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Original Article

A Molecular Study on *Hepatozoon canis* Infection in Dogs in Tehran (Iran)

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ABSTRACT

Hepatozoonosis is a protozoal disease caused by various species of *Hepatozoon*. This parasite is transmitted from tick; the main vector of *Hepatozoon canis* is usually the brown dog tick (*Rhipicephalus sanguineus*). However, several species of ticks are disposed as the alternative vectors. Dogs are usually infected by eating the tick or a part of the tick organ infected by the mature oocysts containing infectious sporozoite. In the current study, a total of 145 blood samples were collected from the cephalic vein of pet, stray, and shelter dogs in Tehran. To conduct this study, first thin blood smears were prepared from all the samples and stained with the Giemsa method. Then, after extraction of DNA from the blood samples, in order to trace *Hepatozoon canis*, the 18S rRNA gene segment of the parasite was amplified using polymerase chain reaction (PCR). To confirm the PCR-positive results, five randomly selected PCR-positive samples were sequenced. According to the results, through direct observation of microscopic slides, no infection of *H. canis* parasite was observed, but according to the PCR results, 32 out of the 145 blood samples were found to be infected by *H. canis*. In this study, infection to *H. canis* in older dogs was higher than in young dogs, and more male dogs were found to be infected by the parasite compared to female dogs; but no significant difference was observed in this regard ($P > 0.05$). Moreover, stray dogs showed a significantly higher rate of infection, compared to the pet and shelter ones ($P < 0.05$).

Keywords: *Hepatozoon canis*, Dog, PCR, Tehran

Une Étude Moléculaire sur l'Infection à l'*Hepatozoon canis* chez les Chiens de Téhéran (Iran)

Résumé: L'hépatozoonose est une maladie à protozoaires causée par diverses espèces d'Hepatozoon. Ce parasite est transmis par des tiques et le principal vecteur d'*Hepatozoon canis* est généralement la tique du chien brun (*Rhipicephalus sanguineus*). Cependant, plusieurs espèces de tiques représentent des vecteurs alternatifs. Les chiens sont généralement infectés après avoir ingérés la tique ou une partie de leur organe infecté par des oocystes matures contenant des sporozoïtes infectieux. Dans cette étude, un total de 145 échantillons de sang a été prélevé à partir de la veine céphalique des chiens de compagnie, des chiens errants et des chiens d'abri dans la ville de Téhéran. Des premiers frottis sanguins ont été préparés à partir de tous les échantillons et colorés avec la méthode Giemsa. Ensuite, dans le but de détecter la présence d'*Hepatozoon canis,* l'ADN des échantillons de sang a été extraite, et le segment du gène de l'ARNr 18S du parasite a été amplifié en utilisant des amorces de réaction en chaîne par polymérase (PCR) spécifiques. Pour confirmer les résultats positifs de la PCR, cinq échantillons positifs ont été sélectionnés au hasard et séquencés. D'après les résultats obtenus, aucune infection reliée directement au parasite *H. canis* n'a été observée à partir des lames microscopiques, alors que les résultats de la PCR montraient que 32 des 145 échantillons desang éxaminés étaient positifs. L'infection à *H. canis* était

plus élevée chez les chiens âgés que chez les jeunes chiens. De plus, il a été constaté que le nombre de chiens infectés par le parasite était supérieur à celui des chiennes; sans pour autant que cela soit statistiquement significatif (p> 0,05). Enfin, les chiens errants présentaient logiquement un taux d'infection significativement plus élevé que les chiens de compagnie et les chiens d'abri (P <0,05).

