Ticks and Tick-borne Diseases 14 (2023) 102192

Contents lists available at ScienceDirect

Ticks and Tick-borne Diseases

journal homepage: www.elsevier.com/locate/ttbdis

Original article

Hepatozoon species infecting domestic cats from countries of the Mediterranean basin

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ARTICLE INFO

Keywords:
Cats
Hepatozoon felis
Hepatozoon silvestris
Clinical-pathological findings

ABSTRACT

Tick-borne diseases (TBDs) are caused by pathogens of human and veterinary concern representing a major public health issue worldwide. Although feline medicine has progressed much in the recent decades, data on feline TBDs (FeTBDs) remain scant. Therefore, this study aimed to assess the prevalence of apicomplexan parasite infections, associated risk factors and clinical-hematological abnormalities in domestic feline populations from countries of the Mediterranean basin. Blood and serum samples from cats (n = 600) living in France, Greece, Israel, Italy, Portugal and Spain were collected along with animal data (i.e., age, sex, breed, housing conditions and geographical origin), clinical signs and laboratory blood test parameters. Cats were grouped according to their age as kitten (up to one year), young (between one and six years), mature (between seven and ten years) and senior (older than ten years). Blood samples were tested for *Hepatozoon* spp. and piroplasmids by conventional PCR targeting 18S rRNA gene. The overall prevalence of *Hepatozoon* spp. infection was 14.5%, being significantly higher in cats from Greece (30%) and Portugal (23%), followed by Spain (15%), Israel (15%) and France (4%). Cats from Italy scored negative. *Hepatozoon felis* was identified in 86 animals, with three different sequence types and *H. silvestris* was detected in one shelter cat from Portugal. No piroplasmid DNA was amplified. The risk of *Hepatozoon* spp. infection was related to feline geographical provenience, housing condition and age. No statistical correlation was reported with any clinical signs, while increased alanine aminotransferase (ALT) activity was the only laboratory abnormality significantly associated (p = 0.03) with the infection. Data suggest a high circulation of *H. felis*, and only occasionally of *H. silvestris*, within domestic feline populations in the Mediterranean basin, mainly in shelter or free roaming and young cats with asymptomatic or subclinical infection.

1. Introduction

Tick-borne diseases (TBDs) are among the most important vector-borne diseases of human and veterinary concern worldwide, second only to those caused by pathogens transmitted by mosquitoes (Otranto et al., 2015; Sgroi et al., 2022). In recent decades, global mobility, urbanization and the growing number of household pets have contributed to the rise of arthropod populations in urban and peri-urban settings (Dantas-Torres et al., 2013; Sgroi et al., 2021). Although TBDs have been mainly studied in dogs, data on feline TBDs (FeTBDs) are scant, being usually characterized by non-specific clinical signs and laboratory alterations (Otranto et al., 2017; Latrofa et al., 2020). A typical example

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https://doi.org/10.1016/j.ttbdis.2023.102192
Received 27 February 2023; Received in revised form 12 April 2023; Accepted 26 April 2023
Available online 16 May 2023

is feline babesiosis by *Babesia felis*, which is known to be endemic in South Africa (Ayoob et al., 2010; Penzhorn and Oosthuizen, 2020), although other species including *Babesia vulpes, Babesia vogeli* and *Babesia canis* have been documented worldwide (Vilhena et al., 2013; Alho et al., 2017; Akram et al., 2019; Remesar et al., 2022;). Cats infected and ill by *B. felis* presented anorexia, lethargy, and exercise intolerance, together with hemolytic anemia and icterus as main clinical-pathological findings (Penzhorn et al., 2004). Conversely, cytauxzoonosis caused by *Cytauxzoon felis* was firstly described in wild felids (i.e., *Felis silvestris silvestris, Lynx lynx*) from North America (Meinkoth and Kocan, 2005). The disease commonly occurs in an acute and often fatal form (i.e., 97% mortality), and is characterized by a febrile illness, depression, tachycardia, vomiting, anorexia and dyspnea along with anemia, leukopenia, thrombocytopenia and lymphopenia (Meinkoth and Kocan, 2005; Birkenheuer et al., 2006). Although *C. felis* is the most common species in North America, three new species, including *Cytauxzoon europaeus, Cytauxzoon otrantorum* and *Cytauxzoon banethi*, were recently described in wild felids and domestic cats from Europe (Panait et al., 2021; Willi et al., 2022) indicating the importance of wild animals in the circulation of tick-borne pathogens (TBPs) causing diseases in domestic mammals (Otranto et al., 2015).

