Molecular Prevalence of Canine Hepatozoonosis in Owned-Dogs in Central Part of Turkey

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ABSTRACT

Canine hepatozoonosis is a tick-borne disease caused by *Hepotozoon canis* and *Hepotozoon americanum*. *H. canis* is widespread almost all over the world, while *H. americanum* exists only in the continental of America. The aim of this study was to determine the prevalence of canine hepatozoonosis from blood samples of 150 owned-dogs in central part of Turkey using polymerase chain reaction (PCR). Sixty seven out of 150 (44.67%) samples were PCR positive for *Hepatozoon* spp. Five out of 67 positive PCR products were sequenced to determine *Hepatozoon* species. Partial nucleotide sequences of 18S small subunit ribosomal RNA gene were compared to *Hepatozoon* spp. sequences registered in GenBank. Nucleotide sequencing resulted in three samples of the Hepatozoon spp.; two samples were determined to be *H. canis*. Five nucleotide sequences detected in this study were deposited in GenBank under accession numbers MW350127, MW350128 (*Hepatozoon canis*), MW350129- MW350131 (*Hepatozoon* spp.). In this study, canine hepatozoonosis infection rate was found to be very high (44.67%), resulting in a high rate of infection among the owned-dog populations in the study areas. To the best of our knowledge, it is the first study on the etiology and epidemiology canine hepatozoonosis in Sivas province in Turkey.

Keywords: Canine Hepatozoonosis; Dog; PCR; DNA Sequences; Sivas; Turkey.

INTRODUCTION

Species of the Hepatozoon genus are apicomplexan parasites belonging to the Hepatozoidae family. This genus has more than 340 *Hepatozoon* species. *Hepatozoon* species infect both domestic and wild animals such as carnivores, reptiles, amphibians, and birds (1, 2). Two species (*Hepatozoon canis* and *Hepotozoon americanum*) of the *Hepatozoon* genus infect domestic and wild canid species causing the disease referred to as canine hepatozoonosis (1, 3).

Hepatozoon canis infection in dogs was first reported from India in 1905. Thereafter this species has been described from almost all parts of the world (4). Other species of canine hepatozoonosis, *H. americanum* was detected in the U.S.A. in 1978. *H. americanum* was thought of as a more virulent form of *H. canis* at that time, but studies revealed that this was a different type of *Hepatozoon* species and named *H. americanum* in 1997 (3).

Hepatozoon canis and *H. americanum* are transmitted to the dogs by hard (Ixodidae) ticks. The main vector of *H. canis* is *Rhipicephalus sanguineus* (brown dog tick). *H. canis* is also transmitted by the tick *R. turanicus* and *Amblyomma ovale* (3, 5). *H. americanum* is transmitted by *A. maculatum* (Gulf Coast tick) in the U.S.A. (6). Most of tick-borne protozoan parasites are transmitted to their host during blood feeding. On the contrary to other tick-borne protozoan parasites, *H. canis* and *H. americanum* are transmitted to the host by

ingestion of ticks or tick parts contaminated with sporozoites (1, 2, 3, 7).

Microscopic, histopathological, serological, and molecular techniques have been used to diagnose canine hepatozoonosis. Microscopic examination is mostly based upon the presence of intracellular ellipsoidal-shaped gamonts within neutrophils and rarely in monocytes in Giemsa- or Wright's-stained blood smears (3, 4, 8). Microscopic examination may be used for identification of *H. canis* infection, but since *H. americanum* gamonts are rarely seen in blood smears, histopathological examination especially immunohistochemical techniques are more sensitive than microscopic technique for the identification of *H. americanum* (2, 3). The histopathology of skeletal muscle of dogs infected with *H. americanum* can be seen in pyogranulomatous myositis and large round or oval "onion skin" cysts containing a central nucleus (2, 3, 4, 7). ELISA and IFAT have been commonly used to diagnose canine hepatozoonosis in large epidemiological studies to determine chronic infections (2, 9, 10).

Molecular identification techniques such as PCR, Real-Time PCR, and DNA sequencing have been preferred for the diagnosis of canine hepatozoonosis (11, 12). These methods have been accepted to be more sensitive and specific than the other methods for the diagnosis of canine hepatozoonosis. Furthermore, molecular methods can detect a small number of pathogen DNA in suspect material (2, 3, 4, 11, 12). Recently, some new genotypes of *Hepatozoon* spp. have been found in dogs using these methods (13).

Canine hepatozoonosis has been described in almost all parts of the world, such as southern Europe, the Middle East, Africa, Southeast Asia, and North and South America (2, 3, 4). The disease has also been detected in different provinces of Turkey (10, 13, 18). To date, to the best knowledge of the authors canine hepatozoonosis has not been investigated in Sivas province of Turkey. The aim of this study was to determine and to identify the presence of *Hepatozoon* species among owned-dogs' population in Sivas province by using PCR and sequencing.

