Molecular characterization of *Hepatozoon* spp. in naturally infected dogs in Aydın province

Metin PEKAĞIRBAŞ[1,a,✉], Muhammed Veli DEMİRBİLEK[1,b], Emrah ŞİMŞEK[2,c], Heycan Berk AYDIN[1,d], Hakan KANLIOĞLU[1,e], Asude Gülçe ORYAŞIN[1,f], Nuran AYSUL[1,g]

[1]Aydın Adnan Menderes University, Faculty of Veterinary Medicine, Department of Parasitology, Aydın, Türkiye; [2]Muğla Sıtkı Koçman University, Faculty of Milas Veterinary Medicine, Department of Preclinical Science, Muğla, Türkiye.

[a]ORCID: 0000-0003-3170-410X; [b]ORCID:0009-0002-0173-7170; [c]ORCID: 0000-0002-0492-9840; [d]ORCID: 0009-0007-9466-3487; [e]ORCID: 0000-0003-4949-944X; [f]ORCID: 0000-0002-7219-2879; [g]ORCID: 0000-0001-6223-058X

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✉Corresponding author
metin.pekagirbas@adu.edu.tr

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ABSTRACT

Canine hepatozoonosis, a disease caused by the protozoan *Hepatozoon canis* and *Hepatozoon americanum*, represents a significant tick-borne disease affecting domestic and wild carnivores. The objective of this study was to detect *Hepatozoon* species in randomly selected dogs from Aydın by PCR and to elucidate their molecular characterization and phylogenetic differences through sequence analysis. In total, 100 blood samples collected from dogs were analyzed, and the prevalence of *Hepatozoon* DNA was determined to be 3%, with only three samples testing positive. Partial sequences of the 18S rRNA gene exhibited 100% similarity with corresponding *H. canis* isolates. Phylogenetic analysis of the 18S rRNA gene region revealed the formation of two primary clusters, one consisting of *H. canis* isolates and the other comprising different *Hepatozoon* species. While *H. canis* isolates formed distinct subclusters, they were all grouped separately from other *Hepatozoon* species. Furthermore, phylogenetic analysis highlighted the presence of multiple *H. canis* haplotypes in Türkiye, with intraspecific nucleotide differences ranging from 0.0% to 2.9%. The nucleotide differences among the isolates identified in this study ranged from 0.0% to 1.6%. All sequences obtained in this study have been submitted to GenBank and assigned accession numbers PQ669652, PQ671331 and PQ669658. These findings underscore the need for further investigations into *Hepatozoon* infections among cats and wild animals in the region. Additionally, the detection of the parasite in vector ticks could offer valuable insights into the genetic diversity and distribution of circulating *Hepatozoon* species.

Introduction

Canine vector-borne diseases (CVBDs) have become a global phenomenon, driven by a combination of factors such as cross-country canine migration, close human-dog interactions, animal transportation, and the growing accessibility of international travel. Additionally, global warming, which affects all aspects of life, plays a crucial role in this context. It may alter the epidemiology of diseases by influencing the presence and mobility of vectors (23). As a result, tick-borne diseases have emerged as significant veterinary and public health concerns, particularly in regions where environmental conditions favor the breeding and survival of vector tick species. Moreover, tick-borne pathogens, which cause both clinical and subclinical infections in domestic and wild animals, are distributed worldwide and can lead to significant morbidity and mortality (28). Among wild carnivores, foxes also may play an important role in the transmission of tick-borne diseases due to their role in the transmission of some infectious diseases in humans and dogs, their suitability as reservoirs of pathogens and also as hosts of vector ticks (25).

