First molecular evidence of *Hepatozoon canis* infection in *Rhipicephalus sanguineus* (sensu lato) ticks in Iran

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Abstract

Background

Hepatozoon canis is a protozoan that is transmitted by the ixodid ticks. Ingesting the tick or a section of the tick organ which is infected by the mature oocysts containing infectious sporozoite is the main source of infection in dogs. Canine hepatozoonosis infects dogs in Iran, but the vector for *Hepatozoon* protozoa from Iran has never been demonstrated. The present study aims to detect *H. canis* in dogs and vector ticks in Iran.

Methods

During the period of 2018–2019, Blood samples and ixodid ticks were collected and examined using microscopical, molecular, and serological methods. A total of 246 blood samples were collected from the cephalic vein of pet, stray, and shelter dogs (103 stray, 99 shelter, and 44 pets) of both genders and varying ages in Northwest of Iran.

Results

Microscopically, infected neutrophils with *Hepatozoon* spp. were detected in 5 of 246 (2.03%) thin stained blood smears with low parasitemia. Indirect immunofluorescent antibody test (IFAT) was used to test the serum samples and antibodies against *H. canis* were detected in 31 (12.6%) of the serum samples. Molecularly, 23 out of 246 (9.34%) blood samples were found to be infected with *H. canis*. A comparison of the results of 3 diagnostic methods demonstrated a good agreement between IFAT and PCR and a poor agreement between microscopical examination with IFAT and PCR. There was no significant difference in different age groups and sex of sampled dogs. However, stray dogs had significant infection rate of than pets and shelter ones. In body inspection, 141 adult ticks (31 partially engorged females, 26 fully engorged female and 84 fed males) were collected from examined dogs and all ticks were belonging to species of *Rhipicephalus sanguineus* (*sensu lato*). Positive reaction to *H. canis* was observed in the genomic DNA of the 7 ticks (4.96%). A BLAST analysis of obtaining sequences isolated from both dogs and ticks indicated a 99–100% similarity with *H. canis* 18S rRNA gene sequences in GenBank. This is the first study in Iran to detect *H. canis* in *R. sanguineus* tick.

Background

Canine hepatozoonosis is a tick-borne disease of domestic and wild canids that is caused by *Hepatozoon canis* and *Hepatozoon americanum* [1]. The first report of hepatozoonosis in canids caused by *H. canis* was in India in 1905 and then it was found in other countries [2]. But *H. americanum* was first noticed predominantly in the southern United States as distinct specie [1]. *H. canis* causes subclinical infection whereas the Canine hepatozoonosis caused by *H. americanum* can be fatal in some cases [3–

5]. Canine hepatozoonosis has been seen in different countries of Asia, Europe, Latin America, and Africa [6].

Dogs are mainly infected by ingesting infected ticks. In the vector, infective oocysts of parasite which has numerous sporocysts with sporozoites was produced in the hemocoel [7]. Among various vectors, *Rhipicephalus sanguineus* is regarded as the main biological vector for *H. canis*, but other species can also be considered as vectors. Several definitive hosts have been approved for *H. canis* among which one can name *Rhipicephalus microplus*, *Haemaphysalis flava*, *Amblyomma ovale*, and *Haemaphysalis longicornis* ticks (de Castro Demoner et al., 2013; De Miranda et al., 2014; Murata et al., 1995; Rubini et al., 2009).

The diagnosis of *H. canis* is conducted by microscopic analysis of blood smears stained with Giemsa and it leads to the recognition of *H. canis* gamonts in the neutrophil's cytoplasm and in some rare cases, it is used to identify in monocytes [12]. Various epidemiological examinations of hepatozoonosis in canids have used different serological tests like IFAT, western blot and ELISA [1]. Recent studies have shown that PCR and sequence analysis are providing accurate information regarding the prevalence of protozoan by detecting the DNA of *H. canis* in both the peripheral blood of infected dogs and in infected ticks [1, 13].