Figure 1. Map of Turkey, location of Sivas. The map was conducted using QGIS (https://qgis.org/en/site/)

MATERIAL AND METHODS

Study Area and Material

Sivas is the second largest province in Turkey with a geographical area of approximately 28,400 km^2, located in the central part of Turkey (Figure 1). The average annual temperature of Sivas is +8.9°C, the average annual precipitation is 432 mm, and the average relative humidity is 65%.

The study was performed on 150 (49 female, 101 male) healthy owned-dogs in five different parts of Sivas (Sivas City Center, Kangal, Yıldızeli, Susehri, and Ulas) (Table 1). The data of the gender and age of each dog were recorded. Approximately eight-milliliter blood samples from the cephalic veins were collected into blood collection tubes containing EDTA for DNA isolation.

Table 1. Comparison of canine hepatozoonosis among gender, age groups, and, sampling area.

	Gander		Age			Sampling Area				
	Female	Male	0-2	3-4	≥ 5	Sivas City Center	Kangal	Ulas	Yıldızeli	Susehir
n	49	101	52	68	30	40	29	28	27	26
p (%)	26 (53.06%)	41 (40.59%)	24 (46.15%)	30 (44.11%)	13 (43.3%)	18 (45.00%)	16 (55.17%)	19 (67.85%)	13 (48.14%)	1 (3.84%)
p-value	p>0.05		p>0.05			p>0.05				p<0.05

n: number of examined dog, p: positive samples,

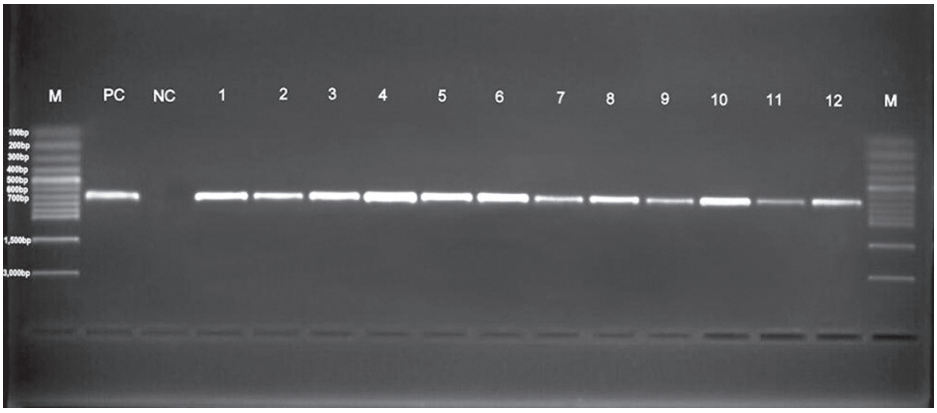

Figure 2. PCR products of 666 bp fragment of the partial region of 18S SSU rRNA, M: Marker, PC: Positive control, NC: Negative control, 1-12: *Hepatozoon* spp. positive dog blood samples.

Total Genomic DNA isolation and Polymerase Chain Reaction (PCR)

Total genomic DNA was obtained from 200 µL blood samples using DNeasy Blood & Tissue Kit (Cat.No.: K0722, Fermentas, Germany) following the manufacturer's instructions. The genomic DNA samples were stored at -20°C until use.

In order to investigate the prevalence of *Hepatozoon* spp., 666 bp fragment of the partial region of 18S SSU rRNA was amplified using Hep-F (5′-ATACATGAGCAAAATCTCAAC-3′) and Hep-R (5′-CTTATTATTCCATGCTGCAG-3′) primers (14). PCR was performed in a final volume of 50 µL of including DNase-RNase-free sterile water (Cat. No.: 129114, Qiagen®, Hilden, Germany), 10× PCR buffer (Thermo Scientific™, Lithuania), 2.5 mM $MgCl_2$ (25 mM) (Thermo Scientific™, Lithuania), 200 µM of each dNTP (Cat. No.: DN0021-1000, GeneDirex®), 1.25 U of Taq DNA polymerase (Cat. No.: EP0402, Thermo Scientific™, Lithuania), 2 µL (10 pmol/µL) of each of the primers, and 5 µL template DNA. The PCR amplification was performed as described by Aktas *et al.* (2013) (15). DNase-RNase-free sterile water (Cat. No.: 129114, Qiagen®, Hilden, Germany) was used as a negative control, the genomic DNA of *H. canis* was used as a positive control for each PCR assay. *H. canis* isolate (accession number: MG917709) obtained in a previous study was used as a positive control DNA in PCR (35). Ten microliters of PCR products were separated on 1.5% agarose gel stained with ethidium bromide and then visualized by UV transilluminator (Figure 2).

