SHORT COMMUNICATION

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A cross sectional study on *Dirofilaria immitis* and *Acanthocheilonema reconditum* in sheepdogs in a western region in Iran

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Abstract

Iran is one of the endemic areas of Dirofilariasis, and also one of the most important zoonotic infections. *Dirofilaria immitis* causes a severe and fatal disease called heartworm disease in dog. It also produces pulmonary nodules in humans. The worm is to be investigated as a potential infection of humans and animals in various provinces in Iran. In this research, the samples were studied with modified Knott's test and molecular method. The results of the modified Knott's test method indicated that 14.00% of sheepdogs were infected with filarial microfiler. The microfilers were characterized with basic morphological features, the length of the infective larva and tail ending. There was an estimated prevalence of 4.45% for *Dirofilaria immitis* and 9.55% for *Acanthocheilonema reconditum* microfiler. To verify the differential diagnosis, molecular method was performed using PCR with *Dirofilaria* specific primers for amplification of ITS2 locus. Gene locus sequencing results of *D. immitis* and sequence alignment recorded in GeneBank showed 97.00% similarity, and relatively 98.00% similarity was observed in *A. reconditum.* The results of the molecular method confirmed the result of modified Knott's test method. Low infection with *D. immitis* was observed the region, probably due to the fact that the annual temperature and precipitation in this area were not suitable for the proliferation of the vector mosquitoes. In general, there was less infection in the region compared to regions with relatively similar climatic conditions. Hence, the results suggested that alternative diagnostic tests are required to determine the occult infections.

Introduction

The distribution of canine filarioids depends on the presence of the vector, climate conditions such as temperature, relative humidity and precipitation, density of human population and the presence of other canid populations that serve as reservoirs for these filarioids.[1]

Dirofilaria immitis in the dog is very important, because this worm can cause severe and lethal disease called heart worm disease and also it has a role as a zoonotic disease.[2] In addition, considering the importance of dogs in animal husbandry, it is important to study the contamination in livestock areas.

Acanthocheilonema reconditum normally resides in the subcutaneous tissue of dogs. A small percentage of adult worms can also be found in the peritoneal cavity of dogs.[3] Adult *A. reconditum* rarely causes clinical signs, although pruritus, alopecia and dermal abrasions may occur in heavy infestations. Many symptoms attributed to this parasite are usually the result of concurrent parasitism.

Disease caused by *Dirofilaria immitis* causes pulmonary and subcutaneous nodules. In Iran, at least 12 cases of human infection have been reported with *Dirofilaria* infection and a case of periocular dirofilariasis has been diagnosed in a young woman.[4] Another report also presents a case of orbital dirofilariasis with lateral rectus muscle involvement.[5] Overall, to date, there have been several reports worldwide of lung and heart infection with *Dirofilaria* including Brazil, Italy, France, Greece, Spain, Ukraine, Russia, USA, Australia, Japan and Thailand.[6]

In suburban Victoria, Australia a case of *Acantho-cheilonema reconditum* in a 62 year-old white man's eye was reported.[7]

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Using modified Knott's test method *D. immitis* microfilers are usually separated from other microfilers. In addition, this technique does not require advanced equipment. Use of molecular methods has been the focus of attention in the diagnosis of *Dirofilaria* microfilers in recent years. These methods are sensitive and accurate for identifying different filarial worm's microfilers that infect dogs. This method is used in cases that microfilers are not detectable in terms of morphology or the numbers of samples are very low. Despite the accuracy and reliability of the method, it is more expensive and requires a longer time compared to other diagnostic tests.

The purpose of this study was to determine *Dirofilaria* distribution in Hamadan province, west of Iran, with modified Knott's test and molecular method.

Materials and Methods

Study Area. Samples were collected from Hamadan province, Iran. Hamadan province is located in the western part of Iran (Fig. 1). Hamadan is located east of the Zagros mountain range at an altitude of 1,800 meters (34.7608° N, 48.3988° E). Hamadan has a steppe climate with large fluctuations in temperatures between day and night and, summer and winter.

Samples collection. A total number of 157 blood samples based on the estimation of 10.00% infection in the dog populations were collected from June 21, 2012 until September 21, 2012 based on the randomized cluster sampling from sheepdogs of Hamadan province during the hours of 17:00-21:00, including the cities of Malayer (55), Nahavand (40), Hamadan (10), Bahar (20), Razan (25) and Tuyserkan (7). Sheepdogs' age and sampling area were recorded then blood samples were collected via cephalic venipuncture and transferred to EDTA tubes.