The recent parasitological, and molecular surveys have revealed *H. canis* infection in stray dogs from the northeastern and central of Iran [14, 15], but the tick vectors were not determined. In the current study, we used 3 different diagnostic methods, microscopic, serologic, and molecular techniques to investigate the occurrence of *H. canis* in dogs, and from the Ixodid ticks removed from the dogs of Urmia, Northwest of Iran.

Methods

Study Area

The study was conducted from 2018 to 2019 in Urmia, West Azerbaijan province located in northwestern Iran (37° 32' N and 45° 04' E). The study area was approximately 8000 km² (Fig. 1). The study area has witnessed varying temperatures in different seasons in the range of -3.8 to +23.4° C. The area is located on the borders of Turkey and Iraq [16].

Sampling

240 dogs (103 stray, 99 shelter, and 44 pets) were randomly selected. The data regarding the sex, age, tick burden and dog's lifestyle were recorded. Blood samples were collected from the cephalic vein of dogs and placed into EDTA and serum tubes. Furthermore, blood smears were prepared from each blood sample. The whole skin of the dogs was carefully examined for ticks and the collected ticks were kept in 70% ethanol. The obtained samples were maintained under cool conditions prior to being sent to the

laboratory of parasitology, Urmia university. Sera were separated by centrifuging the serum tubes. The tubes of sera and blood EDTA were stored at -20° C prior to molecular and serological examinations.

Microscopical Examination

Stained thin blood smears with 10% Giemsa solution were analyzed to find *Hepatozoon* spp. gamonts. Parasitemia was calculated by counting the number of infected neutrophils in at least 50 examined microscopic fields of vision [14].

Identification of Ticks

141 ticks (84 fed males, 26 fully engorged females, and 31 partially engorged females) were obtained from 99 dogs sampled. Then, the ticks were kept in vials with mesh cover which had plaster bottoms. Morphological features of the ticks were used for the identification of their specimen which was formerly explained by Walker et al. and Estrada-Pena et al. [17,18]. Feeding status and the sex (fed males, semi-engorged females, fully engorged females, unfed males) were the criteria used for classification of the ticks. Table 3 presents detailed information about the ticks used in the current study.

Serology for *H. canis*

The indirect fluorescent antibody test (IFAT) was used to determine the serology of *H. canis* [19]. Blood samples were obtained from dogs and allowed to clot. subsequently, they were centrifuged for the separation of the serum. The obtained serum samples were stored at -20 °C until use. *Hepatozoon* spp. gametocyte antigen for IFAT was prepared by collecting 40 ml of heparinized peripheral blood from an infected dog with 18.5% parasitemia. The method described by Baneth et al. was used to separate white blood cells on Ficoll–Hypaque density gradients [20]. The final pellet was resuspended in PBS containing 1.5% bovine serum albumin (BSA), and thin blood smears were prepared on glass slides by the method of Baneth et al. (1998). Sera were diluted at 1:32 as the cut off titer for IgG seropositivity determined earlier [21,22]. Rabbit-antidog IgG fluorescein conjugate (Sigma-Aldrich, St Louis, MO, USA) was used at 1:100 dilution [19].

DNA extraction, PCR and Sequencing

DNA from the dog's blood and ticks were extracted using a commercial kit [Molecular Biological System Transfer (MBST), Tehran, Iran], following the manufacturer's instructions and kept at -20 °C until use. A pair of primers, HepF (5'-ATACATGAGCAAAATCTCAAC-3') and HepR (5'-CTTATTCCATGCTGCAG-3'), were used to amplify a 660 bp fragment of the 18S rRNA gene of *Hepatozoon* species in dog blood samples and tick's specimen. The primer's specificity and sensitivity and the PCR condition have been described previously by Inokuma et al. (2002). PCR was carried out in 20 µl total reaction volume containing 1 µl (10 pmol) of each primer and 1 µl of extracted DNA, and 16 µl of distilled water were added to a Reddy® to use PCR master mix (Sinaclon Co., Tehran, Iran, Cat. No.: MM2061) with a final concentration of 250 µM of each dNTP in 10 mMTris-HCl, pH 9.0, 30 mM KCl, 1.5 mM MgCl₂, and 1 U Taq

