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Emerging Insights into Feline Hepatozoonosis in Türkiye: Molecular Detection and Phylogenetic Characterization from the Aegean Coast

Türkiye'de Kedi Hepatozoonozuna İlişkin Ortaya Çıkan Görüşler: Ege Kıyılarından Moleküler Tespit ve Filogenetik Karakterizasyon

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ABSTRACT

Objective: The aim of this study is to investigate the presence, prevalence, and molecular characteristics of *Hepatozoon* spp. infection in domestic cats living in Aydın and İzmir provinces, located on the Aegean coast of Türkiye. The study specifically focuses on species identification and genotype distribution.

Methods: A total of 203 blood samples from domestic cats were analyzed for *Hepatozoon* spp. using polymerase chain reaction (PCR). Among the 67 PCR-positive samples, 10 (five for each province) were selected for sequencing, and bidirectional Sanger sequencing was performed. The obtained sequences were evaluated using basic local alignment search tool and phylogenetic analyses, and genotype identification was based on the *18S rRNA* gene region. Additionally, potential risk factors such as age, sex, health status and housing type were statistically analyzed for their association with *Hepatozoon* spp. infection.

Results: The overall prevalence of *Hepatozoon* spp. in cats was determined to be 33.0% (67/203). The infection rate was 43.69% in Aydın and 22% in İzmir, and this difference was found to be statistically significant (p=0.001). All 10 sequenced samples were identified as *Hepatozoon felis*, and all isolates belonged to Genotype I. A significant association was found between age and infection, with a particularly high infection rate of 44% observed in cats aged one year or younger (p=0.020).

Conclusion: This study revealed a high prevalence of *Hepatozoon* spp. infection among cats in western Türkiye, with all sequenced isolates identified as Genotype I. The notably high infection rate observed in young cats raises the possibility of transplacental transmission. These findings underscore the need for further investigations to clarify the transmission routes and risk factors associated with this protozoan parasite, the epidemiology of which remains insufficiently understood.

Keywords: Tick-borne disease, *Hepatozoon felis*, domestic cat, genotype, Türkiye

ÖZ

Amaç: Bu çalışmanın amacı, Türkiye'nin Ege kıyısında yer alan Aydın ve İzmir illerinde yaşayan evcil kedilerde *Hepatozoon* spp. enfeksiyonunun varlığını, yaygınlığını ve moleküler özelliklerini araştırmaktır. Çalışma özellikle tür tayini ve genotip dağılımına odaklanmıştır.

Yöntemler: Evcil kedilerden alınan toplam 203 kan örneği, *Hepatozoon* spp. yönünden polimeraz zincir reaksiyon (PZR) yöntemiyle analiz edilmiştir. PZR ile pozitif bulunan 67 örnekten her iki ilden beşer adet olmak üzere toplam 10 örnek dizileme için seçilmiştir ve çift yönlü Sanger dizileme yöntemi uygulanmıştır. Elde edilen diziler temel yerel hizalama arama aracı ve filogenetik analizlerle değerlendirilmiştir; genotip tayini *18S rRNA* gen bölgesine dayalı olarak gerçekleştirilmiştir. Ayrıca, yaş, cinsiyet, sağlık durumu ve barınma şekli gibi potansiyel risk faktörlerinin *Hepatozoon* spp. enfeksiyonu ile ilişkisi istatistiksel olarak analiz edilmiştir.

Bulgular: Kedilerde *Hepatozoon* spp. enfeksiyonunun genel prevalansı %33,0 (67/203) olarak belirlenmiştir. Aydın ilindeki enfeksiyon oranı %43,69, İzmir'de ise %22 olarak bulunmuştur; bu fark istatistiksel olarak anlamlı kabul edilmiştir (p=0,001). Dizilenen 10 örneğin tamamı *Hepatozoon felis* olarak tanımlanmıştır ve tüm izolatların Genotip I'e ait olduğu belirlenmiştir. Enfeksiyon ile yaş arasında anlamlı bir ilişki saptanmıştır, özellikle bir yaş ve altı kedilerde enfeksiyon oranı %44 olarak bulunmuştur (p=0,020).