Hepatozoonosis, mainly caused by *Hepatozoon felis*, was first reported in domestic cats from India (Patton, 1908) and afterwards in many countries worldwide (Allen et al., 2011; Baneth et al., 2013; Giannelli et al., 2017). *Hepatozoon canis* infection is sporadically described in domestic and wild felids (Baneth et al., 2013; Iatta et al., 2020), along with *Hepatozoon silvestris*, firstly detected in European wild cats (*F. silvestris silvestris*) from Bosnia and Herzegovina (Hodžić et al., 2017) and then in domestic cats from Italy (Giannelli et al., 2017; Grillini et al., 2021). Infection with *H. felis* is generally subclinical and the pathogenesis in the feline host is mainly associated with the infection of myocardial and skeletal muscles, with no significant local inflammatory response (Baneth et al., 2013). In addition, stressed or immunocompromised animals may present lethargy, anorexia, and fever as clinical signs (Baneth et al., 1998; Basso et al., 2019), together with anemia and creatinaemia as laboratory abnormalities (Díaz-Regañón et al., 2017). On the other hand, *H. silvestris* was described as the causative agent of a fatal myocarditis (Kegler et al., 2018) and intussusception (Simonato et al., 2022) suggesting a potential high virulence for domestic cats.

The diagnosis of FeTBDs is mainly achieved by blood smear microscopy and molecular techniques, the latter are more sensitive and specific since they also allow an etiological identification at the species level, when combined with sequencing (Jittapalapong et al., 2006; Mosqueda et al., 2012; Sherrill and Cohn, 2015).

Despite a growing scientific evidence on feline infections with TBPs, there is an increased pressure on feline medicine specialists to fulfill gaps in knowledge regarding identification of FeTBPs vectors, geographical distribution and clinical features (Penzhorn et al., 2004; Otranto and Dantas-Torres, 2010; Baneth et al., 2013; Sherrill and Cohn, 2015). Moreover, most of the studies have been carried out on a low number of cats from limited geographic areas (Vilhena et al., 2013; Grillini et al., 2021). Therefore, this study aimed to assess the prevalence of apicomplexan parasite infections, associated risk factors and clinical-hematological abnormalities in feline populations from different countries of the Mediterranean basin.

2. Materials and methods

2.1. Study population

Blood and serum samples of cats (n = 600) were included in the study, as part of a multicenter survey on feline leishmaniosis. One hundred cats per each geographical region (i.e., France, Greece, Israel, Italy, Portugal and Spain) with a history of regular outdoor access (preferably colony or stray cats), not treated with ectoparasiticides or repellents in the previous 6 months, and with clinical-pathological information were primarily included in this study. Data on the animals, including signalment (i.e., age, sex, breed), housing conditions and geographical provenience, were recorded at the enrollment (Table 1). Health status and laboratory parameters, including complete blood cell count (CBC) and serological biochemical parameters (Creatinine, Urea, Alanine-aminotransferase, Albumin, Total proteins, Total globulins and A/G ratio), were recorded when available (Table 2). Cats were grouped according to their age as kitten (up to one year), young (between one and six years), mature (between seven and ten years) and senior (older than ten years) (Quimby et al., 2021).

