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Research Article

# Molecular Characterization of Tick-Borne Blood Protozoa in Stray Dogs from Central Anatolia Region of Turkey with a High-Rate *Hepatozoon* Infection

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## Abstract

Tick-borne blood protozoa play an important role in canine health, especially, *Babesia* and *Hepatozoon* species. In dogs, these species lead to clinical symptoms ranging from mild to severe. This study aimed to investigate the presence of tick-borne blood protozoa in stray dogs using microscopy and molecular methods. While none of the blood smears showed any *Babesia* spp. piroplasms, *Hepatozoon* spp. gametocytes were detected in 3.8% of examined samples. PCR analyses revealed the presence of a *Hepatozoon* infection in 49.5% of dogs. However, the presence of *Babesia* spp. was not found in any dogs. Sequence and phylogenetic analyses revealed that 86% of the positive cases were *Hepatozoon canis* and 13.7% were *Hepatozoon* sp. MF. The positivity rate obtained in this study was higher than the reported rates in many regions in Turkey. This suggests that *Hepatozoon* infections present a risk to dog health in this region. In addition, *Hepatozoon* sp. MF (a new genotype of undetermined pathogenicity) is reported in dogs from this region and detailed pathogenicity and epidemiological studies are thus required for this genotype. This study, therefore, suggests that *H. canis* is common in stray dogs in Ankara. Canine hepatozoonosis should be taken into consideration in pet clinics and the differential diagnosis should not be overlooked.

***Keywords:*** *Dog, Hepatozoon, Babesia, PCR, Molecular phylogeny*

# İç Anadolu Bölgesindeki Sokak Köpeklerinde Kene-Kaynaklı Kan Protozoonları Varlığının Araştırılması ve Yüksek Orandaki *Hepatozoon* Enfeksiyonunun Moleküler Karakterizasyonu

## Öz

Kene kaynaklı kan protozoonları köpek sağlığında önemli bir yer tutmaktadır. Bu protozoonların başında *Babesia* ve *Hepatozoon* türleri yer almaktadır. Mevcut türler köpeklerde ılımlı hastalık tablosundan şiddetli enfeksiyona varan derecede klinik belirtilere neden olmaktadırlar. Bu çalışmada, sokak köpeklerindeki kene kaynaklı kan protozoonlarının varlığı mikroskobik ve moleküler olarak araştırılmıştır. İncelenen kan preparatlarının hiçbirinde *Babesia* spp.'nin piroplazmik formuna rastlanmamıştır. Ancak örneklerin %3.8'inde *Hepatozoon* spp.'nin gametositleri tespit edilmiştir. Yapılan PCR analizleri sonucunda köpeklerde yüksek oranda (%49.5) *Hepatozoon* enfeksiyonu tespit edilmiştir. Buna karşın hiçbir köpekte *Babesia* spp. varlığına rastlanmamıştır. Sekans ve filogenetik analizler sonucunda pozitiflerin %86'sının *Hepatozoon canis,* %13.7'sinin ise *Hepatozoon* sp. MF olduğu belirlenmiştir. Bu çalışmada elde edilen pozitiflik oranın, Türkiye'deki birçok bölgeden rapor edilen oranlardan daha yüksek olduğu görülmüştür. Bu durum bölgedeki köpekler için *Hepatozoon* enfeksiyonu açısından risk teşkil etmektedir. Ayrıca henüz patojenitesi hakkında net bir veri bulunmayan ve yeni bir genotip olan *Hepatozoon* sp. MF varlığı da bölgedeki köpeklerden bildirilmiş olup bu genotip ile ilgili detaylı patojenite ve epidemiyolojik çalışmalara ihtiyaç duyulduğu görülmüştür. Sonuç olarak Ankara'daki sokak köpeklerinde *H. canis*'in yaygın olarak bulunduğu ortaya konmuştur. Bu nedenle, kliniklerde hepatozoonosis enfeksiyonları dikkate alınmalı ve ayırıcı tanıda göz ardı edilmemelidir.

***Anahtar sözcükler:*** *Köpek, Hepatozoon, Babesia, PCR, Moleküler filogeni*

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# INTRODUCTION

In recent years, worldwide interest in canine vector-borne diseases has been increasing due to the social role of dogs in human life and as the cause of zoonotic diseases. Tick-borne diseases are at the top of the list for both animal and public health [1,2]. In addition, tick-borne diseases also take an important place in the "one medicine, one health" concept, which has featured in the news in recent years and has become increasingly popular [3]. Due to this importance, studies on tick-borne pathogens are being carried out, revealing new ecological, epidemiological, and taxonomic data [2]. The primary protozoan tick-borne diseases in dogs are babesiosis and hepatozoonosis [2,4]. Canine babesiosis is an important tick-borne disease that is common worldwide and caused by intra-erythrocytic protozoan species of the *Babesia* genus [4,5]. These *Babesia* species classically occur in two forms, small *(B. gibsoni, B. condrae, B. vulpes)* and large *(B. canis, B. rossi, B. vogeli)* based on intraerythrocytic morphology [5-7]. The clinical picture of babesiosis in dogs ranges from sub-clinical to multi-organ failure and may eventually lead to death [4,5].