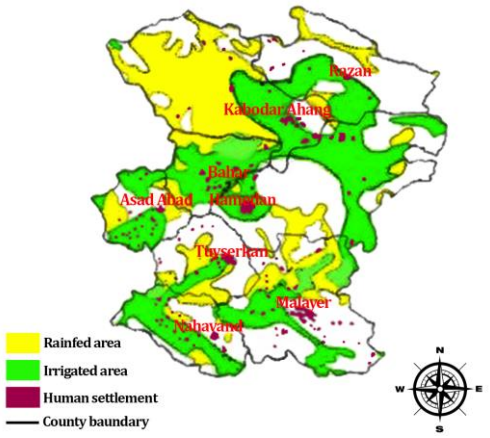

Fig. 1. Hamadan topographic map. Temperature, humidity and precipitation are almost identical in all areas. Sampling was performed in some cities of Hamadan: Malayer, Bahar, Razan, Tuyserkan, and Nahavand. It should be noted that these areas are of great importance in the animal husbandry industry.

Modified Knott's test method. For Knott test, 1.00 mL was taken from blood with 9.00 mL of 2.00% formalin, mixed in a test tube and shaken slowly until blood (containing EDTA) had mixed well with formalin. Then, the samples were centrifuged at 1,500 rpm for 5 min. The supernatant was discarded slowly and the sediment was stained adding one drop of 1.00% methylene blue and examined under light microscope at 40× magnification.[8] Of the positive samples, on average, 10 samples were traced and then measured. The results were then analyzed using SPSS software (version 18.0; SPSS Inc., Chicago, USA).

Molecular method. In the present study polymerase chain reaction (PCR) and sequencing method were used for the differential diagnosis of *Dirofilaria* and *Acantho-cheilonema* species based on the ITS2 Locus sequencing.

DNA extraction. DNA was extracted from 500 µL of blood. Using DNA extraction kit and following the manufacturer's instructions (CinnaGen, Tehran, Iran), isolation of ITS2 genomic DNA from the microfilers of *D. immitis* and *A. reconditum* was performed. For determining the species, PCR amplification and sequence analysis for ITS2 gene were performed in 100 µL volumes containing 2.00 - 2.50 µL DNA sample, 44.50 - 45.00 µL double distilled water and 50.00 µL master mix (Amplicon, Pullman, USA), which contained 1.50 µmol of each primer including F: 5′GGTGAACCTGCGGAAGGAT C3′ and R:5′GC GGGTAATCACGACTGAGT3′ for *D. immitis, and* F:5′CAGGT GATGGTTTGATGTGC3′ and R: 5′CACTCGCACTG CTTCACT T C3′ for *A. reconditum.*

The PCR program was an initial denaturation step at 94.00 ˚C for 5 min followed by 38 cycles of 94.00 ˚C for 45 sec (denaturation), annealing for *D. immitis* at 52.80 ˚C, *A. reconditum* 51.10 ˚C for 45 sec, extension at 72.00 ˚C for 45 sec and final extension at 72.00 ˚C for 5 min. For detection of the PCR amplicons, 8.00 µL of the PCR products was separated by1.50% agarose gel, electrophoresis was done and products were stained with Red safe dye. The PCR products were purified using quick PCR products purification kit (CinnaGen). The DNA sequencing was performed in both directions for each of the PCR products by Takapouzist Co. (Tehran, Iran), based on the Sanger's method. The sequence chromatograms were analyzed using the Chromas software (version 3.1; Technelysium PTY Ltd., Queensland, Australia) and compared to those registered in GeneBank using the Basic Local Alignment Search Tool (BLAST).

Results

The results of the modified Knott's test and molecular analysis are presented in two parts as follow.

In modified Knott's test, out of the 157 samples collected, a total number of 22 cases (14.00%) were

infected with microfiler, 15 out of 22 cases (9.55%) were infected with *A. reconditum* and seven cases (4.45%) were infected with *D. immitis* (Table 1). Sheepdogs were classified according to age into four categories (Table 2). Results were analyzed using SPSS software (version 18.0). Mean length of microfilers for *A. reconditum* was 228.16 ± 12.08 and for *D. immitis* was 311.79 ± 9.83 µm. The minimum length of *A. reconditum and D. immitis* microfilers were 212 to 280 µm, respectively. The maximum length of *A. reconditum and D. immitis* microfilers were 252 and 328 µm, respectively. Comparison of mean lengths of *D. immitis* and *A. reconditum* microfilers indicated statistically significant differences ($p < 0.01$).[9, 10]

In this study, 22 samples that were positive in modified Knott's test were also examined with molecular method. Twenty-five samples which were negative for microfiler with the modified Knott's test were tested again using PCR. Overall, the results of the modified Knott's test were confirmed with the results of DNA sequencing.