2.2. Molecular procedures

DNA was extracted from 200 µl of whole blood using a commercial kit (QIAampDNA Blood Tissue, Qiagen, Hilden, Germany) according to the manufacturer's instructions and analyzed for the detection of piroplasmids and *Hepatozoon* spp. by conventional PCR targeting a partial 18S rRNA gene sequence (520 bp), using primers RLBF (5′-GAGGTAGTGACAAGAAATAACAATA-3′) and RLBR (5′-TCTTCGATCCCCTAACTTTC-3′) (Gubbels et al., 1999). Reaction composition in a 25 µl volume was as follows: 14.3 µl × sterile water; 3 µl $MgCl_2$ solution (25 mM); 2.5 µl 10x PCR buffer (1.5 mM); 2.5 µl dNTPs (1.25 mM); 0.25 µl of each primer (100 pmol/µl); 0.2 µl AmpliTaq Gold DNA™ polymerase (250 U) and 2 µl of DNA sample. The reactions were performed in an automated DNA thermal cycler (BIO-RAD T100™ Thermal Cycler) as follows: 95 °C for 10 min initial denaturation, followed by 38 cycles of 95 °C for 30 s, 54 °C for 30 s, and 72 °C for 1 min, and then 72 °C for 7 min for final elongation. Amplified PCR products were visualized by gel-electrophoresis in 2% agarose gel containing GelRed nucleic acid gel stain (VWR International PBI, Milan, Italy) and viewed on a GelLogic 100 gel documentation system (Kodak, New York, USA). The positive PCR products were purified and sequenced in both directions using the same forward and reverse primers, employing the Big Dye Terminator v.3.1 chemistry in a 3130 Genetic analyzer (Applied Biosystems, California, USA) in an automated sequencer (ABI-PRISM 377). Nucleotide sequences were edited, aligned and analyzed using the Geneious platform version 9.0 (Biomatters Ltd., Auckland, New Zealand) (Kearse et al., 2012), and compared with available sequences in the GenBank database, using the Basic Local Alignment Search Tool (BLAST; http://blast.ncbi.nlm.nih.gov/Blast.cgi). Positive samples sequenced as *Hepatozoon* spp. were further tested for piroplasmids specifically and for detection of possible co-infections, by conventional PCR targeting the partial 18S rRNA gene (410 bp) using primers PiroA (5′-AATACCCAATCCTGACACAGGG-3′) and PiroB (5′-TTAAATACGAATGCCCCCAAC-3′) (Olmeda et al., 1997). The following thermal cycling conditions were used: 95 °C for 10 min initial denaturation, followed by 34 cycles of 95 °C for 30 s, 64 °C for 20 s, and 72 °C for 30 s, and then 72 °C for 7 min for final elongation. Negative (blood sample of a negative cat never exposed to tick bites) and positive controls (i.e., *C. felis* and *H. felis* from cats) were used in all PCR runs.

2.3. Phylogenetic analysis

Phylogenetic analysis was based on 471 bp of the 18S rRNA gene sequences of *Hepatozoon* spp. detected in the study and those available from the GenBank database. Phylogenetic relationship was inferred by the Maximum Likelihood (ML) method based on Tamura's 3-parameter model (Tamura, 1992) and Gamma distribution used to model evolutionary rate differences among sites (+*G*) selected by best-fit model (Nei and Kumar, 2000). Evolutionary analyses were conducted on 8000 bootstrap replications using the MEGA X software (Kumar et al., 2018).

2.4. Statistical analysis

Exact binomial 95% confidence intervals (CIs) were established for proportions herein found. A Chi-squared test ($\chi 2$) was used to assess any

statistical association among TBP infection and signalment data, outdoor activity, geographical origin, health status and laboratory parameters. A p-value (*p*) less than 0.05 was considered significant. Odds ratios (ORs) were calculated to assess the infection risk according to the several variables (i.e., age, sex, breed, housing condition and geographical provenience) of the participants. The 95% CIs, chi-square, *p*-value and OR values were calculated by using the software Epitools - Epidemiological Calculators (Sergeant, 2018).

3. Results

The overall prevalence of *Hepatozoon* spp. infection was 14.5% (87/600, 95% CI: 11.9–17.5), and was significantly higher in cats from Greece (30%, 95% CI: 21.8–39.6) and Portugal (23%, 95% CI: 15.7–32.2), followed by Spain (15%, 95% CI: 9.2–24.4), Israel (15%, 95% CI: 9.2–24.4) and France (4%, 95% CI: 1.2–10.6). All cats from Italy scored negative (Fig. 1). *Hepatozoon felis* was identified in 86 cats and *H. silvestris* in one shelter cat from Portugal. No co-infections and no DNA of piroplasmids were detected. The phylogenetic relationships are depicted in Fig. 2 with three sequence types of *H. felis,* submitted to GenBank under accession numbers OP144204 for ST1, OP144205 for ST2 and OP144206 for ST3, clustering with those feline sequences available in this database, regardless of their geographical provenience and host species. The sequence of *H. silvestris* (Acc. No. OP144219) clusters with those of wild cats (*F. silvestris*) from Bosnia and Herzegovina and with one sequence of a domestic cat from Switzerland. The