Ticks transmit several significant canine infectious diseases, including babesiosis, hepatozoonosis, anaplasmosis, and ehrlichiosis (26). Furthermore, co-infections transmitted by vectors are commonly observed

in dogs, especially in regions with a high prevalence of vector arthropods. In such endemic areas, certain arthropods can carry more than one pathogen. Consequently, dogs are often exposed to vectors infected with different pathogens, which increases the likelihood of co-infections. Moreover, the abundance of arthropods in a specific area plays a critical role in determining the diversity of vector-borne pathogens affecting dogs. For instance, in regions with high arthropod populations, dogs may be infected with multiple pathogens, such as *Ehrlichia canis*, *Anaplasma platys*, *A. phagocytophilum*, *Hepatozoon canis*, *Leishmania infantum*, *Babesia canis vogeli*, *Dirofilaria repens*, and *D. immitis* (12, 26).

Canine hepatozoonosis, caused by the protozoan *H. canis* and *H. americanum* represent a significant tick-borne disease affecting domestic and wild carnivores. *Hepatozoon* species are transmitted by ingestion of ticks. *H. canis* and *H. americanum*, which cause hepatozoonosis, are transmitted by hard ticks (Ixodidae). *H. americanum*, which is more restricted in distribution than *H. canis* but more pathogenic to its hosts, has only been reported from the United States, whereas *H. canis* has been reported from Europe, Asia, Africa, and even the America (8). Although canine hepatozoonosis is typically regarded as a subclinical infection in dogs, it has the potential to manifest a spectrum of clinical findings, ranging from mild to moderately severe on occasion. In severe cases, symptoms may include elevated body temperature, loss of appetite, fatigue, wasting, elevated globulin levels, significant weight loss, and, in extreme instances, anemia (8, 25). The diagnosis of *Hepatozoon* species often involves a combination of clinical signs, laboratory tests, serological tests, and, in some cases, molecular techniques. However, clinical observations may lack sufficient specificity, as the symptoms can overlap with those of other diseases. While microscopic examination is a straightforward and cost-effective method, it has limitations. These include the difficulty of detecting the agent at a microscopic level and the inability to identify species, particularly in infections with low parasitemia. These challenges underscore the importance of molecular methods (9). PCR and sequence analysis provide the opportunity for phylogenetic characterization of *Hepatozoon* sp. isolates. These molecular techniques play a pivotal role in comprehending the detection, distribution, prevalence, and interrelationships of the species, thereby contributing valuable insights to the broader understanding of these infections. In Türkiye, studies on *Hepatozoon* have utilized a range of methodologies in areas with diverse ecological and geographical characteristics (2-5, 10, 13, 24, 25). Unlike other pathogen detection studies on *Hepatozoon* spp. in dogs and ticks, a previous study showed the presence of *Hepatozoon canis* in foxes in Türkiye. This indicates that both domestic and wild carnivores living in similar habitats are infected and underlines the necessity to examine the disease distribution by considering ecological factors (25). Despite this, the study region lacks information about the phylogenetic characterization of the species detected. Of the three studies conducted in the region, two are case reports, while the third employs both serological and molecular methods (17, 31, 36).

The objective of this study was to detect *Hepatozoon* species in dogs randomly selected from the study area by PCR and to uncover their molecular characterization and phylogenetic differences through sequence analysis.

Materials and Methods

Sample Collection and Microscopic Examination: Blood samples were collected from 100 dogs taken to Aydın Adnan Menderes University Faculty of Veterinary Medicine Animal Hospital clinics for treatment between 2018-2019. Five ml of blood was collected from the *vena cephalica antebrachii* of each dog into tubes coated with ethylenediaminetetraacetic acid (EDTA). For microscopic examination, blood was taken from the dog's ear tips. Prepared smears were dried, fixed in methanol for five minutes, stained with 5% May-Grünwald Giemsa for 30 minutes, and examined under a microscope (21). Additionally, anticoagulated blood samples were stored at -20°C until DNA extraction.

Genomic DNA Extraction and PCR: Genomic DNA was extracted from blood samples using the GeneJET™ Whole Blood Genomic DNA Purification Mini Kit (Thermo Fisher Scientific Cat. No K0781, USA) following to the manufacturer's instructions. *Hepatozoon* spp. was diagnosed by PCR using the primers Hep-F (5'-ATACATATGAGCAAAATCTCAAC-3') and Hep-R (5'-CTTTATTATTCCATGCTGCAG-3'), which amplify a 666 bp conserved region of the lineage-specific 18S rRNA gene. (16).