DNA polymerase. Cycling condition for *Hepatozoon* was 95°C for 5 min, followed by 34 cycles at 95°C for 30 sec, 72°C for 5 min and 72°C for 5 min with a final extension step of 72°C for 10 min. The PCR products were separated by electrophoresis on 1% agarose gel in TAE (Tris-acetate-EDTA) buffer and visualized using ethidium bromide and UV illumination [14]. The positive control for *H. canis* was provided from faculty of Veterinary Medicine, University of Tehran, Iran. Distilled water used as negative control in PCR amplification.

To confirm the results and identify species, positive PCR products detected in dogs and ticks were purified and subjected to sequencing with both forward and reverse primers by Sinaclon Co., Tehran, Iran. The sequencing results were analysed using MEGA 6 software and compared with each other and the reference sequence in the GenBank database by Basic Local Alignment Search Tool (BLAST) analysis (www.ncbi.nlm.nih.gov). The phylogenetic tree was constructed using MEGA 6 software based on the maximum likelihood method based on the Tamura-Nei model [23], with bootstrap 1,000 (1,000 replicates). The nucleotide sequences of *Babesia canis* were used as an outgroup (Fig. 2).

Statistical Analysis

The results were analyzed by SPSS software (ver. 21.0). The Chi-square test was used to analyze the relationship between several risk factors including sex, age, and tick burden with the rate of infection in dogs and the values less than 0.05 was considered significant ($P < 0.05$). Cohen's Kappa test was used to evaluate the agreement of three diagnostic methods and the values obtained for Kappa were categorized as low ($0.2 < \kappa < 0.4$), moderate ($0.4 < \kappa < 0.6$), good ($0.6 < \kappa < 0.8$), or excellent ($\kappa > 0.8$) [24]. The method proposed by Kahl et al. was used to analyze the prevalence of infestation, the mean density, and the intensity of infestation of adult ticks on dogs [25].

Results

The results of the study showed that the gamonts of *Hepatozoon* spp. were found by microscopic methods in 5 out of 246 (2.03%) blood smears of dogs and the percentage of neutrophils infected with gamonts was between 0.001 and 0.003 (Fig. 2). IFAT revealed the seropositive reaction in 31 dogs (12.6%). The results of PCR also showed the infection of 9.34% (23/246) dogs with *H. canis* (Fig. 3).

A comparison of results obtained by PCR and IFAT indicated that 21 (67.74%) IFAT-positive sample were also positive by PCR (Table 1). While 10 (32.25%) samples were positive only by IFAT and 2 (9.30%) samples were positive only by PCR. The rate of *Hepatozoon* positive dogs determined by microscopy, PCR and IFAT differed significantly between the three tests ($P < 0.05$). A poor agreement (κ values, 0.252 and 0.335) with a highly significant difference was observed between microscopic examination with IFAT and PCR methods ($P = 0.000$), whereas a good agreement (κ values, 0.751) with a highly significant difference was observed between IFAT and PCR ($P = 0.000$) (Table 1).

Table 1
Comparison of microscopy, PCR, and IFAT results for the detection of *H. canis* infection in dogs from Urmia, Iran

Microscopical examination and serology	PCR		Total No
	Positive No	Negative No	
M(+), S(+)	5	0	31
M(-), S(+)	16	10	26
M(+), S(-)	0	0	0
M(-), S(-)	2	213	215
Total	23	223	246
M, microscopical examination; S, serology.			