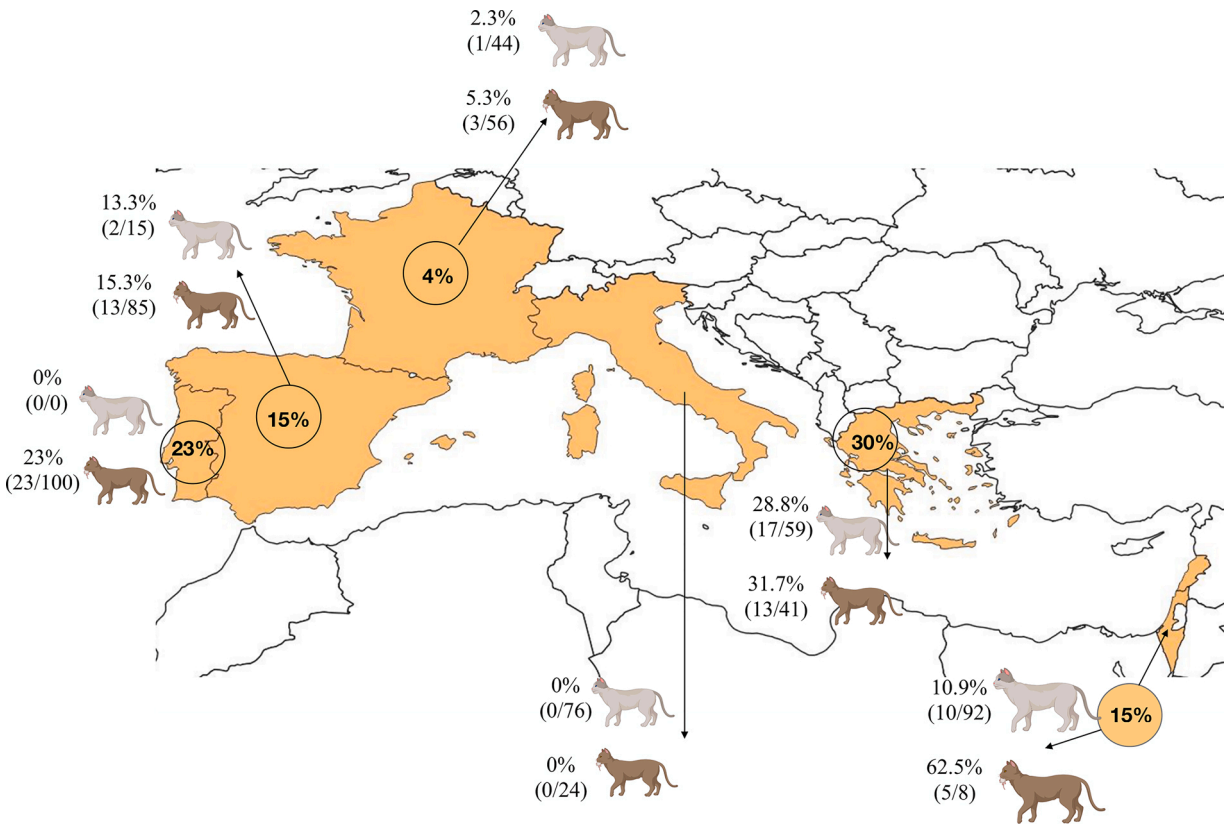

Fig. 1. Prevalence of *Hepatozoon* spp. infections in shelter/free roaming (brown cats) and owned animals (light colored cats) per geographical area (orange).