Sonuç: Bu çalışma, Batı Türkiye'de kedilerde *Hepatozoon* spp. enfeksiyonunun yüksek prevalansa sahip olduğunu ve dizilenen tüm örneklerin Genotip I'e ait olduğunu ortaya koymuştur. Özellikle genç kedilerde gözlemlenen yüksek enfeksiyon oranı, transplasental bulaş ihtimalini gündeme getirmektedir. Bu bulgular, henüz tam olarak aydınlatılamamış olan bu protozoon parazitin bulaş yolları ve risk faktörlerinin daha iyi anlaşılması için ileri çalışmalara duyulan ihtiyacı vurgulamaktadır.

Anahtar Kelimeler: Kene kaynaklı hastalık, *Hepatozoon felis*, evcil kedi, genotip, Türkiye

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Türkiye PARAZİTOLOJİ Dergisi

INTRODUCTION

Hepatozoonosis is a disease caused by *Hepatozoon* species which are apicomplexan protozoan parasites belonging to the family Hepatozoidae. These parasites infect mammals, birds, reptiles, and amphibians and are transmitted by various arthropod vectors. The transmission of *Hepatozoon* species occurs through ingestion of infected invertebrates, such as ticks, mites, mosquitoes, or fleas, which act as definitive hosts (1,2). Although *Hepatozoon* infection in dogs is well documented, data on feline hepatozoonosis remain limited (1). The prevalence of infection has been found to vary between 0.6% and 83.3% in various regions depending on the lifestyle of the cats, the geographical area, the diagnostic method used, and the type of samples tested (3). It has been noted that infection in cats is predominantly subclinical, with the potential for exacerbation in animals with compromised immune systems or co-infections (4,5).

Three different *Hepatozoon* species have been reported in felids so far, namely *Hepatozoon felis* (*H. felis*), *Hepatozoon canis* and *Hepatozoon silvestris* (6). Among them, *H. felis* is the earliest identified and most widespread species, affecting both wild and domestic felids worldwide (7). This species, which is the primary causative agent of feline hepatozoonosis, a disease of veterinary importance, was first detected in domestic cats in India (8). Although *H. canis* infections have only been described sporadically in domestic and wild felids, *H. felis* infections are subclinical without a significant local inflammatory response (5,7).

It has been hypothesized by some researchers that different species or genotypes may possess distinct biological characteristics and pathogenic potential (5,9). However, the most prevalent *H. felis* variant in the world is believed to be a species complex comprising multiple variants, including a genotype on a global scale. Furthermore, it has been hypothesized that multiple genotypes or species may circulate among cats in the same country or a more limited area (3,10). Phylogenetic analyses of *H. felis*, the most prevalent species infecting cats, utilizing partial *18S rRNA* gene sequences, have identified two distinct genotypes: The more common genotype I and genotype II, characterized by a lower prevalence (11). Also, (12) reported a high genetic diversity with the existence of two different genotypes of *H. felis* as a result of *18S rRNA* gene sequence analysis. Additionally, Panda et al. (12) reported high genetic diversity, identifying two distinct genotypes of *H. felis* based on *18S rRNA* gene sequence analysis.

In a recent study, *H. silvestris* was identified as a new species infecting cats. Subsequently, this species has been detected in several European wild and domestic cats, including those in Türkiye (6,9,13,14). In contrast to the typically subclinical nature of *H. felis*, *H. silvestris* has been implicated as the causative agent of severe clinical conditions such as fatal myocarditis and intussusception suggesting a potentially higher virulence to domestic cats (15,16).

The majority of research on *Hepatozoon* infections in Türkiye has focused on canine hosts and tick vectors, and comprehensive study data on cats are quite limited (14,17-27). In particular, information regarding the prevalence, species and genotype distribution, as well as potential risk factors such as age, sex, health status, and housing conditions, is notably scarce. Addressing these gaps is essential for elucidating transmission dynamics and developing effective preventive veterinary strategies. The current findings emphasize the need for further research to better understand the epidemiology of *Hepatozoon* spp. infections in cats. Accordingly, the present study was conducted to investigate the presence, prevalence, molecular characteristics, and phylogenetic structure of *Hepatozoon* species in domestic cats from Aydın and İzmir provinces, located on the Aegean coast of Türkiye, while also evaluating potential risk factors associated with infection.

METHODS

Ethically Approval

The study was performed under the instructions and approval of the Institutional Animal Ethics Committee of Aydın Adnan Menderes University (protocol no: 64583101/2024/019, date: 18/01/2024). Animal owners participating in the study were informed about the purpose and procedures of the research, and written informed consent was obtained before blood sample collection.