Canine hepatozoonosis is a tick-borne disease caused by the introduction of *Hepatozoon* protozoan species into dogs. It is known that two hepatozoan species *(H. canis and H. americanum)* cause this infection [8]. However, the existence of new genotypes has emerged in recent years, thanks to developing molecular methods [9]. The disease is widespread in Europe, Asia, Africa, and South America [2,5,10]. *H. canis* infection is mainly transmitted by *Rhipicephalus sanguineus* ticks. *H. canis*, which usually induces mild clinical symptoms, causes anemia and lethargy by infecting hemolymphatic tissues in dogs. However, in case of high parasitemia, it has also been reported to cause severe clinical outcomes [5,10].

Both *H. canis* and *H. americanum* are transmitted by hard (Ixodidae) ticks. However, the *Babesia* species are transmitted by ticks during feeding [4], while the *Hepatozoon* species are transmitted by ingestion of ticks [8]. The distribution of these diseases is determined by their vector ticks [5,10].

Published studies of babesiosis in Turkey have identified *B. canis* [11-16], *B. vogeli* [13,15,17-19], *B. gibsoni* [20], and an unnamed *Babesia* spp. [15] in dogs. In addition, the presence of *B. rossi* has been reported in *Haemaphysalis parva* ticks collected from a human and wild boars [21,22].

Printed articles regarding the existence of *Hepatozoon* spp. in Turkey show that the disease was first reported in a dog in 1933 [23]. Subsequently, the presence of *H. canis* was determined morphologically [24-29], molecularly [9,13,15,16,19,25,26,28-30], and serologically [26] in dogs. *Hepatozoon* spp. were also identified in unfed and semi-fed *Rh. sanguineus* ticks obtained from dogs [28,31] and *Rh. sanguineus, Dermacentor marginatus, Haemaphysalis sulcata, Haemaphysalis* spp. (nymph), *Ixodes ricinus* and *Ixodes* spp. (nymph) collected from humans [32]. In addition, a new *Hepatozoon* spp. genotype has been reported in dogs from Central Turkey [9].

In this study, we aimed to investigate the presence of tick-borne blood protozoa and focus on high-rate hepatozoonosis infections that were confirmed in stray dogs in Ankara.

# MATERIAL and METHODS

Blood samples were collected from 103 stray dogs living in a shelter in Ankara. Study materials were obtained from blood taken from dogs during routine examinations by the veterinarian in charge of the shelter. It has been recorded that the examined dogs are asymptomatic as clinical appearance. Blood samples were collected in EDTA-containing tubes. One half of the blood sample was set aside for examination by microscopy and the rest was stored at -20°C until PCR analysis. Blood smears were immediately (1-2 min) prepared for microscopic examination, fixed with methanol for 5 min, and stained with 5% Giemsa for 45 min. After Giemsa staining, slides were examined for the presence of piroplasmic forms of *Babesia* spp. and gamonts of *Hepatozoon* spp. by light microscopy at 100x magnification. A Nikon Eclipse 80*i* (Nikon, Tokyo, Japan) light microscope equipped with a Leica MC170 HD (Leica, Heerbrugg, Switzerland) digital camera and LAS (V4.11.0) software was used for microscopic examination.

Genomic DNA was extracted from blood samples using a DNeasy blood and tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Purified DNA samples were stored at -20°C until PCR analysis was performed. To determine the presence of *Babesia* spp. in blood samples, genus-specific PCR analyses were performed using BJ1 and BN2 primers [33], which amplify the partial 18S ribosomal RNA *(18S rRNA)* gene of *Babesia* spp. In addition, genus-specific PCR analyses were performed on all blood samples using HepF and HepR primers [34], which amplify the partial *18S rRNA* region of *Hepatozoon* spp. DNase-RNase-free sterile water was added to each reaction as a negative control and DNA samples from *B. bigemina* (isolated from naturally infected cattle) and *H. canis* (isolated from naturally infected dog) were included as positive controls. Positive PCRs were purified using a QIAquick Extraction Kit (Qiagen) and sequenced using the BigDye Terminator V3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, USA).