Mots-clés: *Hepatozoon canis*, Chien, PCR, Téhéran

INTRODUCTION

Hepatozoonosis is a protozoal disease caused by the various species of Hepatozoon; Hepatozoids are parasitic protozoa that infect a wide range of pet and wild carnivorous, birds, reptiles and amphibians (Aktas et al., 2015). More than 300 species of Hepatozoon have been so far identified that 46 species of them are pathogenic for mammals. *H. canis* and *Hepatozoon americanum* are shown in pet and wild canines, but *H. canis* has longer been known as the Hepatozoonosis factor in dogs (Aydin et al., 2015). Compared to the *H. americanum, H. canis* causes few clinical symptoms; however, it is the most common species related to dog's Hepatozoonosis in Europe, Asia, Africa, and Latin America (Eiraset al., 2007; Kaewkong et al., 2014; Mohanchandra et al., 2012). The incidence of Hepatozoon in canines depends on tick; that is to say, it is transmitted by tick. According to some studies, the main cause of *H. canis* infection in dogs is *Rhipicephalus sanguineus* but *Haemaphysalis longicornus* & *Haemaphysalis flava* also may be potential carriers (Baneth & Shkap 1996). The main route of transmission through eating the tick contains mature oocyct of the parasite by dog. The infected dogs mostly show clinical symptoms like fever, lethargy, lack of appetite, pale mucous membranes, anemia, diarrhea, severe wasting, difficulty in moving and severe parasitemia may appear (Cardoso et al. 2014; Kaewkong et al. 2014; Karagenic et al. 2006). The main reason for selecting *H. canis* in this study was the certain nature of infectious diseases in animals and human, and variety of the incidences of these diseases as well as the prevalence of these species related to dog's hepatozoonosis. The mentioned species have not been so far investigated in Tehran in any way. The main advantage of molecular diagnosis is its higher sensitivity in pathogen detection in the peripheral blood and arthropod conveyor compared to the other methods (Andre et al. 2010; Baneth & Aroch 1997). The aim of conducting this study is to examine *H. canis* in the blood samples of dogs in Tehran province by PCR molecular method.

MATERIAL AND METHODS

Blood collection and blood smear preparation. As respects, access to dogs (pets, stray and shelter) isn't easy in Iran, so the best it could be done was collecting 145 dogs, including 42 pets, 28 shelters, 75 strays, 55 males and 90 females during 4 months (from 22.10.2015 until 20.02.2016). According to the studies, another influential factor except of age, gender and the lifestyle, have not been yet known as the main factors that causing infection.They were investigated in terms of the incidence of clinical symptoms of disease and in none of them, the clinical symptoms of disease were observed. About 3 ml of blood was collected from the cephalic vein of all 145 pet, shelter and stray dogs. First, the thin blood smears were prepared from all samples and stained with Giemsa method.

DNA extraction. 100 µl of the blood samples was transmitted to the numbered micro tubes, the same volume of distilled water was added, and after vortex, it was put in a centrifuge device with speed of 2000 RPM for 10 min. Then, the superntant was thrown out (this stage repeated for 3 times). In this stage, 700 µl of DNG-PLUS kit was added to the micro tubes and after vortex shaking and adding 500 µl of propanol then, re-shaking down. It was put in a centrifuge device with

speed of 12000 RPM for 10 min. After the centrifuging has stopped, the supernatant was thrown out. Then, 1 ml of 75% alcohol was added to micro tubes; after vortex shaking, they were put in a centrifuge device with speed of 12000 RPM for 10 min. After that, the micro tube was pouring out (the alcohol adding stage was repeated for 3 times), then the micro tubes were put in incubators in temperature of 95 °C for 5 min and finally, 50 µl of deionized distilled water was added to them and they were put in a freezer in temperature of -20 °C.