Table 2 shows the prevalence of *H. canis* in dogs in relation to their sex, tick burden, age, and the lifestyle. No significant association with sex and age was found for *Hepatozoon* ($P > 0.05$). Positive serology and PCR had correlation with the presence of ticks on dogs ($P < 0.05$). The statistical analysis indicated that the prevalence of *H. canis* in stray, shelter and pet dogs was 15.53%, 5.05% and 4.54%, respectively. As shown in Table 2, the prevalence of *H. canis* is the highest in stray dogs and the statistical analysis revealed significant difference between them ($P < 0.05$).

Table 2
Frequency of *Hepatozoon* infection in dogs from Urmia, Iran, in relation to possible risk factors

Risk factors	IFAT No(%)	PCR No(%)	Total No
Age			
6 m to 1 yr	8 (9.87)	5(6.17)	81
≥ 1 yr	23(13.93)	18(10.90)	165
P value	$\chi^2 = 0.814$, P = 0.367	$\chi^2 = 1.438$, P = 0.230	
Gender			
Male	21(14.58)	14(9.72) b	144
Female	10(12.82)	9(11.53)	78
P value	$\chi^2 = 1.238$, P = 0.266	$\chi^2 = 0.057$, P = 0.811	
Tick infestation status			
Positive	19(19.19)	18(18.18)	99
Negative	12(8.16)	5(3.40)	147
P value	$\chi^2 = 6.533$, P = 0.011	$\chi^2 = 15.249$, P = 0.000	
Dog Lifestyle			
Pet	1(2.27)	2(4.54)	44
Shelter	8(8.08)	5(5.05)	99
Stray	22(21.35)	16(15.53)	103
P value	$\chi^2 = 13.272$, P = 0.001	$\chi^2 = 8.005$, P = 0.018	
Total	31	23	246

During the visual examination, a total of 141 adult ticks were taken from 99 dogs and all of them were morphologically identified as *R. sanguineus (sensu lato)*. 57(40.4%) of collected ticks were females, 84 (59.5%) were males. The prevalence of adult tick infestation, the mean density of adult ticks per examined dogs, and the intensity of adult tick infestation on infested dogs were determined as 40.24%, 0.57, and 1.42, respectively. Table 3 shows the frequency and infection rates of *H. canis* for each tick. Of the 141 tick tested, only 7 ticks (4.96%) were infected with *H. canis* (Fig .3).

The partial sequences of the 18S rRNA gene for *H. canis* which was isolated in this study were submitted to GenBank database and they are recorded with MT810118 (dog), and MT810115 (tick) accession

numbers. A BLAST search performed with the 18S rRNA gene sequences of *Hepatozoon* species isolated from dogs and ticks showed 99–100% similarity with the corresponding *H. canis* isolates deposited in the GenBank database. Phylogenetic analyses revealed that the obtained *H. canis* sequences clustered in the group with *H. canis* sequences and sequences were clearly distinct from *Hepatozoon americanum*, and *Hepatozoon felis* (Fig. 4).

Discussion

H. canis has long been recognized to cause disease in dogs in all over the world [26, 27]. *H. Canis* infection was first reported by Khoshnegah et al. (2009) in an eleven-year-old male dog in Iran. They observed *H. canis* gametocytes inside neutrophils of peripheral blood smears and bone marrow smear in Giemsa stain [28]. Previous studies used molecular and microscopic techniques to investigate the presence of *H. Canis* in Iran [14, 15]. It should be noted that, the vector ticks of *Hepatozoon* species are completely understood in Iran. This study used serological and molecular survey based on PCR amplification and DNA sequencing to recognize the species of *Hepatozoon* in dogs and ticks feeding on the dogs. This is the first report of *H. canis* in dogs and the ticks collected from dogs in northwest of Iran.