After ITS2-rDNA amplification, nucleotide sequence of ITS2 gene locus was identified in *D. immitis* and *A. reconditum* species (Fig. 2).

After sequencing, the chromatogram of the samples was analyzed by Chromas software and the results were compared to information released in GeneBank. Sequences results showed the difference between *D. immitis* and *A. reconditum,* because the size of the bands was different, therefore, could be distinguished with simple PCR.

The Comparison result map of BLAST sequence of *D. immitis* of Hamadan province was compared to China and India ITS2 locus. The alignment showed high sequence homology between sequences. *D. immitis* sequenced in this study showed 99.00% homology with GeneBank records. It should be noted that, sequences recorded in GeneBank had about 5.00 to 8.00% differences (Fig. 3).

The Comparison result map of BLAST sequence of *A. reconditum* in Hamadan province and India, Taiwan, Tunisia and Turkey and other data from Iran showed 98.00% sequence homology between sequences. Only five sequences of *A. reconditum* have been deposited in GeneBank (Fig. 3). The part of sequencing from *Acanthocheilonema* ITS2 with sequences recorded in GeneBank showed 98.00% similarity.

Table 2. Number of infected sheepdogs with *D. immitis* and *A. reconditum* in the various cities of Hamadan province.

Samples	Age (year)			
	< 1	1 - 2	2 - 3	> 4
Infected cases	-	4	8	10
Non-infected cases	29	36	40	30

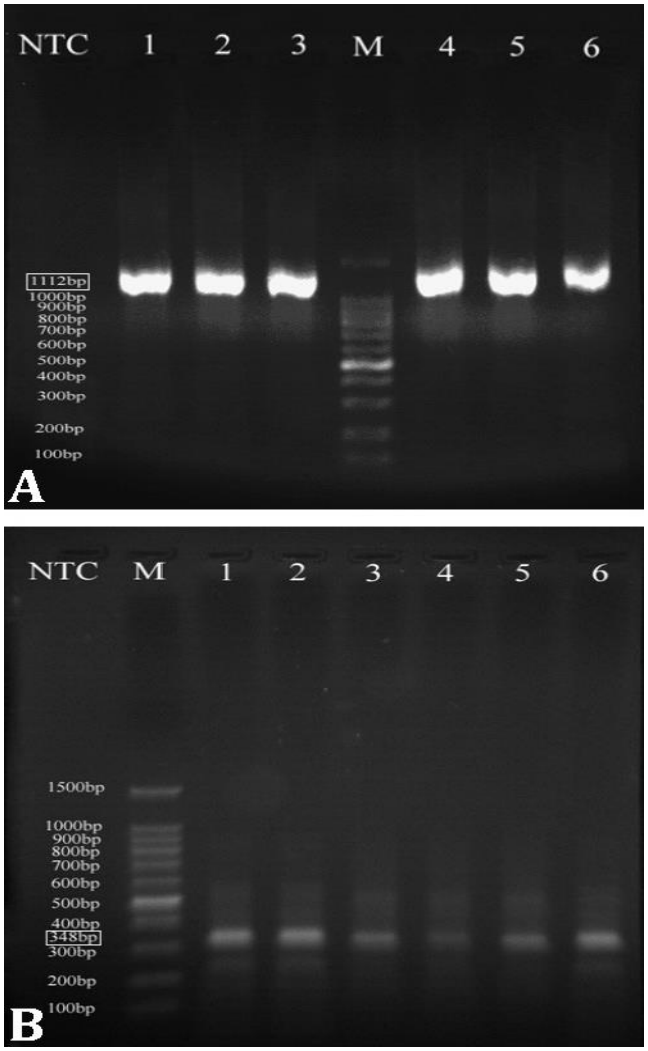

Fig. 2. A) Gel electrophoresis of 1112-bp PCR product of ITS2 locus *D. immitis*. NTC: no template control, lanes 1, 2 and 3: samples number show *D. immitis* positive results, M: 100-bp DNA marker, lanes 4, 5 and 6: samples number show *D. immitis positive results.* **B)** Gel electrophoresis of a 348-bp PCR product of ITS2 locus *A. reconditum*. NTC: no template control, M: 100-bp DNA marker, Lanes 1 - 6 show *A. reconditum* positive results.

Table 1. The percentage of infection in the various cities of Hamadan province.