DNA Sequencing and Phylogenetic Analysis: To confirm the PCR results, *Hepatozoon* positive PCR products were sequenced using Sanger sequencing by a commercial company. Subsequently, the obtained sequences were edited according to sequence chromatograms and aligned using Geneious 11.0.2 software, to generate the final sequences. In addition, the BLASTn program (http://www.ncbi.nlm.nih.gov/BLAST) was used to compare isolates and to make species identifications, and to create a quality data set. Moreover, the isolates were compared with *Hepatozoon* isolates from different geographical regions of Türkiye and the world, as registered in GenBank. To reveal intraspecific and interspecific nucleotide differences among isolates, the Kimura 2-parameter model (18) was performed using the MEGA X bioinformatics program (19). Additionally, the Maximum Likelihood (ML) method was applied within

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the same program to determine phylogenetic relationships (19). At this stage, MEGA X was also employed to identify the most suitable DNA evolution model for sequence analysis, based on Akaike Information Criterion. Thus, the Hasegawa-Kishino-Yano + Gamma distribution (HKY+G) model was determined to construct the phylogenetic tree (15, 19). Finally, *Babesia canis canis* (KF499115) was included as an outgroup.

Results

All dogs included in this study had been regularly treated for ectoparasites, and no ticks were detected during the clinical examinations. Additionally, none of the 100 blood smears tested positive for *Hepatozoon* gametocytes. However, PCR analysis revealed that only three out of 100 samples (3%) were positive for *Hepatozoon*. The positive samples were subsequently confirmed through sequence comparisons. Notably, all three positive dogs had a history of residing in outdoor environments, such as streets, shelters, and gardens.

The 666-bp-long sequences of the 18S rRNA gene region from the isolates identified in this study were successfully obtained. BLASTn analysis confirmed that these isolates belonged to *H. canis*, which were designated as ADU-HC1, ADU-HC2, and ADU-HC3. Additionally, the sequences were submitted to GenBank (NCBI), and accession numbers PQ669652, PQ671331, and PQ669658 were assigned.

The analysis of the dataset revealed that the ADU-HC isolates showed 0–1.6% nucleotide differences among themselves. The ADU-HC1 and ADU-HC2 isolates found in the study showed 100% similarity to isolates from dogs in Ankara (accession number: MG254603-20; KX588232) and the isolate from red fox in Türkiye (accession number: MG077087). ADU-HC2 isolate was also 100% similar to a dog isolate from Kayseri (accession number: KJ513193) and another dog isolate (accession number: JQ867390). In terms of intraspecies variation, the nucleotide differences among *H. canis* isolates from different hosts in Türkiye, included in the dataset, were determined to range between 0.0% and 2.9%.

In the phylogenetic tree of the 18S rRNA gene region, two main clusters were formed, including *H. canis* and other species. Although *H. canis* isolates formed different cluster among themselves, all *H. canis* isolates grouped separately from other *Hepatozoon* species (Figure 1). Specifically, the ADU-HC1 and ADU-HC2 isolates were grouped with *H. canis* isolates previously reported from Ankara and Konya, whereas the ADU-HC3 isolate clustered with isolates from the Aegean region.