PCR reaction with gene-specific primers. the following specific primers were used in order to trace the *H.canis* parasite with the PCR method (Dalimi et al., 2017): Hep F: (5-CAG CAA AAC TGC AAA TGG CTC A-3)

Hep R: (5-GGC AAA TGC TTT CGC AGT AGT TT-3)

The result of amplification is expeced to be 897 bp band of 18srRNA gene-segment of *H. canis*. To perform a PCR reaction, 12 µl of master mix Ampliqon, 0.5µl forward primer, 0.5 µl of reverse primer, 0.5 µl of the extracted DNA, and 11.5 µl of deionized distilled water were added to each microtube obtaining volume of 25 µl. The denaturing process was started in 94 °C for 4 min. Then, 40 cycles: denaturing in 90 °C for 30 s, anealing in 57 °C for 30 s, elongation in 72 °C for 60 s, and final elongation stage in 72 °C for 10 min were conducted and after that, the PCR product was loaded on agarose gel 1%.

Data analysis: in this study, the SPSS v.16 software and X^2 statistical method was conducted for statistical analysis of the research's variables. All data were compared by using Chi-square test with a confidence level of 95% and P-value of at most equal to 0.05 that was statistically significant.

Determining the amplified nucleotide sequences and drawing phylogenetic tree: the obtained sequence through BLAST software (https://blast.ncbi. nlm.nih. gov) was compared with the recorded sequences in GenBank. The MEGA6 software was employed by using Maximum Likelihood and Clustal W2 in order to draw a phylogenic tree and the data extracted from it.

RESULTS

Blood smears. No infection with *the H.canis* was identifiedin the blood smear of the dogs.

PCR. The results of the PCR product indicated that among 145 dogs, 32 dogs were found to be infected by *H.canis*. Following PCR amplification, a positive band with the approximate length of 897 bp appeared in the electrophoresis (Figure 1).

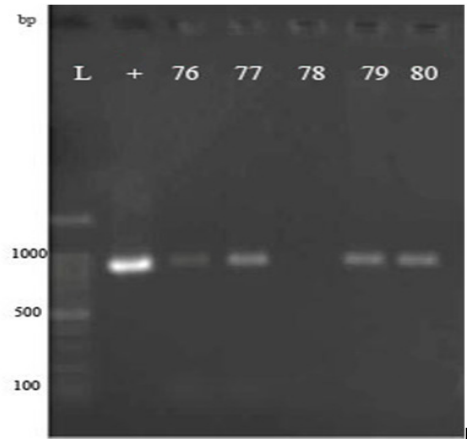

Figure1. PCR products of 18srRNA gene-segment of *H.canis* on agar gel: Lane L: 100bp DNA ladder, Lane +: Positive Control, Lanes 76, 77, 79 & 80:positive samples, Lane 78: negative sample

Dog's gender. The statistical analysis indicated that the prevalence of *H.canis* in male dogs was 30.9% and in female dogs, 16.7 % (Table1). The statistical analysis of data at the significance level of 0.05 was not shown any significant difference (P>0.05).

Table 1. Relative and absolute frequency of the *H.canis*infection in the dogs bygender

Gender	No.	Infected dogs		Uninfected dogs		Significant difference (P value)
		Number	Percent	Number	Percent	
Male	55	17	30.9	38	69.1	
Female	90	15	16.7	75	83.3	0.159
Total	145	32	22.1	113	77.9	

Dog's age. Age was estimated based on dentition and body size, and dogs were classified as young (1 to 12 months old) and old (1-16 years old). The statistical analysis indicated that the prevalence of *H. canis* in old dogs was 26.3 % and for young dogs, 19.3 % (Table2). The statistical analysis of data at significance level of 0.05 was not shown any significant difference (P>0.05).

Table 2. Relative and absolute frequency of the *H.canis*infection inthe dogs by age of dogs

Age*	No.	Infected dogs		Uninfected dogs		Significant difference (P value)
		Number	Percent	Number	Percent	
Young	88	17	19.3	71	80.7	
Old	57	15	26.3	42	73.7	0.159
Total	145	32	22.1	113	77.9	

*young: up to one year old *old: more than one year old

Dog's lifestyle. The statistical analysis indicated that the prevalence of *H.canis*in stray dogs was 32%, in shelter dogs 14.3%, and for pet dogs, 9.5% (Table3). As it is shown in Table 3, *H. canis* in stray dogs had the most frequency among all types. The statistical analysis showed significant difference between them (P<0.05).