Sequence analysis was performed bidirectionally using the ABI PRISM 3100 Genetic Analyzer (Applied Biosystems). Obtained nucleotide sequences were compared to sequences registered in the GenBank database using BLAST (www.ncbi.nlmn.nih.gov/BLAST). The sequences were then edited and aligned using the BioEdit program [35]. We used jModeltest version 0.1.1 [36] to determine the most

appropriate phylogenetic model for our sequences. Phylogenetic and molecular evolutionary analyses were carried out using the MEGA version 7.0 [37] with the maximum likelihood method based on the general-time reversible model (GTR + I + G) and a phylogenetic tree was constructed. The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The nucleotide sequences obtained in this study were stored in the GenBank database with accession numbers MG254573-MG254623.

## RESULTS

Blood samples were collected from 103 dogs in total. While none of the smears obtained from the blood samples showed any *Babesia* spp. piroplasms, the presence of *Hepatozoon* spp. gametocytes was detected in 4 (3.8%) samples *(Fig. 1)*. PCR analysis of the DNA collected from the blood samples confirmed the absence of *Babesia* spp. However, *Hepatozoon* spp. were identified in 51 (49.5%) blood samples. Sequence analysis revealed that 44 (86.2%) of the positive samples were *H. canis* and 7 (13.7%) were *Hepatozoon* sp. MF. BLAST analyses showed that the *H. canis* sequences were 99.2-100% similar to *H. canis* isolates obtained from a dog (accession number FJ497022) and a fox (accession number HM212626) in Croatia, from dogs (accession numbers KU360326, KU360328, and KX880506) in Iran, from a dog (accession number KX588232) in Samsun, from foxes (accession numbers KX879135 and KX887327) in Slovakia, and a Golden Jackal (accession number KX712124) in the Czech Republic. The *Hepatozoon* spp. sequences were also determined to be 100% identical to *Hepatozoon* sp. MF identified in dogs from Karaman (accession number KF439864) and Konya (accession number KF439865). Phylogenetic trees were constructed using *Hepatozoon* spp. sequences obtained in this study and published *Hepatozoon* spp. sequences. The data were registered in GenBank and the phylogenetic tree is illustrated in *Fig. 2*.

## DISCUSSION

Canine vector-borne diseases directly threaten the health of dogs. In addition, there are zoonotic vector-borne disease agents that affect human health, for which dogs are known reservoir hosts [1]. The life cycle of these microorganisms is maintained between host, pathogen, and vector. It is well known that ticks are one of the most important vector arthropods. Ticks are responsible for the transmission of a number of pathogens to dogs; therefore, the distribution of these pathogens is determined by the distribution of their vector ticks [2]. Given that dogs have an important place in human life, both their vector-borne diseases and their zoonotic pathogens are an issue [1]. *Babesia* and *Hepatozoon* species are the leading blood protozoa that cause infections in dogs. These species can lead to clinical symptoms ranging from mild to severe and even death. Both diseases are globally distributed [2,5,10] and wild canids are known reservoir host for some of these pathogens [5,10].

The aim of this study was to determine the presence of tick-borne blood protozoa in stray dogs. *Hepatozoon* infections were detected at high levels in 49.5% dogs by PCR, while 3.8% positivity was detected by microscopy. Additionally, we detected very low parasitemia level in blood smears of 4 microscopy-positive dogs. This situation indicated that PCR is much more sensitive than microscopy and all positive dogs are chronically infected with *Hepatozoon* spp. However, *Babesia* spp. were not identified in any dogs. Sequence analysis of the 51 *Hepatozoon* spp., positive samples revealed that 44 (86.2%) were *H. canis* and 7 (13.7%) were *Hepatozoon* sp. MF. *H. canis* isolates obtained in this study were similar to *H. canis* isolates reported in