Blood Samples

In this study, a total of 203 blood samples were used from domestic cats as part of the MSc thesis titled *"Investigation of the Prevalence of Ehrlichia Species in Cats Living in İzmir and Aydın Provinces"*. These samples were collected from cats brought to veterinary hospitals or clinics in the Aydın and İzmir provinces, located in the Aegean coast of Türkiye, between 2024 and 2025. The age of the animals was recorded at the time of sampling and categorized into three groups: One year or younger, 1-4 years, 4 and over 4 years. Blood samples were collected in tubes containing ethylene diamine tetraacetic acid (EDTA) (BD Vacutainer®, Franklin Lakes, NJ, USA) for polymerase chain reaction (PCR) analysis and were stored at -20 °C until further processing.

Genomic DNA Extraction, PCR Amplification and Sequencing

Genomic DNA was extracted from the collected blood samples (200 µL) using the Invitrogen Genomic DNA Extraction kit (Invitrogen by Life Technologies, Thermo Fisher Scientific, USA), following the manufacturer's protocol. A total of 200 µL of each blood sample was used for DNA extraction. Amplification of an approximately 600 bp fragment of the *18S rRNA* gene of *Hepatozoon* spp. was performed using the primer pair HepF300: 5′-GTT TCT GAC CTA TCA GCT TTC GAC G-3′ and Hep900: 5′-CAA ATC TAA GAA TTC ACC TCT GAC-3′, following the protocol described by Ujvari et al. (28). PCR reactions were carried out using a Techne TC-512 thermocycler (Techne, UK), in a final volume of 50 µL, containing 1X PCR buffer, 2 mM $MgCl_2$, 0.2 mM of each deoxynucleotide triphosphate, 0.25 U of VitaTaq DNA polymerase, 0.5 µM of each primer, and 2 µL of DNA template. The amplification process included an initial denaturation at 94 °C for 3 min, followed by 35 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. A positive and a negative control were included in all PCR reactions. The positive control consisted of DNA extracted from a naturally infected dog, while double-distilled water was used as the negative control. A total of 10 µL of each PCR product was electrophoresed on a 1.5% agarose gel containing SafeView™ nucleic acid stain (10 µL/mL; ABM Inc., Richmond, Canada) in Tris-acetate-EDTA buffer. Electrophoresis was conducted using a horizontal gel system at 120 V for 1 hour. For species identification, PCR products from *Hepatozoon*-positive

samples were sequenced bidirectionally using the same primers through Sanger sequencing at Atlas Biotechnology (Ankara, Türkiye). However, due to financial constraints, only ten positive samples (five from each province) were selected for sequencing. The resulting sequences were aligned and assembled into consensus sequences using Geneious Prime 2025.1.2 software (https://www.geneious.com). These consensus sequences were subsequently compared with reference sequences in the GenBank database using the basic local alignment search tool (BLAST) algorithm (https://blast.ncbi.nlm.nih.gov/Blast.cgi). The resulting sequence data were submitted to the GenBank database under the following accession numbers: PV765406- PV765415.

Phylogenetic Analysis

A phylogenetic tree was constructed using the online tool available at https://www.phylogeny.fr. The reference sequences retrieved from the NCBI GenBank database (https://www.ncbi.nlm.nih.gov) AY628681.1, KC138533.1, and LC179794.1 (*H. felis*); KY649446.1 and KC138531.2 (*H. canis*); and AF130361.1 (*H. catesbianae*) (11); OQ862293-OQ862310 (22); OM214003-OM214012 (14) were used in the analysis. GenBank accession number, host and country of origin were included in the phylogenetic tree.

Statistical Analysis

All statistical analyses were performed using the SPSS software package (IBM SPSS for Windows, version 25). Associations between potential risk factors and the presence of infection were assessed using the chi-square (χ^2) test. A p-value less than 0.05 was considered statistically significant.

RESULTS

Demographic Characteristics and Molecular Prevalence of *Hepatozoon* spp.

A total of 203 domestic cats were included in this study, comprising 100 from İzmir and 103 from Aydın provinces. Of these, 129 were female and 74 were male. Based on age distribution, 75 cats were younger than 1 year, 118 were between 1 and 4 years old, and 10 were older than 4 years. Regarding housing conditions, the majority were outdoor cats (n=161), followed by cats with both indoor and outdoor access (n=12), while only 30 were strictly indoor cats. Importantly, all animals included in the study had no prior history of antiparasitic treatment.