In this study, peripheral blood smears were used for identifying *Hepatozoon* spp. gamonts. *H. canis* was microscopically detected in only a small number of blood smears (2.03%) of dogs, which could be due to the low sensitivity of microscopic method. Based on the microscopic examination of blood smears, *Hepatozoon* spp. infection in sampled dogs in the present study (2.03%) is higher than the previous study conducted in Iran (1.6%) [29] which it may be related to the parasitemia rate in sampled dogs [14]. There were some similar studies conducted in different countries which used microscopic examination of dog's blood smears and they showed *Hepatozoon* spp. infection in 2.7% of stray dogs in Thailand [30], 2.3% of stray dogs in India [31], 4.3% of dogs in Colombia [32], 1–10.6% of stray dogs in Turkey [19, 33], 11.6% of pet and stray dogs in Pakistan [34], 23.7% of hunting dogs in Japan [35], 11.3% of rural dogs in Brazil [13], and 43.9% of young dogs that lived in a shelter located in Italy [36].

The seroprevalance of *H. canis* infection in this study was 12.6%. The study conducted by Karagenc et al. (2006) delineated that IFAT was able to detect more positive animals to *Hepatozoon* compared to microscopic methods and PCR. The existing evidence show lower sensitivity of direct observation compared to IFAT, because the investigation of *H. canis* prevalence by blood smear parasitology was only 1%, while IFAT yielded the result of 33.1% [37]. The absence of gamonts in the animals identified as positive with IFAT may be due to low or intermittent parasitemia or arrest of parasite development at the meront stage in visceral organs [3, 37, 38]. It should be noted that antibodies against *Hepatozoon* spp. might be present for several months after parasitemia can no longer be detected [21, 39].

In the present study, *H. canis* was found in 9.34% of the dogs investigated with molecular analysis. The study conducted by Barati and Razmi (2018) in northeast of Iran using molecular analysis found *H. canis* in 8% of the dogs investigated. Climatic difference can justify the difference in prevalence in the two regions. Different occurrence rates of *Hepatozoon* infection in dogs have also been reported in other

countries. Using PCR, the prevalence of *H. canis* infection in dogs has been reported at 9.3% in Haiti [40], 0.7% in the United States [41], 31.8% in Colombia [32], 53.3–79.2% in Brazil [8, 13, 42], 3.6–25.8% in Turkey [19, 33, 43], 5.5% in Palestine [44], 49.7% in India [31], 42.9% in Japan [35], 36.0% in Thailand [30], 20.3% in Nigeria [45], 57.8% in Italy [36], 11.8% in Croatia [46], 50.0% in Czech Republic [47], and 36.0% in Hungary [48]. The differences in reported prevalence might be related to several factors such as the population of tick vector, the methods used for animal management, and the climate [14].

This study is the first in Iran to record *H. canis* in *R. sanguineus* (s.l.) ticks from dogs. It should be noted that the previous studies have mentioned *R. sanguineus* s.l. as the most prevalent species of tick to infest the dogs in Iran [29], but the vector competence of *R. sanguineus* s.l. for *Hepatozoon* protozoa from Iran has never been demonstrated. In the present study, *R. sanguineus* (s.l.) ticks were the only species found in sampled dogs of the study area which could be related to its huge capability to adapt [49–52]. The molecular screening of tick specimens collected in the study area reveals the importance of vector *R. sanguineus* (7/141 ticks positive to *H. canis*). The percentage of positive ticks in a previously conducted study by Aktas et al. [53] was higher (20.58%) compared to that of ours (4.96%; 7/141). In this study, fed males, partially and fully engorged adult females were the only life stages positive to *H. canis*. The results of this study are in line with that of Aktas et al. (2013), but it differs with the results reported by study conducted in Italy which showed the highest positivity to *H. canis* in unfed adults [51].