DNA purification and sequencing

To confirm and determine the *Hepatozoon* species, five positive PCR products (one product originating from each of the five different sampling areas) were purified with the PCR Clean-Up & Gel Extraction Kit (Cat.No.: NA006-0300, GeneDireX®) fallowing to the manufacturer's instructions. The purified PCR products were sequenced in a commercial Company (BM Labosis, Ankara, Turkey). DNA sequences of partial 18S SSU rRNA gene of *Hepatozoon* spp. were performed using the MUSCLE algorithm of MEGA-X (16). The consensus sequences were compared for similarity with the sequences available in the GenBank database using basic local alignment search tool (BLAST). After the sequences were identified, all consensus sequences were uploaded to GenBank, and accession numbers were obtained.

Ethic statement

Permission was obtained from the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approved number:10.11.2020-454).

Statistical evaluation

Statistical analyses among various parameters were performed using the chi-square test. $p \leq 0.05$ was accepted to be statistically significant.

RESULTS

Hepatozoon spp. was found in 44.67% (67/150) in blood samples of the examined dogs by PCR. The prevalence among female dogs was 53.06% (26/49), among the male dogs was 40.59% (41/101). The prevalence of canine hepatozoonosis was found 46.15% (24/52) between 0 and 2 years of age; 44.11% (30/68) between 3 and 4 years of age and 43.33% (13/30) in dogs ≥ 5 years old (Table 1).

The distribution of canine hepatozoonosis in the sampling area was determined to be 45.00% (18/40) in Sivas city center, 55.17% (16/29) Kangal, 67.85% (19/28) Ulas,

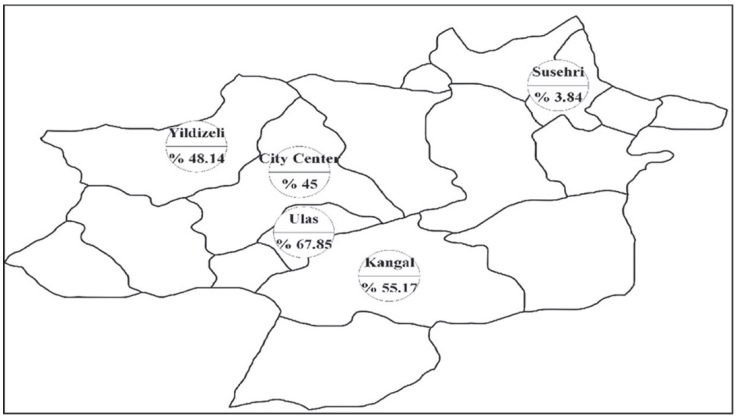

Figure 3. Distribution and prevalence of canine hepatozoonosis in the sampling area. The map was conducted using QGIS (https://qgis.org/en/site/).

48.14% (13/27) Yildizeli, 3.84% (1/26) Susehri (Figure 3). There were found statistically significant differences between Susehri and other sampling areas ($p<0.05$).

Five positive PCR products were sequenced to identify as *Hepatozoon* species. Nucleotide sequences of *Hepatozoon* spp. were aligned with *Hepatozoon* species registered to the GenBank. The results of three of five nucleotide sequences were found to be *Hepatozoon* spp., whereas two of them were identified as *H. canis*. These partial sequences of *H. canis* and *Hepatozoon* spp. 18S rRNA genes were deposited in the GenBank following accession numbers; MW350127, MW350128 (*H. canis*), MW350129-MW350131 (*Hepatozoon* spp.).

BLAST analyses revealed that *Hepatozoon* spp. sequences were 97.65-100% similar to *Hepatozoon* spp. MF identified from Karaman (dog isolate, accession number KF439864), Konya (dog isolate, accession numbers KF439865, KX641901), and Ankara (dog isolates, accession numbers MG254588, MG254589, MG254593, MG254601, MG254612, MG254616, MG254617). *H. canis* sequences were found to be 99.01-99.83% similar to the sequences of dog samples from Turkey (accession numbers KX588232 in Samsun and MG254611 in Ankara), Slovakia (accession number KX761384), Germany (accession number MK757808), Iran (accession number KX880506), Croatia (accession number KT692038), Czechia (accession number KY021184), South Korea (accession number MK238384) and Ireland (accession number LS453286).

There were statistically significant differences ($p<0.05$) between Susehri and other sampling areas. But there were no statistically significant differences ($p\geq0.05$) between male and female dogs and between age groups in terms of canine hepatozoonosis.

DISCUSSION

Canine hepatozoonosis caused by *H. canis* and *H. americanum* is one of the most important tick-borne diseases in both domestic and wild carnivores (2, 3, 7). *H. canis* was found in Turkey for the first time in 1933 (17). *H. americanum* has not been reported so far in Turkey. The aim of this study was to determine the prevalence of canine hepatozoonosis in Sivas province from the central part of Turkey using PCR and identify the etiological agents of canine hepatozoonosis with DNA sequencing.