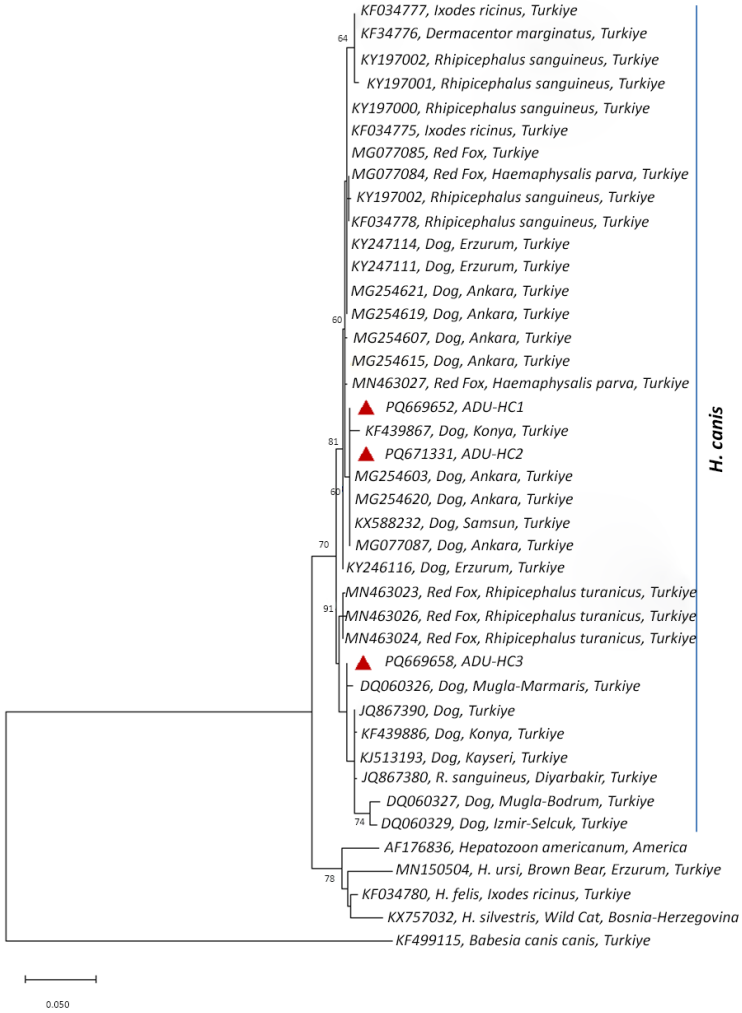

Figure 1. Phylogenetic relationships among *Hepatozoon* isolates. The Maximum-Likelihood (ML) analysis method based on the HKY+G model was used to analyze the 18S rRNA dataset of *Hepatozoon* species. *Babesia canis* (KF499115) was used as an outgroup. Isolates are listed with their GenBank accession numbers, hosts, and locations. Isolates detected in this study are marked with a triangle symbol. Scale bar represents nucleotide substitutions per position.

Discussion and Conclusion

Hepatozoonosis is one of the important tick-borne diseases affecting the health of domestic and wild canids worldwide. It can present in a variety of forms, ranging from subclinical to severe (8). In dogs with suspected *H. canis* infection, the presence of the parasite in Giemsa stains of peripheral blood slides within neutrophils and rarely in monocytes is considered sufficient for diagnosis (8). However, the absence of detectable parasites in animals with low parasitemia does not necessarily indicate the absence of the disease. In such cases, microscopic examination may still be effective when samples are prepared from the buffy coat layer (11, 27). Serological tests, such as indirect immunofluorescence assay (IFA) and enzyme-linked immunosorbent assay (ELISA), are also employed for disease detection. Molecular methods such as polymerase chain reaction (PCR), real-time PCR and sequence analysis are widely used to detect the disease in dogs (17, 27).

