North Khorasan Province.

by the Vector-borne diseases research center (VDRC), Bojnurd, Iran, after examination for leishmaniasis as part of another research project carried out in that center. Samples were collected from October 2013 to March 2014. The study was reviewed and approved by the Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran.

Parasitological examination

Carcasses were first examined for the presence of any cutaneous or subcutaneous nodules. Next, they were necropsied at the VDRC Laboratory, and tissue samples were taken and stored in 70% ethanol in separate containers and shipped to the School of Public Health, Tehran University of Medical Sciences (TUMS) for examination, detection, and identification of helminths. Tissue samples were examined grossly for the presence of adult *Dirofilaria* species.

In the event of finding filarial nematodes, these were removed gently and washed in normal saline. After clearing of filarial worms in lacto-phenol solution, the morphological and morphometric characteristics of the males and females were determined, using a light-calibrated microscope equipped with a camera lucida drawing tube[8].

Molecular analysis

For DNA extraction, adult filarids preserved in 70% ethanol were used. A sample from each isolate was first thoroughly washed twice in sterile distilled water to remove the ethanol. Total genomic DNA was extracted using the DNA Extraction Mini kit (GeneAll, Korea) according to the manufacturer's instructions. Genomic DNAs were eluted and stored at –20°C until PCR amplification.

For PCR amplification, one primer set was used in this study, including the CO1 forward primer intF (5'-TGA TTG GTG GTT TTG GTA A-3') and the CO1 reverse primer intR (5'-ATA AGT ACG AGT ATC AAT ATC- 3') for amplification of the cytochrome oxidase 1 gene (cox1) in the mitochondrial genome[9]. All PCRs were carried out in a final reaction volume of 25 µl, consisting of 12.5 µl of PCR mix (2x Master Mix RED Ampliqon, Denmark), which included 1.25 U Taq DNA polymerase, 0.5 µM of dNTPs, and 1.5 mM $MgCl_2$; 25 pmol of each primer, and 5 µl of template DNA. The temperature profile included one cycle of 94°C for 5 min (primary denaturation), followed by 30 cycles of 94°C for 30 sec (denaturation), 52°C for 45 sec (annealing), and 72°C for 60 sec (extension), followed by a final extension step of 72°C for 7 min. A sample containing water instead of template DNA was included in each run as negative control. PCR products were analyzed using a 1.5% TBE (Tris 0.09M, Borate 0.09M, EDTA 0.02M) agarose gel and stained with FluoroDye Fluorescent DNA Loading Dye for loading and detecting DNA markers (SMOBiO DM3100). Electrophoresis was carried out at 90 V for 1 h. PCR products were visualized using a UV Transilluminator (UVItec, EEC) and digitally photographed.

All PCR products representing the *cox1* gene were purified using the AccuPrep Gel purification kit (Bioneer, Korea) according to the manufacturer's instructions. Purified products were subjected to unidirectional sequencing using the forward primer employed in the PCR. Nucleotide sequences were compared with NCBI GenBank sequences using BLAST queries (*http://www.ncbi.nlm.nih.gov/*). Multiple alignments of sequences of the *cox1* gene were compared to related reference sequences. Identity and similarity of the sequences were determined using the BioEdit software version 7.0.9[10].

RESULTS

Parasitology

Overall, 77 canids from North Khorasan Province, including 45 jackals (*Canis aureus*), 16 foxes (*Vulpes vulpes*), 15 dogs (*Canis familiaris*), and one wolf (*Canis lupus*) were necropsied in the study. Among these, four of the jackals (8.9%) were found infected with *D. immitis* (Table 1). No filariae were found in any of the other canids. In all infected jackals, heartworms were localized in the right ventricle and atrium of the heart (Fig. 2).

The rate of infectivity in male and female jackals was 9.1% (3 out of 33) and 8.3% (1 out of 12), respectively, and so there was no statistically significant difference between males and females of jackals in terms of infection with *D. immitis*.