Area	No. samples	Positive samples infected with *D. immitis*	Positive samples infected with *A. reconditum*
Malayer	55	4 (7.27%)	9 (16.00%)
Razan	25	1 (4.00%)	4 (16.00%)
Hamadan	10	1 (4.00%)	-
Bahar	20	1 (4.00%)	-
Nahavand	40	0(0.00%)	2 (5.00%)
Tuyserkan	7	-	-
Total	157	7 (19.27%)	15 (37.00%)

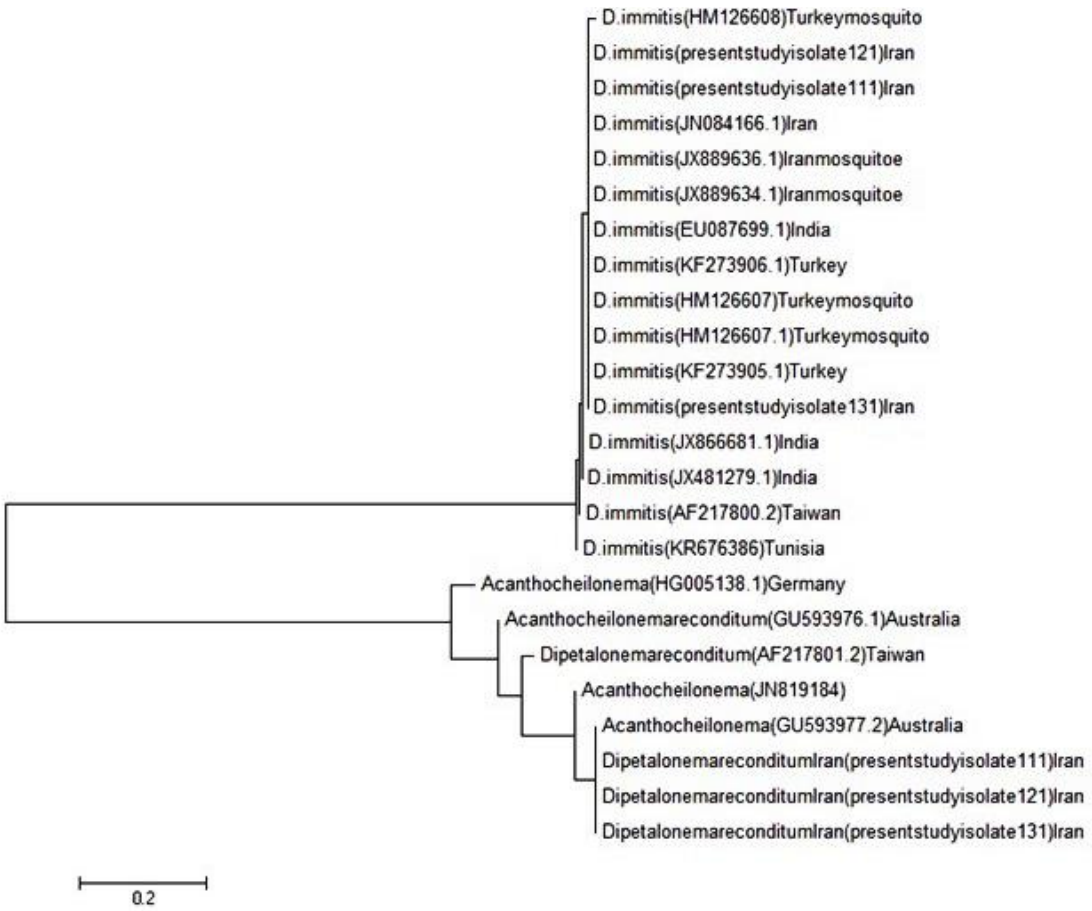

Fig. 3. Phylogram of data of locus ITS2 of *D. immitis* and *A. reconditum* using Genetic Distance method and NJ algorithm.

Discussion

According to zoonotic infection between human being and animals, these parasites, *A. reconditum* and *D. immitis* are very important in endemic areas and annual testing is essential for detection, treatment and infection control.[11]

Fundamentally, a *D. immitis* adult stage is pathogenic. It is the most common infection in tropical, subtropical and temperate climates.[12] Dirofilariasis is one of the most important vector-borne infections by more than 60 species of mosquitoes.

Dirofilaria infection in endemic areas is expanding. Iran is one of the endemic areas of this infection. In Iran, *D. immitis* infection of a dog was first reported in 1970. So far, in different areas of Iran, *D. immitis* has been determined using the parasitology (i.e. Knott method) and serology methods. Infection with this parasite has been detected in most regions except for Mashhad. Microfiler is present at night (11:00 PM till 1:00 AM) in the blood, however, there is a kind of alternation that varies according to different countries, hours and seasons, which is usually most viewed in the afternoon and summer season. In Iran, in a study carried out by Ranjbar Bahadori and Eslami, an increase in the number of microfilers counted in 1 mL of blood at 1:00 AM (3316 microfilers) and decrease at 12:00 noon (668 microfilers) were observed.[13] These results were consistent with the studies conducted by Eslami and Meshgi on infected dogs in Tehran and Tabriz.[14] In this study, samples were collected in the afternoon as well as in the summer season.