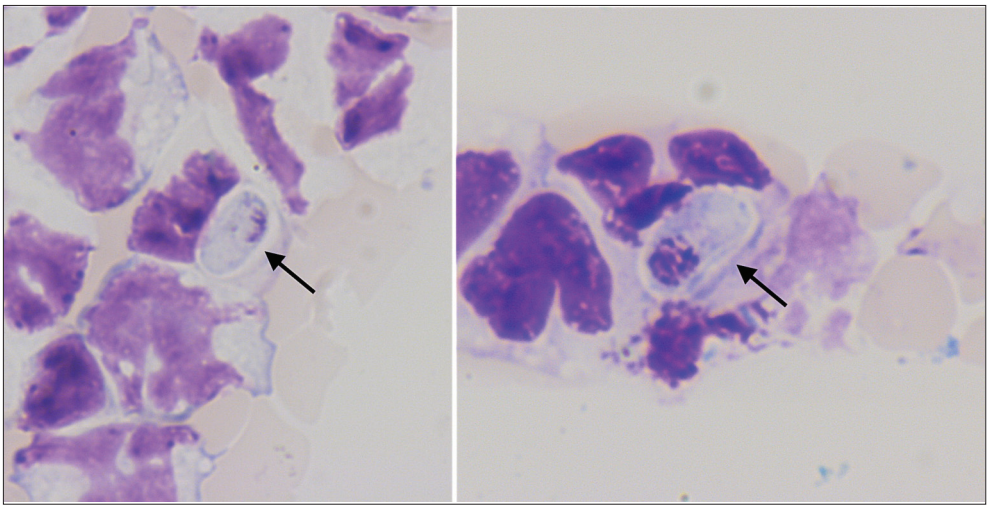

**Fig 1.** Ellipsoidal-shaped gamonts of *Hepatozoon* spp. (arrows) in peripheral blood from naturally infected dogs

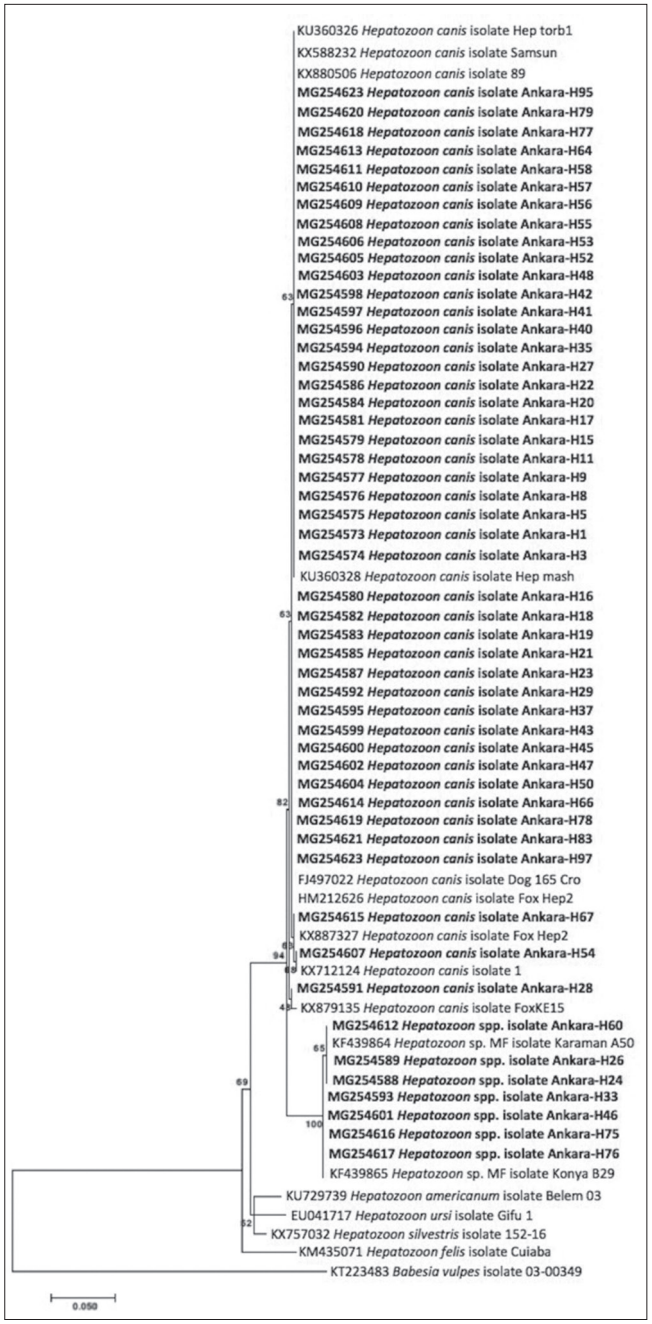


**Fig 2.** Phylogenetic tree based on aligned sequences of *18S rRNA* of *Hepatozoon* spp. with *Babesia vulpes* as outgroup and constructed by using Maximum Likelihood method calculated under the GTR+I+G substitution model. The *Hepatozoon* sequences obtained in this study are shown in bold. GenBank accession numbers of sequences and names of lineages are given before species names

dogs, foxes and a jackal. In addition, *Hepatozoon* sp. MF isolates were identical to isolates obtained from dogs in the Central Anatolia region.