Considering the age of the jackals, none of the cubs (< 2 yr-old) were infected with heartworm; however, three out of 35 young jackals (2–5 yr-old) and one out of 5 old

Table 1. Gender, age group, and worm burdens of jackals infected with *Dirofilaria immitis*

S. No.	Gender	Age group (yr)	Total number of *D. immitis* recovered	Sex of *D. immitis*	
				Males	Females
1.	Male	≥ 6	5	1	4
2.	Male	2–5	10	4	6
3.	Male	2–5	5	1	4
4.	Female	2–5	1	0	1

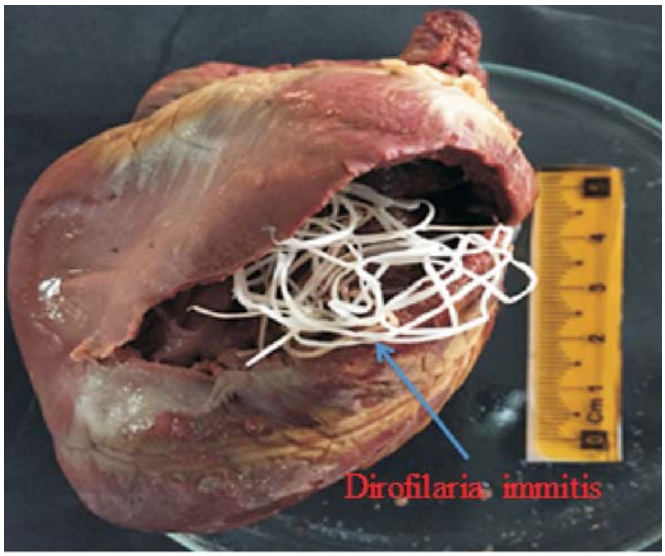

Fig. 2: Adult heartworm (*Dirofilaria immitis*) in a jackal heart.

jackals (≥ 6 yr-old) were found infected with *D. immitis* (Table 1).

Male and female heartworms were distinguished based on morphometric and morphological characteristics[8] (Figs. 3–5). As indicated in Table 1, the range in total worm burden was 1–10, with a mean of 5.25 for each infected jackal. In all four infected jackals, the number of female *D. immitis* was higher than that of males (Table 1).

Molecular characterization

The four isolates of *D. immitis* were characterized by sequencing of the *cox1* gene. For all isolates, amplicons of about 689 base pairs (bp) were successfully produced by PCR. Sequences were compared with other available sequences in GenBank. All four isolates were identified as *D. immitis*, being identical with each other, and showing 100% homology with sequences available in the NCBI database representing isolates from other geographical areas in the world, *e.g.* Hungary (KM452920, KM452921), Bangladesh (KC1078050), China

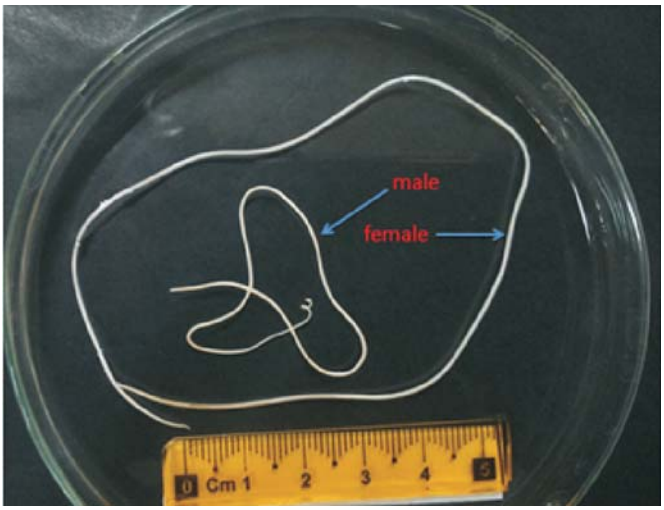

Fig. 3: Adult *Dirofilaria immitis*—Male is shorter, with a spirally coiled posterior end; the female is larger and straight on both ends.

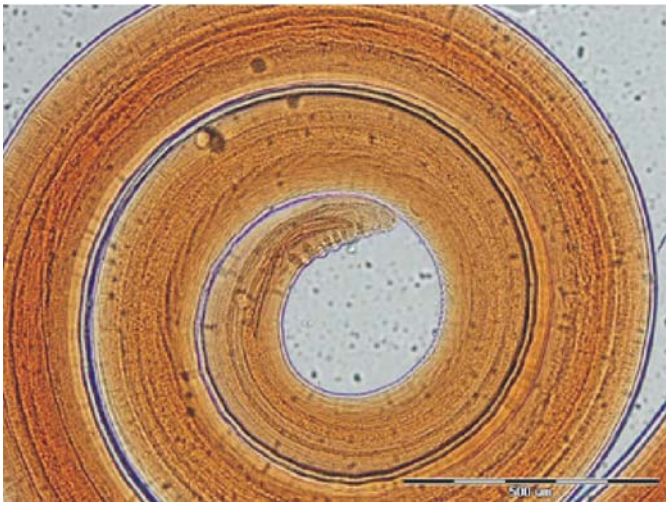

Fig. 4: Spirally coiled posterior end of a male *Dirofilaria immitis* showing spicules and pre-anal papillae.