Also, the percentage of infection with *D. immitis* in dogs with different uses and in different regions of Iran is as follows: In the west of the Caspian Sea (51.40%), the east of the Caspian Sea (7.69%), Urmia, West Azarbaijan province (24.80% in sheepdogs), Tabriz, East Azarbaijan province (8.40% in urban and rural dogs), Meshkinshahr, Ardabil province (34.60% in sheepdogs), Shiraz Fars province (9.50%) and Ahvaz, Khuzestan province (11.70% in sheepdogs), Tehran, Tehran province (2.00%), and Garmsar, Semnan province (12.29% in stray dogs) infection has been seen.[9,15-19] As it can be seen, the percentage of infected sheepdogs with *D. immitis* in Hamedan province was lower than that of the sheepdogs of West Azarbaijan province (Urmia), Ardabil (Meshkinshahr) and Khuzestan provinces (Ahvaz).

The most important factor in the epidemiology of this parasite is the weather conditions. Comparison of the weather of the mentioned areas indicated that the rate of

temperature in Hamadan province was less than Tabriz city (8.40%), with cold weather and dry climate, however, it was greater than Meshkinshahr city (34.60%), with cold and relatively dry weather, while the annual precipitation and humidity in Meshkinshahr was higher than Hamadan.

It seems that among the factors mentioned, precipitation and humidity levels are more important than the other climatic factors. This topic compares infection in the east of the Caspian Sea (7.69%) with temperate weather, wet climate and less annual precipitation and to the west of the Caspian Sea (51.40%) with temperate weather, wet climate and more annual precipitation.[9,16,17]

In this study, out of 157 samples taken a total number of 22 cases (14.00%) were infected with microfiler, out of which 15 cases (9.55%) were infected with *A. reconditum* and seven cases (4.45%) with *D. immitis*.

In morphometric results, mean length of *D. immitis* microfilarias was 311.79 ± 9.83 µm and *A. reconditum* was 228.16 ± 12.08 µm. The results of microfiler length in this study were consistent with other studies in Iran and other countries. The results showed the same sensitivity of both methods. However, the specificity of the molecular method was higher.

In general, the age of infection was different in dogs, in most of the areas the infection was determined between 3 and 15 years old. In this study, dogs examined, in terms of age groups were divided into four categories (< 1, 1-2, 2-3, ≥ 4), the highest percentage of infection was seen in the age group of four years.

The presence of microfiler in the blood represents infection, however, the important point is that about 30.00% of infected dogs with adult worms did not have any microfiler in their circulatory system. Thus, the sensitivity of modified Knott's test and molecular tests was not enough and showed false negative result. There can be a definite reason for the absence of infection. However, modified Knott's test is cheap and a relatively fast, and remains to be used as a good diagnostic method.

The ITS2 sequences between *D. immitis* and *A. reconditum* have common sequences as well as different sequences, in this study an ITS2 sequence was used for differentiation. Due to the differences, the existence of strains within *D. immitis* was possible, which requires further evaluation and the results of sequencing has confirmed that *A. reconditum* has the same genus and species in different parts of the world. So far, in GeneBank, ITS2 gene sequences of *Dirofilaria* and *Acanthocheilonema* from Iran have not been reported. Infection levels in Iran are different in the various areas. The highest rate of infection with *D. immitis* is from Gilan province (51.40%) in stray dogs and the lowest prevalence in Mashhad city (0.00%) has been reported. Also, infections in tropical regions such as Shiraz and Ahvaz cities in sheepdogs or rural dogs with different uses were reported to be 9.50% and 11.70%, respectively.[9,18]

Nevertheless, infection with *Dirofilaria* in carnivores has not been studied in many parts of Iran. Infection rate, 8.40%, in dogs in Tabriz city with lower temperatures and humidity are almost identical compared to Hamadan province. Also, in Meshkinshahr infection rate of 34.60% in dogs was determined. Contamination of the above mentioned areas in Hamadan province was considerably low. However, further sampling is needed to establish a more accurate status of this infection. Having established the importance of this issue, greater research is needed with regard to the prevalence of infection in the country and its transmission and distribution.

Conflict of interest

The authors declare that there is no conflict of interest.

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