Table 3. Relative and absolute frequency of the *H.canis*infection by lifestyleof dogs

Dog Lifestyle	No.	Infected dogs		Uninfected dogs		Significant difference (P value)
		Number	Percent	Number	Percent	
Pet	42	4	9.5	38	90.5	
Shelter	28	4	14.3	24	85.7	
Stray	75	24	32	51	68	0.03
Total	145	32	22.1	113	---	

Phylogenic tree. To confirm the PCR-positive results, five randomly selected representative PCR positive samples were sequenced. A BLAST search performed with the 18S rRNA gene sequences of *H. canis* isolates shared 99–100% similarity to various GenBank sequences (Figure 2). The evolutionary history was inferred using the Maximum Likelihood method, supported by 1000 bootstrap replicates. The numbers above the branches indicate the percentage of bootstrap samplings percentages. The phylogenic tree of *H. canis* drawn for pet, shelter, and stray dogs of Tehran by gene 18srRNA indicated that there was 99% consistency between Number 4 (pet) with KT736298, Number 58 (pet) with KT736298, sample Number 71 (stray) with Number KT736298, sample Number 89 (stray) with Number KT736298, and sample Number 57 (shelter) with the recorded sample Number KC138532. However, all these samples have been reported in the arrangement of phylogenic tree aside *H. canis* from the other parts of the world and are different from *Hepatozoon felis*. Also, these sequences have been submitted with accession numbers KX880502- KX880503- KX880504- KX880505- KX880506 in GenBank.

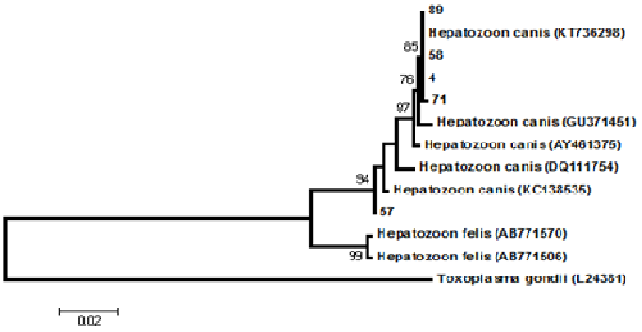

Figure 2. Phylogenetic tree of 4 *H.canis* isolates (4, 57, 58, 71 and 89) of the present study compared to the selected isolates from GenBank, based on 18srRNA gene.

DISCUSSION

The current study was conducted to investigate the infection and prevalence of *H. canis* parasite in the population of dogs in Tehran city. This study is the first research in the field of prevalence of infection with *H. canis* parasite in dogs of city of Tehran. In this research, the prevalence of infection with the *H. canis* parasite was carried out through using PCR technique and preparation of blood smears; totally, 22.1% of the studied dogs were diagnosed infected by PCR technique; while in the examination of blood smears, no infection to *H. canis* parasite was shown. In the similar studies conducted by Aktas et al.(2015), among 285 blood smears of dogs with no clinical symptoms,