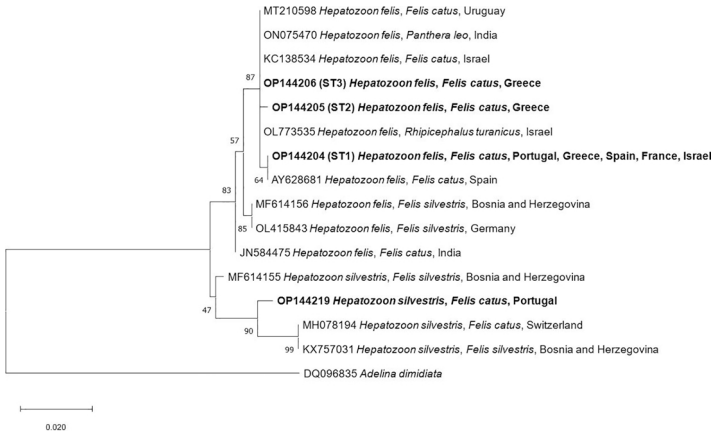

Fig. 2. Phylogenetic relationships between the 18S rRNA *Hepatozoon* spp. sequences herein obtained (consensus sequences of this study are bold) and those available in the GenBank database. *Adelina dimidiata*. (DQ096835) was used as outgroup.

Table 1
Prevalence of infections according to animal data.

	Cats enrolled (n = 600) (%)	Positive cats (n = 87) (%, 95% CI)
Age		
<1 year	118 (19.6)	17 (14.4, 9.1–21.96)
1- 6 years	332 (55.3)	56 (16.8, 13.2–21.3)
7–10 years	66 (11)	7 (10.6, 4.9–20.6)
>10 years	83 (13.8)	7 (8.4, 3.8–16.6)
Gender		
Female	315 (52.5)	41 (13, 9.7–17.2)
Male	285 (47.5)	46 (16.3, 12.3–20.8)
Housing condition		
Sheltered/free roaming	314 (52.3)	58 (18.5, 14.5–23.1)
Owned	286 (47.6)	29 (10.1, 7.1–14.2)
Breed		
European	569 (94.8)	83 (14.6, 11.9–17.1)
Non-European	31 (5.2)	4 (12.9, 4.5–29.4)

prevalence of infections correlated with age, gender, housing condition and breed is reported in Table 1. Infection by *Hepatozoon* spp. was significantly associated with the geographical area, the housing conditions and the age. In particular, cats living in Greece had a higher risk of infection than those from Spain (p = 0.01, OR= 2.4), Israel (p = 0.01, OR= 2.4) and France (p<0.01, OR= 10.3). Cats from Portugal (p<0.01, OR= 7.2), Spain (p = 0.01, OR=4.2) and Israel (p = 0.01, OR=4.2) had a significantly higher risk than those from France. Young cats were more exposed to *Hepatozoon* spp. infection than adults (p<0.01, OR= 1.7), as well as and shelter/free roaming cats compared to owned cats (p<0.01, OR=2). No correlation with gender and breed was found.

No statistically significant association was found between infection and clinical signs (p>0.05), but at least one clinical manifestation was described in 27.6% of infected cats (Table 2), with systemic (i.e., fever, pale mucous membranes, jaundice, lymphadenomegaly and malnutrition) and gastro-intestinal signs (i.e., vomiting and diarrhea) being the most common.

Out of 87 positive cats, laboratory parameters (Table 2) were available for 53 animals, of which 98.8% (52/53) presented at least one clinical-pathological alteration. Leukocytosis, hyperproteinemia and increased ALT values were the most frequently observed laboratory findings, and the latter was significantly associated with *Hepatozoon* infection (p = 0.03).

Table 2
Clinical (a) and laboratory (b) data of cats negative and positive to *Hepatozoon* spp.