Among the total 203 blood samples screened, 67 (33.0%) were found to be positive for *Hepatozoon* spp. by PCR. Region-specific prevalence rates were 22.0% (22/100) in İzmir and 43.6% (45/103) in Aydın.

Sequence and Phylogenetic Analysis

Phylogenetic analysis of *Hepatozoon*-positive samples was conducted based on the *18S rRNA* gene sequences obtained in this study, along with reference sequences retrieved from the GenBank database. Both BLAST results and phylogenetic tree reconstruction confirmed that all positive samples belonged to *H.* felis (Figure 1). Nucleotide sequence analysis of the *H. felis 18S rRNA* gene revealed a sequence similarity ranging from 98.58% to 100%. Moreover, the *H. felis* sequences obtained in this study clustered with previously reported sequences from Türkiye (11,22), which have been classified as *H. felis* genotype I.

Risk Factor Analysis

Out of 203 cats examined, 67 (33.00%) were positive for *Hepatozoon* spp. by PCR. Statistical analysis revealed a significant association between infection and age (p=0.020), with the highest positivity observed in cats ≤1 year (44.00%) (Table 1). A significant difference was also found between provinces, with higher prevalence in Aydın (43.6%) compared to İzmir (22.00%) (p=0.001) (Table 1). No significant associations were detected for sex (p=0.288), housing type (p=0.363), or clinical status (p=0.625), although slight differences in infection rates were noted across categories (Table 1). These findings suggest that younger age and geographic location may act as potential risk factors for *Hepatozoon* spp. infection in the studied cat population, while sex, housing conditions, and clinical status showed no statistically significant associations.

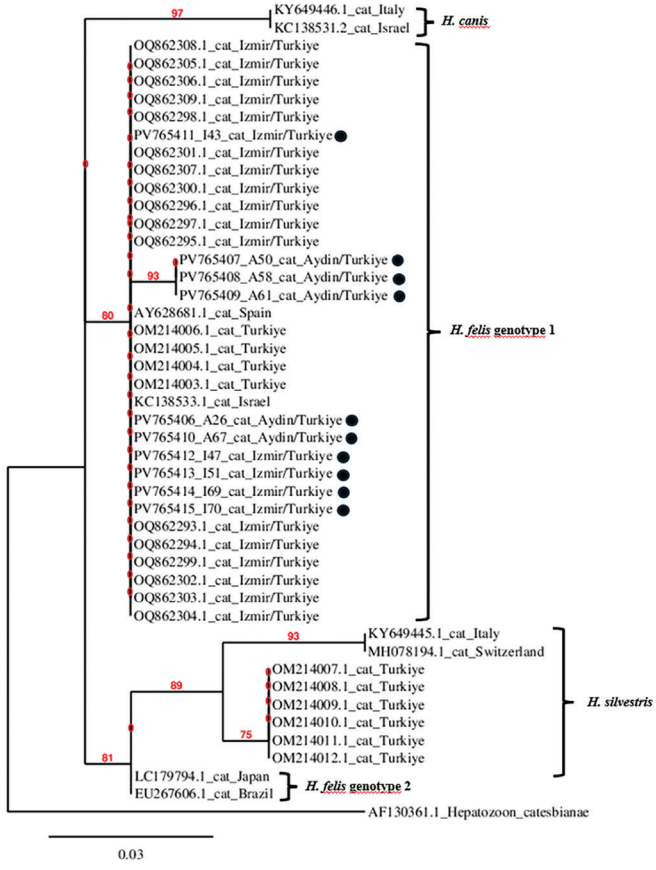

Figure 1. Phylogenetic tree based on the *18S rRNA* gene sequences of *Hepatozoon felis* detected in cats from Türkiye, along with representative sequences of *H. felis*, *H. canis*, and *H. silvestris* obtained from felids. Additionally, all *H. felis* and *H. silvestris* isolates previously reported from Türkiye and available in GenBank were included in the analysis. *H. catesbianae* was used as the outgroup. Molecular evolutionary genetic analysis was performed using the online platform available at https://www.phylogeny.fr. The phylogenetic tree includes information on the host species, country of origin, and GenBank accession number for each sequence. Black circles indicates the *H. felis* sequences obtained in the present study from cats in İzmir (coded with "i") and Aydın (coded with "A"), Türkiye

Table 1. Association between *Hepatozoon* spp. infection and potential risk factors in cats