The frequency of *Hepatozoon* infection in dogs did not significantly vary regardless of age and gender in this study. The results of the study are in agreement with other studies done in the east of Iran [14], Turkey [33], in Japan [35, 54], in Thailand [30], in Pakistan [34], and in India [55], but the results were not in line with two other studies. The study conducted by Aktas et al. (2015) in Turkey claimed that the frequency of *Hepatozoon* infection was significantly related to age of the sampled dogs. Another study in Brazil by de Miranda et al. (2014) showed the influence of gender and age on frequency of *Hepatozoon* infection.

The results of this study showed a correlation between presence of *H. canis* DNA with the presence of *R. sanguineus* in dogs ($P < 0.05$). The results obtained during this study are in agreement with the previous studies and all report that *Hepatozoon* infection has positive correlation with tick's presence [53, 56, 57]. It was also shown that *H. canis* infection in stray dogs (15.53%) assessed by PCR was significantly higher compared to pet and shelter dogs which were 4.54% and 5.05%, respectively. This study proved the relationship between the prevalence of *H. canis* and the dogs' lifestyle. This finding is in line with that of Soltani and Dalimi (2018).

A phylogenetic tree based on 18S rRNA gene in the present study showed that the *H. canis* sequences obtained in this study from both the ticks and dogs (with accession numbers MT810118 and MT810115) clustered with *H. canis* sequences of dogs from eastern Iran and other countries and it was different from the *H. americanum* and *H. felis* clades. This was the first study of the type conducted in ticks of Iran.

Conclusions

In conclusion, this study shows the predominance of *R. sanguineus (s.l.)* in infesting the domestic dogs living in the studied area and it also shows the existence of *H. canis* in these tick populations. As far as we know, our study is the first molecular examination of tick vectors of *H. canis* which has been conducted in Iran. It should be noted that performing transmission evaluations is necessary to confirm the vector capacity of a given tick species. It is also necessary to conduct further studies to identify the epidemiology, and vector ticks of this infection in other parts of Iran.

Abbreviations

IFAT: Indirect fluorescent antibody test; PCR: polymerase chain reaction; BLAST: basic local alignment search tool; ELISA: enzyme-linked immunosorbent assay; EDTA: Ethylenediamine tetraacetic acid; BSA: bovine serum albumin; IgG: Immunoglobulin G; MBST: Molecular Biological System Transfer; TAE: Tris-acetate-EDTA

Declarations

Declaration of Competing Interest

Authors disclose no potential conflict of interests.

Abbreviations

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Authors' contributions

FM and AR contributed in conception, design, data collection, statistical analysis and drafting of the manuscript. FM, AR, MZ and MY, contributed in conception, design, supervision of the study and drafting of the manuscript. All authors approved the final version for submission.

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Availability of data and materials

The datasets analyzed during the present study are available from the corresponding authors upon reasonable request.

Ethics approval and consent to participate

Collection of blood samples from dogs was performed by skilled and trained veterinarians following proper physical restraint of dogs to reduce the potential painful stimuli and ensure both animal safety and personnel. Ethical approval for this study was obtained from Ethics Committee of the Faculty of Veterinary Medicine, Urmia University (approval number: IR-UU-AEC-3/618/AD-6/9/2020).

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

Figure 1

Map of sample collection sites in Urmia, Northwest of Iran

Figure 2

Gamont of *Hepatozoon canis* in the stained blood smear ($\times 1000$)

Figure 3

PCR detection of *H. canis* in dogs and ticks of Urmia, Northwest of Iran. M: 100 bp DNA marker (Fermentas); Lane1, Positive control; Lane 2, *H. canis* positive tick samples; Lane 3, *H. canis* positive dog blood samples; Lane 4, Negative control.

Figure 4

Phylogenetic analysis of *Hepatozoon canis* isolates from dogs and ticks in present study and other isolates based on the partial sequence of the 18S rRNA gene in GenBank. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model. *Babesia canis* was used as an outgroup. The phylogenetic tree was plotted by the neighbor joining method with bootstrap 1,000 (1,000 replicates). Evolutionary analyses were conducted in MEGA6.