(a)	Negative cats (%)	Positive cats (%)
Clinical signs	513	87
Yes	224 (43.6)	24 (27.6)
No	289 (56.3)	63 (72.4)
Systemic signs	133 (25.9)	11 (12.6)
Skin lesions	60 (11.7)	0 (0)
Ocular signs	23 (4.5)	1 (1.1)
Oral signs	56 (10.9)	6 (6.9)
Gastro-intestinal signs	36 (7)	8 (9.2)
Respiratory signs	44 (8.6)	6 (6.9)
Urinary signs	21 (4)	2 (2.3)
(b)	**Negative cats (%)**	**Positive cats (%)**
Clinic pathological alterations	284	53
Yes	273 (96.1)	52 (98.8)
No	11 (3.9)	1 (1.2)
Hematology		
Haematocrit (28–43%)	284	53
High	47 (16.5)	12 (22.6)
Low	52 (18.3)	5 (9.4)
Normal	185 (65.1)	36 (67.9)
Leukocytes (5.5–12×1000/µl)	284	53
High	123 (43.3)	27 (50.9)
Low	26 (9.1)	1 (1.9)
Normal	135 (47.5)	25 (47.2)
Platelets (130–400×10,000/µl)	273	52
High	58 (21.2)	12 (23)
Low	37 (13.5)	5 (9.6)
Normal	178 (65.2)	35 (67.3)
Biochemistry		
Total proteins (5.8–7.7 g/dl)	269	53
High	99 (36.8)	24 (45.3)
Low	9 (3.3)	2 (3.7)
Normal	161 (59.8)	27 (50.9)
Albumins (2.8–3.7 g/dl)	284	53
High	69 (24.2)	8 (15)
Low	74 (26)	18 (33.9)
Normal	141 (49.6)	27 (50.9)
Total globulins (2.9–4.3 g/dl)	199	40
High	102 (51.2)	23 (57.5)
Low	8 (4)	0
Normal	89 (44.7)	17 (42.5)
A/G Ratio (0.6–1.3)	199	40
High	7 (3.5)	0
Low	74 (37.2)	17 (42.5)
Normal	118 (59.2)	23 (57.5)
Urea (29–60 mg/dl)	267	53
High	51 (19.1)	6 (11.3)
Low	48 (17.9)	15 (28.3)
Normal	168 (62.9)	32 (60.3)
Creatinine (0.93–1.7 mg/dl)	279	49
High	68 (24.3)	12 (24.4)
Low	69 (24.7)	7 (14.3)
Normal	142 (50.8)	30 (61.2)
Alanine transaminase (33–70 UI/l)	258	52
High	80 (31)	24 (46.1)
Low	38 (14.7)	4 (7.7)
Normal	140 (54.2)	24 (46.1)

4. Discussion

The results indicate a high prevalence of *Hepatozoon* spp. infection in the feline populations enrolled, highlighting its occurrence in the Mediterranean basin (Panait et al., 2023). Indeed, the large circulation of *Rhipicephalus sanguineus* sensu lato (Lorusso et al., 2010; Aktas, 2014), *Rhipicephalus turanicus* (Maia et al., 2014) and *Ixodes ricinus* (Karasartova et al., 2018) in the Mediterranean countries may support the high prevalence of feline infection herein reported (Baneth et al., 1998; ECDC, 2022; Dantas-Torres et al., 2013; Estrada-Peña et al., 2017).

The prevalence of *H. felis* infection from Greece (30%) was similar to that reported in a single study carried out in domestic cats from insular and continental regions of Greece (i.e., 25.5%; Morelli et al., 2021). Conversely, the percentage of infection described in free roaming cats from Portugal (i.e., 23%) was slightly higher than that reported in the northern and central regions of the country (i.e., 16.5%; Vilhena et al., 2013), probably due to the different source of sampled animals (i.e., owned *vs* shelter cats). Indeed, the outdoor lifestyle is a risk factor for *Hepatozoon* spp. infection (Baneth et al., 2013; Lloret et al., 2015), since stray/shelter cats may be highly exposed to ectoparasites, have marked grooming activity, higher reproduction rates and predatory behavior, which may also favor the alternative transmission routes hypothesized for this pathogen (i.e., transplacental and carnivorism) (Baneth et al., 2013; Schäfer et al., 2022; Vitale, 2022). Different composition of feline populations and tick distributions may also justify the higher prevalence recorded in cats from Spain (15%) than that described in the literature (i.e., 0.6–4%) (Criado-Fornelio et al., 2006; Tabar et al., 2008). The percentage of infection detected in Israel (15%) suggests a high exposure of stray/shelter cats from this country, as previously reported (Baneth et al., 2013). Conversely, the absence of PCR positive cats from Italy, where mainly privately owned were enrolled, suggests a spotted distribution in specific areas (Giannelli et al., 2017; Grillini et al., 2021). Likewise, the low infection rate detected in cats from France (4%) agrees with the single epidemiological study reporting similar data from this country (1.7%; Criado-Fornelio et al., 2009).

