A Molecular survey of *Hepatozoon canis* in dogs in the Siirt province of Turkey

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Abstract

This study aimed to determine *Hepatozoon canis* prevalence in dogs in the Siirt province of Turkey by the molecular method. The animal material of the study consisted of a total of 75 dogs that appeared clinically healthy. Two ml of blood sample were taken from the vena cephalica antebrachii. Then, DNA extraction was performed. Polymerase chain reaction (PCR) was performed to amplify the 666 bp 18S rRNA gene region of *Hepatozoon canis*. Two positive PCR products were purified and sequenced. As a result of Nested-PCR, *H. canis* specific bands in 666 bp size were obtained in 7 (9.33%) out of 75 dogs. The result of sequence analysis, the nucleotide sequence was registered in the NCBI GenBank database with accession numbers OL467380.1-OL467538.1. *Hepatozoon canis* registered in GenBank of sequence OL467380.1 was found to be similar with other *H. canis* strains of registration numbers MW684292.1 with 99.69% and MH615006.1-MK091085.1-MF797806.1 with 99.53% rates; and the sequence with registration number OL467538.1 was found to be similar to the series MW684291.1 with 99.09% and MH615006.1-MK091085.1-KX 818220.1 with 99.08% rates by BLAST analysis. *Hepatozoon canis* prevalence of dogs in the Siirt province was determined as a result of this study. It is of great importance to take preventive measures, especially to fight ticks with appropriate acaricides, since there is no vaccine to prevent the disease.

Hepatozoon canis, PCR, DNA extraction, sequence

Hepatozoon canis is a tick-borne protozoan parasite classified in the phylum Apicomplexa, closely related to Plasmodium species and pyroplasms (Baneth et al. 2003; Gonen et al. 2004). Canine hepatozoonosis is a protozoal disease that primarily affects dogs, and is caused by *Hepatozoon canis* (O'Dwyer et al. 2001; Baneth et al. 2007). *Hepatozoon canis and H. americanum* are identified as two hepatozoa species that infect dogs (Rubini et al. 2009). Common species associated with canine hepatozoonosis are *H. canis* in Europe, Asia, Africa, and South America (O'Dwyer et al. 2001; Gonen et al. 2004), and *H. americanum* in the United States (Inokuma et al. 2002; Little et al. 2009; Baneth, 2011; Altay et al. 2013). The vector of *H. canis* is *Rhipicephalus sanguineus* ticks, and vectors play an important role in the transmission of the disease (Baneth et al. 2003; Gavazza et al. 2003; Little et al. 2009; Baneth 2011).

Transmission of the infection to dogs occurs by ingestion of ticks or parts of ticks that contain mature *H. canis* oocysts (Gonen et al. 2004). *Hepatozoon canis* infection can range from an asymptomatic condition in dogs with low parasitaemia rates to a serious

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life-threatening illness with fever, lethargy, anaemia, and emaciation in dogs with high parasitaemia (Baneth et al. 2003; Gonen et al. 2004; Baneth 2011). Clinical signs of *H. canis* infection vary depending on the age of the host, the degree of infection, and the presence of concomitant infections, although fever, lethargy, and weight loss are among the most prominent symptoms (Paşa et al. 2009; Altay et al. 2013; Aktas et al. 2015).

Microscopic examination (Elias and Homans 1988; Paşa et al. 2009; Baneth 2011), serological **(**IFAT, ELISA) (Baneth et al. 1996; Gonen et al. 2004; Karagenc et al. 2006; Baneth 2011) and molecular methods (Inokuma et al. 2002; Rubini et al. 2005; O'Dwyer 2011) are used in the diagnosis of the disease. It is reported that molecular methods are more sensitive and specific than microscopic and serological methods in the diagnosis of hepatozoonosis and other blood parasites (Altay et al. 2019).

This study aims to determine *H. canis* prevalence in dogs of the Siirt province by the molecular method.

Materials and Methods

The study area

The Siirt province is located in the Southeastern Anatolia Region of Turkey (Fig. 1). The province is in a semi-arid climate region, where the average highest and lowest temperatures range between 36.9 °C and 18.9 °C in summer, and 8.7 °C and −0.5 °C in winter.