The first study on Hepatozoonosis in Türkiye was conducted by Tüzdil (32) in 1933, followed by Voyvoda et al. (36), who presented a comprehensive clinical description of the disease in 2004. Since then, numerous contributions to the scientific literature have been made using various diagnostic methods in different geographical regions of Türkiye (3, 5, 10 13, 14, 17, 24, 25, 31). Despite the high infection rates found in most previous studies in Türkiye (3, 13, 17, 24), a very low infection rate (3%) was found in the present study, similar to that of studies conducted in Aydın et al. (5) and Bolukbas et al. (10). The fact that one of the highest infection rates detected in dogs in Türkiye to date was found in Aydın by Karagenç et al (17) and that, despite the prominence of the study area in relation to tick-borne diseases (6, 7), one of the lowest infection rates detected in Türkiye in this study was found in Aydın. This may be due to several reasons, such as the successful use of ectoparasiticides in the region over time, the season during which samples were collected, host immune status, vector presence/density (27), small sample size, and the fact that samples were collected from a single site. Moreover, when analyzing discrepancies in infection prevalence, it is essential to consider multiple factors that influence regional differences. These factors include the DNA extraction methodology, the primers used, and the targeted gene region, all of which may exhibit varying sensitivities. Host-related variables, such as age and breed, and, most importantly, tick infestations (34) are also critical contributors. Additionally, the discrepancy between PCR-positive and microscopically negative results observed in three dogs may be explained by the chronic nature of the infection or the inherent limitations of microscopic examination. Although the positivity rate found in the study was low, the possibility that street dogs are exposed to ticks and therefore to tick-borne diseases should not be ignored.

As illustrated in the phylogenetic tree, the *H. canis* isolates identified in red foxes were grouped within the same or analogous clusters as those recovered from dogs and ticks. In a previous study, *H. canis* isolates obtained from dogs, wolves, and jackals were identified as belonging to the same group in the phylogenetic tree (22). Additionally, it was emphasized that wolves and jackals may play a significant role in the dissemination and distribution of this agent (20). The intraspecific nucleotide differences of the *H. canis* isolates obtained from disparate hosts in Türkiye were determined to range from 0.0 to 2.9%. A review of the phylogenetic tree of the 18S rRNA gene region reveals the presence of numerous *H. canis* haplotypes in Türkiye. In addition to *H. canis*, two new species/genotypes (*Hepatozoon* sp. MF) were identified in another study (5) conducted in Türkiye and isolates identical to these new cryptic species were reported in other studies conducted in different province (25). In a previous study conducted in Pakistan, it was revealed that only one species, *H. canis*, is present, but that 15 different genotypes exist. It was also stated that genetic diversity among *H. canis* isolates is quite high (1). Additionally, the isolates identified in this study exhibited similarities with those from different geographical regions and were classified within the same group. It has also been proposed that the genetic diversity of this parasite is likely due to the widespread presence of dogs in diverse geographical regions, countries, and continents, which is facilitated by human migration (22, 33, 35) Finally, it is underscored that this situation may contribute significantly to the dissemination of vectors and vector-borne diseases, highlighting the need for continued vigilance (29, 30).

This study represents the molecular characterization of *Hepatozoon canis* in dogs in the Aydın province of Türkiye and the positivity rate of a relatively small sample group in the study area. Isolates obtained from positive dogs in the study exhibited high similarity with those obtained from different regions. Additionally, although a low positivity rate (3%) was observed in dogs in this study, high positivity rates were reported in previous studies conducted in the study area. This discrepancy highlights the need for further investigation into *Hepatozoon* infections, particularly among cats and wild animals other than dogs in the region. Furthermore, the detection of the parasite in vector ticks within the study area could offer valuable insights into the genetic diversity and distribution of circulating *Hepatozoon* species understanding of the current situation.

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Ethical Statement

Ethical approval for this study was obtained from the Aydın Adnan Menderes University Local Ethics Committee (Decision number 64583101/2018/069).

Conflict of Interest

The authors declared that there is no conflict of interest.

Author Contributions

NA, MVD, AGO and MP conceived and planned the experiments. MVD, MP, HK, HBA and ES carried out the study, NA, ES, MP, MVD contributed to the interpretation of the results. MP, ES, NA, HBA took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis and manuscript.

Data Availability Statement

The accession numbers given by NCBI for the isolates identified in this study (https://www.ncbi.nlm.nih.gov/) are PQ669652, PQ671331, and PQ669658.

Animal Welfare

The authors confirm that they have adhered to the ARRIVE Guidelines to protect animals used for scientific purposes.

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