Canine hepatozoonosis, which is the worldwide protozoan parasitic disease, has been determined from different countries, including Turkey. In Turkey, the prevalence of canine hepatozoonosis was researched using different techniques such as microscopic, serological, and molecular methods. Studies revealed that the prevalence of canine hepatozoonosis in dogs varies between 0.5 and 54.3% in different parts of Turkey (10, 13, 15, 18, 19, 20, 21, 22, 23, 24, 25). The prevalence of canine hepatozoonosis was found 31.8% in Colombia (26), 79.2% in Brazil (27), 54.3% in Romania (28), 26% in Hungary (29), 11.8% in Croatia (30), 14% in Italy (31), 1.57% in Iran (32), 11.9% in Pakistan (33), 11.4% in Thailand (34), 28.8% in Kyrgyzstan (35). In the present study, prevalence of canine hepatozoonosis was found 44.67% (67/150) with *Hepatozoon*-genus specific PCR. The prevalence of canine hepatozoonosis in Sivas was lower than in Diyarbakir 54.3% (18), and in Ankara 49.5% (24), but was higher than from other studies in Turkey (10, 13, 19, 20, 21, 22, 23, 24, 25). The prevalence of canine hepatozoonosis may be related to the distribution and abundance of vector tick species and the origin of dogs (owned or stray) (18, 19, 24). Studies that were performed in Diyarbakır (18) and Ankara (24) were conducted using stray dog samples, whereas our study was done using owned-dog samples. Stray dogs are more exposed to the ectoparasites such as tick compare to owned-dogs. Therefore, tick-borne pathogens like canine hepatozoonosis can be more prevalent in stray dogs compared to owned-dogs. We speculate that for this reason, the prevalence of canine hepatozoonosis studies that were

conducted in Diyarbakır (54.5%) and in Ankara (49.5%) was higher than in our study (44.67%). In this study, the positive infection rate of canine hepatozoonosis was found very high. This infection rate revealed that canine hepatozoonosis prevalent among the owned-dog population. These infected dogs might be served as a reservoir for other healthy dogs. Because canine hepatozoonosis is an important tick-borne pathogen, veterinarians should take this disease into account.

In this study, there were no statistically significant differences between male and female dogs, similar to research studies that were conducted in Turkey (19), Hungary (29), Italy (31) and Thailand (34). We didn't also find statistically significant differences between age groups in terms of canine hepatozoonosis. The results were similar to studies that were conducted in Italy (31, 37), Pakistan (33), Costa Rica (34).

In our study, canine hepatozoonosis was found in all sampling areas with a prevalence varies between 3.84 % (Susehri) and 67.85% (Ulas). According to our results, Ulas was found as the most prevalent region of canine hepatozoonosis, while Susehri was found to be the lowest region. We found statistically significant differences between Susehri and other sampling areas in terms of canine hepatozoonosis. Probably, this situation could be related to the successful tick control program in Susehri.

Molecular identification techniques such as PCR and DNA sequencing have been preferred techniques for the diagnosis of canine hepatozoonosis due to its greater sensitivity and specificity for the determination of canine hepatozoonosis and detection of new *Hepatozoon* spp. genotype (10, 13, 19, 34). The nucleotide sequence analyses of five *Hepatozoon* spp. positive isolates obtained in the study revealed that two samples were *H. canis* (but the other three samples were not *H. canis* and *H. americanum*). The three samples were found as *Hepatozoon* spp. MF with BLAST analysis. Nucleotide sequencing results were revealed that two species/genotype (*H. canis* and *Hepatozoon* spp.) are circulating among the owned-dog population in Sivas province. To the best of our knowledge, that is the first report of *Hepatozoon* spp. and *H. canis* in dogs in Sivas.

In conclusion, this study revealed that canine hepatozoonosis is prevalent in owned-dogs in Sivas province. Additionally, our results suggest that there are other species or genotypes than the *H. canis* in dogs. The results of this study will contribute to understanding the epidemiology of canine hepatozoonosis in Turkey and the world. On the other hand, different wild carnivore species such as red fox, gray wolf, and golden jackal can be lived in Sivas province due to geographical and climatic features. For this reason, there is still a need for comprehensive epidemiological and clinical studies on *H. canis* and *Hepatozoon* spp. in Sivas province and Turkey, including both domestic and wild carnivores.

CONFLICT OF INTEREST:

The authors declare that they have no conflict of interest.

ACKNOWLEDGMENTS

The authors would like to thank all veterinarians and technicians for their kind help during sample collection. The authors also thank Prof Dr. A. Kürşat AZKUR for proofreading the manuscript.

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