A number of publications have documented the presence of *Hepatozoon* spp. in dogs in Turkey. Karagenc et al.[26] took samples from 349 dogs in the Aegean region and found *Hepatozoon* spp. in 10.6% of samples by microscopy and 25.8% of samples by PCR. All of the sequenced samples were *H. canis*. In addition, they reported that 36.8% of the blood samples obtained from these dogs had antibodies against this disease. A study carried out in Diyarbakır Province identified the presence of *H. canis* infection in 15.87% of the 63 dogs sampled [28]. In a study including a total of 694 dogs from Sakarya, Kocaeli, Mersin, Giresun, İzmir, Elazığ, Erzurum, Ankara and Nevşehir, 3 (1.05%) of the 285 dogs examined were determined to be positive by microscopy and 155 (22.3%) of the 694 dogs were positive for *H. canis* by PCR. From Ankara, 49 dogs were tested and 2 dogs (4%) were *H. canis*-positive [29]. In Erzurum Province, 43 (32.3%) of 133 asymptomatic dogs were positive for *Hepatozoon* spp. by PCR. Seven of the positive samples were sequenced and were all confirmed as *H. canis* [16]. In Kayseri Province, 21 (5.3%) of 400 dogs were reported as positive for *H. canis* by real-time PCR [13]. In Samsun Province, only one dog (0.5%) of 200 dogs examined was reported as positive for *H. canis* by PCR [30]. By comparing these reports from many parts of Turkey with our results, it is evident that the hepatozoan infection rate is higher in stray dogs in Ankara. Moreover, the molecular method of identification is much more sensitive than microscopy. In addition, Aktas et al.[29] found a 4% positive rate in dogs in Ankara, which is much lower than our rate (49.5%). We believe that this is due to the limited number of samples in their study.

In a study conducted in Diyarbakır, samples from 219 street dogs were examined by microscopy and PCR for the presence of *Hepatozoon* spp. No samples were positive by microscopy, but 54.3% of the samples were determined to be infected with *H. canis* by PCR [15]. Thus, the data obtained in Diyarbakır are compatible with our study and the infection rate is very similar in both regions.

In a study in the Central Anatolia region, Aydin et al.[9] took blood samples from 221 dogs in Konya and Karaman Provinces and tested for *Hepatozoon* spp. by PCR. *Hepatozoon* spp. were found in eight dogs (3.61%). Of these, *H. canis* was found in 6 dogs (2.71%) in Konya Province and an unclassified *Hepatozoon* spp. *(Hepatozoon* sp. MF) was reported in 2 dogs in both Konya and Karaman Provinces. Two species/genotypes were identified in our study. *Hepatozoon* sp. MF was detected more frequently than in previous reports and is only the third such report of this genotype to-date. Sequence and phylogenetic analyses show that these isolates are identical to isolates obtained in this study. This is the first identification of this hepatozoan genotype (of unknown pathogenicity) in dogs in Ankara. In addition, the existence of *H. canis* infection was much higher in our work than in Konya and Karaman Provinces. In another study conducted in Konya Province, blood samples were taken from 192 dogs and *Hepatozoon* spp. were identified in only 8 dogs (4.2%). Sequence analysis revealed that 7 samples were *H. canis* and one sample was *Hepatozoon* sp. MF [19]. This study is consistent with the other study in the same region [9]

but the infection rate is very low compared to our study. Thus, the hepatozoonosis infection rate in dogs in the Central Anatolia region is very different and the infection is more common in the Ankara than other Provinces. We speculate that the reason is related to the distribution of vector ticks. Moreover, studies show that *Hepatozoon* sp. MF is circulating in dogs in the Central Anatolia region. Therefore, more studies of this genotype are needed.

To our knowledge, there are no reports of *Babesia* infection in dogs in Ankara Province. However, *B. rossi* was observed in *Ha. parva* collected from a human and wild boars in Ankara [21,22]. In this study, *Babesia* spp. were not found in any dogs. However, more epidemiological studies are needed to determine the prevalence of babesiosis in dogs in this region. *B. rossi*, causes severe clinical manifestations in dogs [6], is circulating in ticks and thus poses a considerable risk to the dogs of this region.

A high rate of *Hepatozoon* infection (49.5%) was detected in stray dogs in Ankara. In positive dogs, *H. canis* (a cosmopolitan species) and *Hepatozoon* sp. MF genotypes were identified. Detailed pathogenicity and epidemiological studies are required for *Hepatozoon* sp. MF because this genotype is circulating in dogs and the pathogenicity is not clear. Together, these results show that hepatozoonosis is common in dogs in Ankara and this infection should be taken into account in clinical differential diagnoses.

## Conflicts of Interest

The authors declare no conflict of interest.

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