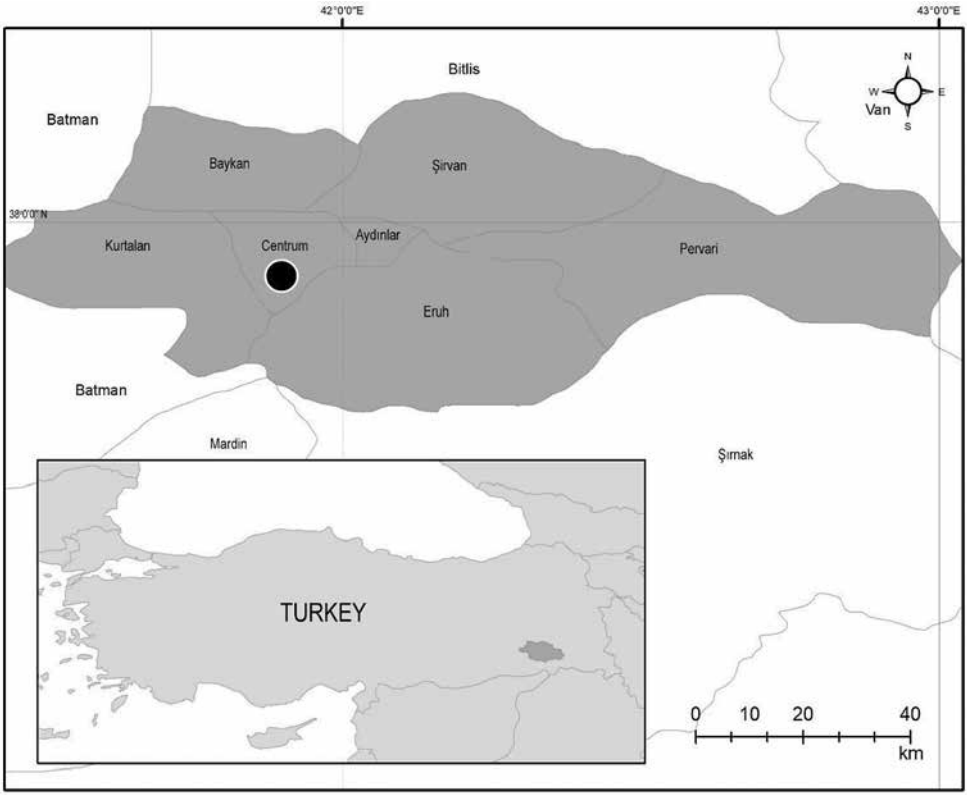

Fig. 1. The Siirt province map. The black circle represents the study area.

Animal material and sample collection

The animal material of the study consists of a total of 75 dogs that appeared clinically healthy. The animals were clinically examined and their age, sex, and presence of ticks were recorded. Blood samples of 2 ml were taken from the vena cephalica antebrachii of dogs into EDTA tubes and brought to the laboratory in a cold chain.

DNA extraction and PCR amplification

DNA extraction was performed according to the kit protocol using the PureLink™ Genomic DNA Mini Kit (USA, K182002). The obtained DNAs were stored at −20 °C until PCR.

Polymerase chain reaction was performed to amplify the 666 bp 18S rRNA gene region of *Hepatozoon canis*. The primers HepF (5'-ATACATGAGCAAAATCTCAAC-3') and HepR (5'- CTTATTATTCCATGCTGCAG-3') were used (Otranto et al. 2011; Dantas-Torres et al. 2012). Two hundred and fifty µM dNTPs, 2.5 mM MgCl in 25 µl mastermix$_2$, 25 pmol forward and reverse primer, 1 U Taq Polymerase, 10X PCR buffer (0.8 M Tris-HCl, 0.2 M $(NH_4)_2SO_4$, 0.2% w/v Tween-20), Nuclease Free Water and 2 µl of DNA were used. The reaction was created by pre-denaturation for 15 min at 95 °C, followed by 34 cycles of denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s, and elongation at 72 °C for 1 min and 30 s with a final elongation of 7 min at 72 °C. The reaction was performed on Gradient PCR, Super Cycler (Kyratec, Australia). Subsequently, 1.5% agarose gel was prepared and stained with RedSafe™ Nucleic Acid Staining Solution. The PCR products were run on agarose gel afterward, and images were obtained on a gel imaging device (Syngene bioimaging system).

Sequence analysis

Two positive PCR products were purified and sequenced. The sequences of each amplicon were manually aligned and edited. 16S rRNA gene sequences were subjected to GenBank's BLAST analysis. The nucleotide sequence was registered in the NCBI GenBank database with access numbers OL467380.1-OL467538.1.

Statistical analysis

The relationship between grouped variables was analyzed using an SPSS V16.0 for the chi-square test. A value of $P < 0.05$ obtained through the analysis was considered significant.

Ethical approval

Ethical approval for this study was obtained from the Siirt University Local Ethics Committee for Animal Experiments (Decision number 2021/01/05).

Results

As a result of Nested-PCR, positivity was detected in 11.11% (4/36) in male dogs and 7.69% (3/39) in females (Table 1). *Hepatozoon canis* specific bands in 666 bp size were obtained in 7 (9.33%) out of 75 dogs in total (Fig. 2). Tick infestation was detected in 41 (54.66%) dogs in clinical examinations. Ticks collected were identified as *R. sanguineus* ticks.