Variable		Total (n)	PCR positive (%)	p-value
Age	≤1 year	75	33 (44.00)	0.020*
	1-4 years	118	33 (27.97)	
	>4 years	10	1 (10.00)	
Sex	Female	129	46 (35.66)	0.288
	Male	74	21 (28.38)	
Housing type	Indoor	30	11 (36.67)	0.363
	Indoor-outdoor	12	6 (50.00)	
	Outdoor	161	50 (31.06)	
Clinical status**	Viral, bacterial, or parasitic infections	34	10 (29.41)	0.625
	Unknown	169	57 (33.73)	
Province	İzmir	100	22 (22.00)	0.001*
	Aydın	103	45 (43.69)	

*: Indicates p-values considered as statistically significant (<0.05) based on the chi-square test, **: Indicates clinical status refers to 34 of the 203 cats examined that were diagnosed with other infectious diseases, including 30 viral infections (Feline Leukemia Virus, Feline Herpesvirus Type 1, Feline Calicivirus, Feline Infectious Peritonitis, and Feline Immunodeficiency Virus); one parasitic infection (*Toxocara cati*); and three bacterial infections of unknown etiology, PCR: Polymerase chain reaction

DISCUSSION

This study primarily aimed to investigate the presence and molecular characterization of *Hepatozoon* spp. in domestic cats from two provinces in the Aegean coast of Türkiye, with a particular focus on species identification and genotype distribution. Of the 203 cats tested, 33.0% (67/203) were found to be positive for *Hepatozoon* spp. by PCR analysis. This notably high prevalence highlights the widespread presence of this protozoan parasite in feline populations in the western part of Türkiye. Among the 67 *Hepatozoon*-positive cats, 10 samples were successfully sequenced. Subsequent BLAST and phylogenetic analyses confirmed that all sequenced samples belonged to *H. felis* (Figure 1).

Previous studies have demonstrated the presence of *Hepatozoon* spp. infection in cats in Türkiye, with varying prevalence rates reported across different regions. The prevalence observed in this study (33.0%) is considerably higher than those reported in previous molecular studies conducted in other regions of Türkiye, which documented infection rates of 2.37%, 10.8%, and 18.64% (14,22,23). In a study conducted on 1012 stray cats from İzmir province, which is located in the same region as the current study (23%), reported a much lower prevalence of *H. felis* (2.37%) compared to our findings. Notably, the samples used in that study were collected approximately a decade earlier than those analyzed in the present investigation. It is possible that ecological changes in recent years such as global warming, rapid urban population growth, and the increased transcontinental movement of companion animals have contributed to a rise in the transmission rates of vector-borne pathogens worldwide (29), potentially influencing the current distribution of *Hepatozoon* spp. infections.

The prevalence of *Hepatozoon* spp. detected in Aydın (43.69%) was significantly higher than that observed in İzmir (22%) (p=0.001). A review of the existing literature reveals that the prevalence of *Hepatozoon* spp. infection in cats shows considerable variation across different geographical regions worldwide, with reported rates ranging wide 0.6-83.3% (3,5,10,11,30-33). The findings of the present study are in agreement with this broad global variability in prevalence rates. Although the samples in both provinces were selected in equal numbers and from cats with similar characteristics, specifically stray cats without a history of antiparasitic treatment, the results varied. This discrepancy may be attributed to the fact that some of the sampling areas in Aydın shifted from urban to rural settings.

Although biological and epidemiological knowledge about *H. felis* remains limited, *Rhipicephalus sanguineus* is considered one of its main potential vectors (34). The ingestion of ticks during grooming behavior in cats may also contribute to the transmission of *Hepatozoon* species (2). In the present study, the previously reported presence of tick species belonging to the genus *Rhipicephalus* in the study area (35) supports the infection rates observed.

In the current study, all sequenced samples were identified as *H. felis*. These findings are consistent with previous molecular investigations conducted in Türkiye, particularly in İzmir and Tekirdağ, where *H. felis* was the only *Hepatozoon* species detected in cats, with prevalence rates of 2.37% and 10.8%, respectively (22,23). However, a recently published study from the northern part of Türkiye reported the presence of both *H. felis* and *H. silvestris* in feline hosts (14). Additionally, a case report from Aydın documented the detection of *H. canis* in a cat by both microscopic examination and PCR analysis; however, sequence analysis was not performed in the study (26).