Among risk factors, young cats were more exposed than adults to *Hepatozoon* spp. infection probably because they are more prone to predatory behavior that favors the parasite transmission through tick ingestion and they have a less efficient immune response (Baneth et al., 1998; Overall et al., 2005).

Furthermore, the results confirm *H. felis* as the main species circulating in cat populations (Baneth et al., 2013), followed by *H. silvestris* (Hodžić et al., 2017, 2018; Giannelli et al., 2017), showing an increased distribution in some European countries (Hodžić and Alić, 2023). On sequencing analysis, *H. felis* had three sequence types, with ST1 as the most represented (95.4%) in all regions except two (i.e., ST2 and ST3) in Greece, likely justified by the vector population (Harris et al., 2019).

The minor clinical impact of feline *Hepatozoon* spp. infection found in this study is supported by previous data (Baneth et al., 2013; Grillini et al., 2021), with only 27.6% of positive cats presenting at least one clinical manifestation, without any statistically significant association with the infection. On the other hand, the increased ALT values (>70 UI/l) correlated positively with *Hepatozoon* spp. infection, may be related to the muscle damage caused by the parasite in feline hosts (Aroch et al., 2010; Baneth et al., 2013), even though low hematocrit values and hypercreatininemia were described in the literature (Díaz-Regañón et al., 2017). However, clinical signs and hematological alterations should be interpreted with caution considering the possible occurrence of concomitant diseases and/or coinfections within the studied feline population.

5. Conclusions

This study provides data on the current epidemiological status of feline *Hepatozoon* spp. infection in different countries of the Mediterranean basin, suggesting that *H. felis* mainly circulates in shelter and free roaming cats, and young cats being more likely infected. Overall, *Hepatozoon* spp. infection usually occurs asymptomatically or with occasional subclinical manifestations.

Ethics approval

A written consent for patient enrolment was obtained from owners or animal protection societies' responsible persons. Animals were handled with regard for their well-being. The protocol of this study was approved by the ethical committees of the Department of Veterinary Medicine, University of Bari, Italy (Prot. Uniba 7/17), the Aristotle University of Thessaloniki, Greece (Prot. 192,746/2022), the Koret School of Veterinary Medicine Teaching Hospital, Hebrew University, Israel (Prot. KSVMVTH/7_2018), the Complutense Univeristy of Madrid, Spain (Prot.60–29,102,019), Organism Responsible for Animal Welfare from the Institute of Biomedical Sciences Abel Salazar, University of Porto, Portugal (Prot. 385/2020/ORBEA).

Funding

This work was partially supported by Elanco Animal Health GmbH (Germany, Europe). The funders had no role in data collection, data analysis, decision to publish, or preparation of the manuscript.

CRediT authorship contribution statement

Mariaelisa Carbonara: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Writing – original draft, Writing – review & editing. **Roberta Iatta:** Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing. **Giovanni Sgroi:** Data curation, Formal analysis, Investigation, Methodology, Writing – review & editing. **Elias Papadopoulos:** Methodology, Investigation, Writing – review & editing. **Clara Lima:** Methodology, Investigation, Writing – review & editing. **Emilie Bouhsira:** Methodology, Investigation, Writing – review & editing. **Guadalupe Miró:** Methodology, Investigation, Writing – review & editing. **Yaarit Nachum-Biala:** Methodology, Writing – review & editing. **Gad Baneth:** Methodology, Investigation, Writing – review & editing. **Domenico Otranto:** Data curation, Investigation, Methodology, Supervision, Writing – original draft, Writing – review & editing.

Declaration of Competing Interest

None.

Data availability

Data will be made available on request.

Acknowledgements

The authors would like to thank all the practitioners who contributed to the collection of samples that were analysed in this study. The graphical abstract was created using Biorender.

This research was supported by EU funding within the Next Generation EU-MUR PNRR Extended Partnership initiative on Emerging Infectious Diseases (Project no. PE00000007, INF-ACT).

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