Table 1. Distribution of *H. canis* infection according to age, sex, and presence of ticks.

Variable	Number of dogs (n)	Positive (n)	(%)	P
Sex				
Female	39	3	7.69	NS
Male	36	4	11.11	
Age				
< 1	29	2	6.90	
1–3	30	3	10.00	NS
> 3	16	2	12.50	
Presence of ticks				
Evet	41	5	12.20	NS
Hayır	34	2	5.88	
Total	75	7	9.33	

NS - Non-significant

As a result of sequence analysis, the nucleotide sequence was registered in the NCBI GenBank database with accession numbers OL467380.1-OL467538.1. *Hepatozoon canis* registered in GenBank of sequence OL467380.1 was found to be similar with other *H. canis* strains of registration numbers MW684292.1 with 99.69% and MH615006.1-MK091085.1-MF797806.1 with 99.53% rates; and the sequence with registration number OL467538.1 was found to be similar to the series MW684291.1 with 99.09% and MH615006.1-MK091085.1-KX818220.1 with 99.08% rates by BLAST analysis.

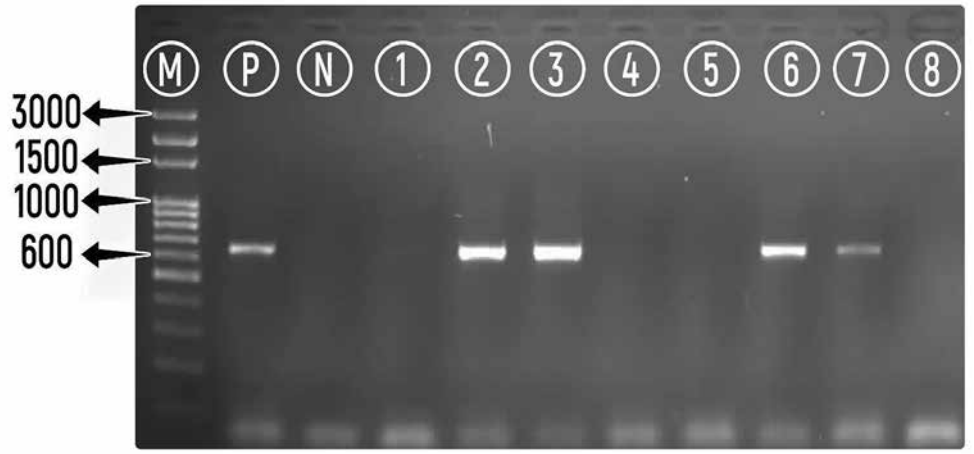

Fig. 2. 18S rRNA amplification of H. canis in dogs using gradient PCR. Lanes: M - marker, P - positive control, N - negative control; lanes 2, 3, 6, and 7 are positive for H. canis (666 bp); lanes 1, 4, 5, and 8 are negative amplifications.

Discussion

Canine hepatozoonosis is a common disease worldwide and has been reported in many countries (Altay et al. 2019). The geographical distribution of the disease-causing species differs depending on the type of tick that transmits the parasite even though canine hepatozoonosis is common all over the world (Altay et al. 2013). *Rhipicephalus sanguineus* type ticks that vectorize *H. canis* (Baneth et al. 2003; Baneth 2011) are common worldwide and they are frequently found in tropical and subtropical regions (Aguirre et al. 2004).

In studies carried out to determine the rate of *H. canis* infection in the world; 22.6% prevalence has been reported for blood smear examination and 67.7% prevalence has been reported for PCR tests in Brazil (Rubini et al. 2005). In other locations, 28.8% prevalence was reported in Kyrgyzstan (Altay et al. 2019), whereas this number was 41.4% in Nigeria (Kamani et al. 2013), 57.8% in Italy (Otranto et al. 2011), 33.1% in Israel (Baneth et al. 1996), and 11.4% in Thailand (Jittapalapong et al. 2006).