only 3 cases were infected by the parasite; while 694 dogs without clinical symptoms, 155 detected positive with PCR molecular method. Aydin et al. (2015), collected 221 blood samples of the stray dogs in Turkey during a three-year period to investigate the *H. canis* in PCR molecular method; 8 blood samples (3.61%) were detected infected by *H. canis* parasite, Cardoso et al. (2014), investigated *H. canis* in 90 red foxes by PCR and histopathologic methods in Portugal; by PCR method, it was found that 68 foxes were infected by *H. canis*. Miranda et al. (2014), collected 346 blood samples from the urban and rural dogs in Brazil in order to evalute the infection to *H. canis*; 17 samples of the urban dogs and 13 samples of the rural dogs were diagnosed positive through PCR molecular method. Farkas et al. (2014), tested the blood samples of 334 red foxes and 15 golden jackals to investigate *H. canis* parasite by PCR method; the obtained results indicated that 26 red foxes (8%) and 9 golden jackals (60%) had been infected by *H. canis*. Amoli et al. (2012), tested 254 blood samples of 51 stray dogs and 203 pet dogs and only 4 cases were diagnosed infected by *H. canis*. In Dalimi et al. (2017) study, twenty-four out of the 104 (23.07%) samples were found to be positive for *H. canis* by molecular detection in dogs of Ardabil province. Hernandez et al. (2013), investigated 91 dogs in terms of infection to *H. canis* and the results of PCR method indicated that 25 dogs were diagnosed positive and by using the direct microscopic method, only 3 dogs were diagnosed positive. Gomez et al.(2010), investigated the blood sample of 300 dogs through microscopic method, including 120 dogs transferred to Hospital of Federal University Veterinary Medicine, 80 dogs transferred to private clinics and 100 shelter dogs which only 7.66 % were diagnosed infected by *Hepatozoon*. Dezctek et al. (2010), studied the protozoals transmitted by tick in the bodies of 191 red foxes by PCR method in Croatia; the results indicated that 30% of samples were infected by the blood protozoa and 23% were infected by *H. canis*. Andre et al. (2010), investigated the probability of infection to *Hepatozoon* species in 165 feliformia cases and 100 canines by PCR molecular method which the results shown that 6 feliformia and 5 canine cases were infected to *Hepatozoon* species. Gimenez et al. (2009), investigated red foxes to determine the probability of infection to *H. canis* by PCR method which the results indicated that 28% of the red foxes were infected to *H. canis* parasite. Sasaki et al. (2007), investigated 400 dogs by PCR method and 81 dogs, 20.3%, were infected by *H. canis*. Karagenc et al. (2004), investigated 349 dogs in terms of infection to *H. canis* through three methods of blood smears, PCR, and IFAT; the obtained results shown that 25.8% of the studied dogs were infected to *Hepatozoon*. In this study, the infection to *H. canis* in old dogs (26.3%) was higher than the young dogs (19.3%); however, no significant difference was observed (P>0.05). While in performed studies, by Aktas et al. (2015), there was a significant difference and the results indicated that the infection of old dogs was significantly more than young dogs. Examinations indicated that the age of the dogs is not influential on the probability of the infection to *H. canis* and this fact confirmed by the study of Rojas et al. (2014) concerning to the lack of significant difference in prevalence of *H. canis* infection in young and old dogs. Thus, factors like the vectors, geographical distribution and the host immune play significant role in the prevalence of infection to *Hepatozoon canis*; So that, infection to *H. canis* in the stray dogs (32%) is higher than pet dogs (9.5%) and shelter dogs (14.3%). The results of the statistical analysis confirms the obtained findings at significance level of 0.05. In this study, however the prevalence of infection in male dogs (30.9%) was more than female ones (16.7%), but difference in terms of gender is not significant and the result is consistent with the studies of Aktas et al. (2015) and Gomez et al. (2010). Regarding to the findings of this study and the other studies, it could be explained that the prevalence of *H. canis* is related to the distribution and density of the conveyor population. Therefore, infection with *H.*

canis parasite in the dogs of Tehran is common and shows that this infection is related to the lifestyle of dogs and has no relationship with their age and gender. Although the research has reached its aims, but there have been some unavoidable restrictions. Because of the time limit, this research was conducted only in small size of population. We collect 145 samples during 4 months, from 22/10/2015 until 20/2/2016, just in Tehran; therefore in order to generalize the results to whole country level was a limitation of the study.

Ethics

We hereby declare all ethical standards have been respected in preparation of the submitted article.

Conflict of Interest

The authors declare that they have no conflict of interest.

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