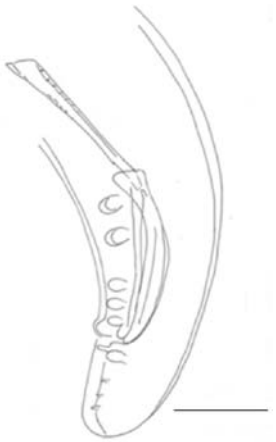

Fig. 5: Camera lucida drawing of the tail end of a male *Dirofilaria immitis* showing spicules and pre-anal papillae. Scale bar = 100 µm.

(EU159111), Italy (DQ358815), and Australia (AJ537512). The cox1 sequences of North Khorasan Province isolates obtained in the present study were registered in NCBI GenBank with accession numbers KT351849–KT351852.

DISCUSSION

Dirofilaria immitis is a cosmopolitan parasite, infecting domestic dogs, cats, and humans, as well as wild animals. From an epidemiological perspective, dirofilariasis is considered as an emergent parasitic disease of humans and animals[1]. It is more prevalent in areas with temperate and tropical climates. There are also reports on the existence of heartworm in jackals in some European countries[1]. This parasite has previously been reported from canines in different regions of Iran with variable rate of infections[3-5], and also from humans in sporadic case reports [3, 6], from the same areas from which animal infections have been reported. However, this study provides the first report on the occurrence and molecular characterization of *D. immitis* isolated from wildlife in North Khorasan Province, which has favourable climatic conditions for sustaining the life cycle of the parasite.

Overall, 8.9% of the jackals were found to be infected with adult heartworm. No filariae were found in the 16 foxes, 15 stray dogs, and one wolf necropsied, may be partly owing to lower numbers of carcasses available for study from each of these canid species. In a similar study on adult *D. immitis* in wild carnivores from Serbia, 7.32% of the jackals studied were infected; rates of infection in red foxes and wolves were 1.55 and 1.43%, respectively[11].

With respect to the sex of the jackals and infectivity with *D. immitis*, no statistically significant difference was found between males and females (9.1% *vs* 8.3%). Similarly, in a study in north Iran, microfilaraemic stray dogs did not exhibit any statistically significant difference with regard to sex[4]. Likewise, in northeastern India, infectivity with *D. immitis* in dogs was independent of the sex of the dogs, as evidenced by microscopy and immunological tests[12]. Meanwhile, the prevalence of adult *D. immitis* in jackals in Serbia was higher in males (10%) than in females (4.06%)[11].

In the present study, the worm burden in jackals ranged from 1 to 10; and in all four infected jackals, the number of female *D. immitis* was higher than that of males. The mean worm intensity among infected jackals was 5.25, with a sex ratio of 2.5:1 in favour of females. These results are in concordance with the results of a study carried out on sex ratio in *D. immitis* obtained from naturally infected dogs[13]. Accordingly, in *D. immitis*, sex disequilibrium exists at lower worm intensities (presumably conferring a species survival advantage), but disappears at higher worm intensities[13].

Molecular characterization of all four North Khorasan isolates of *D. immitis* involving the *cox1* gene showed 100% homology with sequences already deposited in NCBI database from several other geographical areas in the world. Cox1 sequences of *D. immitis* from dogs in Serbia showed 100% identity to Italian isolates[14]. Characterization of *D. immitis* isolates from dogs in a northeastern state in India based on the ITS-2 region also showed close identity to isolates from South Asia[12]. These findings indicate low genetic variability among *D. immitis* isolated to date; on the contrary, *Dirofilaria repens* shows more pronounced intra-species variability, which fits with its biological feature[14].

CONCLUSION

In conclusion, *D. immitis* is circulating in wildlife of the study area, and initiation of control programmes to prevent transmission of the disease to human and domestic animals appears relevant. Similar to the emergence of cardiopulmonary dirofilariasis in European countries[15] and other geographical areas in the world[1], reports of dirofilariasis in Iran during last decade have increased[4-6]. Global warming and its impact on the spread of vector-borne parasites is an issue of concern. Further, research on animal reservoirs and appropriate entomological studies for identification of mosquito vector species and their potential for transmitting the disease to domestic animals and humans, in areas with suitable climate for the maintenance of the parasite life cycle, are recommended.

Conflict of interest

The authors declare that there is no conflict of interest.

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