First data on canine hepatozoonosis in Turkey was reported in a dog by Tüzdil (1933). In other studies carried out in Turkey, a prevalence of 10.8% was reported in Mersin (Aktas et al. 2015), 2.44% in Hatay (Aslantaş et al. 2020), 2% and 4.9% in Konya and Karaman provinces, respectively (Aydin et al. 2015), 5.3% in Kayseri (Düzlü et al. 2014), 3.9% in Nevşehir (Aktas et al. 2015), 0.5% in Samsun (Bölükbaş et al. 2016), and 18% in Giresun (Aktas et al. 2015). In Sakarya and Kocaeli, 21.5% and 17.4% prevalence rates were reported, respectively (Aktas et al. 2015). A prevalence of 42.8% was reported in Erzurum (Aktas et al. 2015) and 5.3% (Guven et al. 2017), while this number was 25.3% in Elazığ (Aktas et al. 2015). Aktas et al. (2013) also reported 15.87% prevalence in Diyarbakır. Some other results include 3.8% and 49.5% prevalence in Ankara determined by microscopic and PCR methods (Orkun et al. 2018), and 38.46%, 61.54%, and 69.23% in İzmir by microscopic, PCR, and IFAT methods, respectively (Karagenc et al. 2006). Similarly, 3.7%, 14.81%, and 20.37% prevalence was reported in Aydın (Karagenc et al. 2006) for microscopic, PCR, and IFAT methods, respectively. Karagenc et al. (2006) also reported 4.35%, 8.7%, 26.09% prevalence in Manisa and 5.05%, 24.24%, 37.37% prevalence in Muğla for the said methods, respectively.

Microscopic, serological and molecular methods are used in the diagnosis of the disease (Rubini et al. 2005; Karagenc et al. 2006; Baneth 2011). Among these, molecular methods are reported to be more sensitive and specific compared to microscopic and serological methods in the diagnosis of hepatozoonosis, and in other blood parasites as well (O'Dwyer et al. 2001; Otranto et al. 2011; Altay et al. 2019). As a result of this study, *H. canis* specific bands of the size of 666 bp were obtained in 7 (9.33%) of 75 dogs, by the PCR method. The results of this study are similar to the results of other authors (Düzlü et al. 2014; Aydin et al. 2015; Guven et al. 2017). Geographical conditions, vector population, sample size, sampling period, and methods used can be counted among the reasons for the differences observed between studies.

Epidemiological factors such as habitat, environmental conditions, and the presence of the vector are the main factors in the development of *H. canis* infection (Tuna et al. 2020). *Rhipicephalus sanguineus* ticks are the most important vector for *H. canis* in the Mediterranean basin (Baneth 2011; Dantas-Torres et al. 2012; Aktas et al. 2013). The Siirt province is under the influence of a typical Mediterranean climate with mild and rainy winters and hot and dry summers. This situation provides a suitable environment for vector ticks. In this study, a total of 41 (54.66%) dogs were found to be infested with *R. sanguineus* type ticks. No significant difference was found between the presence of ticks and the result on the other hand ($P > 0.05$).

It has been reported that the disease can be seen in both sexes (Gavazza et al. 2003). Some researchers (Paşa et al. 2009) reported that females are more sensitive than males, while others (Gomes et al. 2010; Aktas et al. 2015; Tuna et al. 2020) reported that males are more sensitive than females. In these studies, it is reported that there is no significant difference between the sexes (Paşa et al. 2009; Gomes et al. 2010; Aktas et al. 2015; Tuna et al. 2020). The positivity obtained in this study was found to be higher in males (11.11%) compared to females (7.69%), and no significant difference was detected ($P > 0.05$). The obtained results support the results of other studies (Gomes et al. 2010; Aktas et al. 2015; Tuna et al. 2020).

Canine hepatozoonosis is a chronic disease that can infect dogs of all ages. In the study carried out by Paşa et al. (2009), *H. canis* infected dogs were reported to be between the ages of 2 and 8 years. In a study by Baneth and Weigler (1997), it is reported that the *H. canis* infection is most common in dogs younger than 6 months and dogs aged 5–10 years. In some studies, it has been reported that more positivity is detected in adult dogs than in young dogs (Gomes et al. 2010; Aktas et al. 2015). In this study, dogs were divided into three age groups and the highest positivity was found in those older than 3 years. There was no significant difference between age groups ($P > 0.05$). These results are similar to the findings of other researchers (Gomes et al. 2010; Aktas et al. 2015). The reasons for the higher positivity in elderly dogs compared to young ones can be explained by increased exposure to the vector and cumulative exposure of older animals to the parasite (Aktas et al. 2015).

As a result of the sequence analysis, the sequence registered in the NCBI GenBank database with the accession number OL467380.1 were found to be similar with other *H. canis* strains 99.69% (MW684292.1) and 99.53% (MH615006.1, MK091085.1, MF797806.1) rates and the sequence recorded with the accession number OL467538.1 with 99.09% (MW684291.1) and 99.08% (MH615006.1, MK091085.1, KX818220.1) rates.

In conclusion, *H. canis* prevalence of dogs in the Siirt province has been determined in this study. It is of great importance to take preventive measures, especially to fight ticks with appropriate acaricides, since there is no vaccine to prevent the disease. Clinicians should be aware of this disease as well as others in the differential diagnosis of patients with ticks or tick infection anamnesis. It should be remembered that the elimination

of gametocytes from the peripheral blood is slow and an 8-week treatment is required in the treatment of the disease.

Conflict of Interest

The authors declare no conflict of interest.

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