The taxonomic status of *Hepatozoon* spp. infecting felids is not fully established (10). *H. felis* has been identified as the predominant species infecting cat populations worldwide (7). Due to the high genetic variability observed across various studies, *H. felis* has been proposed to represent a species complex (36). To date, phylogenetic analysis based on 18S rRNA sequences revealed that two distinct genotypes of *H. felis* genotypes I and II have been reported in domestic cats (11). Among these, genotype I is recognized as the most common genotype infecting domestic cats. Pereira et al. (11) reported that *H. felis* genotype I has been detected in cats from Europe, Asia, Africa, and South America. Phylogenetic analysis in this study revealed that all sequenced isolates clustered within *H. felis* Genotype I. Similarly, studies conducted in Türkiye have also identified *H. felis* genotype I (14,22), supporting the notion that this genotype is the most prevalent one in the country.

In a study conducted by Attipa et al. (31), non-healthy cats were found to have a threefold higher risk of *Hepatozoon* spp. infection compared to healthy individuals (p=0.010); however, the authors noted that the observed health problems might not be directly caused by hepatozoonosis and could instead result from coinfections with other pathogens. In the present study, no statistically significant association was found between *Hepatozoon* spp. infection and the presence of viral, bacterial, or other parasitic agents, possibly due to the limited number of infected animals (34 out of 203). Additionally, in line with previous findings (2,14,31), no significant association with sex was observed.

To date, studies have not identified a significant association between age and *Hepatozoon* infection in cats (2,7,14,31). However, in the present study, a statistically significant difference was observed in cats aged one year or younger (p=0.020). Similarly, Attipa et al. (31) reported that 21% of kittens under six months of age tested positive for *Hepatozoon* spp. In line with these findings, the present study found a *Hepatozoon* spp. positivity rate of 44% among cats aged one year or younger, which was statistically significant. When comparing overall age distributions, it appears that differences in age group classifications among studies may lead to inconsistent results. Nonetheless, the notably high infection rates detected in young cats in this study are of particular importance. These findings raise the possibility of vertical (transplacental) transmission from queen to offspring. Indeed, studies on *H. canis* in dogs have demonstrated that transplacental transmission is possible (37,38). Vertical transmission is an important adaptation in many parasites that enables the persistence of infection within a population.

Previous studies have reported a significant association between infection and outdoor access (7,14,31). In the present study, a statistically significant difference was initially detected in cats reported to live indoors. However, interviews conducted with cat owners revealed that these animals had frequent contact with other animals that had access to outdoor environments. Upon reevaluation of the living conditions, it became evident that these cats were indirectly exposed to external sources. These findings support the vector–host transmission route and indicate that animals living outdoors or in contact with the outdoor environment are at increased risk of infection.

The present study provides valuable insights into the current epidemiological status of *Hepatozoon* spp. infections in domestic cats from two provinces located in the Aegean cost of Türkiye. Molecular analyses confirmed that *H. felis* is the predominant species circulating in these areas, particularly among free-roaming cats.

Hepatozoon spp. infections in cats appear to be largely asymptomatic or present with occasional subclinical manifestations, which may contribute to underdiagnosis in routine clinical practice (7). Given the relatively high prevalence of *Hepatozoon* spp. observed in present study and its widespread distribution among free-roaming feline populations (22,33), this parasitic infection warrants further attention.

CONCLUSION

In conclusion, this study provides up-to-date epidemiological data on feline hepatozoonosis in western Türkiye and confirms *H. felis* genotype I as the predominant species. Raising awareness among veterinarians and incorporating *Hepatozoon* spp. into routine diagnostic panels is essential, particularly in areas with high stray cat populations. Further molecular surveillance and larger-scale studies are needed to better understand transmission dynamics and potential risk factors.

*Ethics

Ethics Committee Approval: The study was performed under the instructions and approval of the Institutional Animal Ethics Committee of Aydın Adnan Menderes University (protocol no: 64583101/2024/019, date: 18/01/2024).

Informed Consent: Animal owners participating in the study were informed about the purpose and procedures of the research, and written informed consent was obtained before blood sample collection.

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Footnotes

*Authorship Contributions

Concept: S.H., M.P., Design: S.H., M.P., Data Collection or Processing: S.H., M.P., Analysis or Interpretation: S.H., M.P., Literature Search: S.H., M.P., Writing: S.H., M.P.

Conflict of Interest: No conflict of interest was declared by the authors.

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