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PROTOZOOLOGY - ORIGINAL PAPER

Preliminary study of *Cytauxzoon felis* infection in outdoor cats in Mashhad, Iran

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The objectives of the current study were to assess the preliminary status of *Cytauxzoon felis (C. felis)* infection among outdoor cats in Mashhad, Iran and also to compare clinicopathological findings between *C. felis* infected and non-infected cats. Blood samples were collected from 100 outdoor domestic cats between April and September in 2019. Infection with *C. felis* was determined using microscopic observation of giemsa-stained blood smears and molecular analysis. The piroplasms was microscopically detected in 5 (5%) of the blood smears with low parasitemia. The presence of *C. felis* was confirmed in one positive microscopy sample by PCR. The molecular assay revealed that 19 cats (19%) were infected with *C. felis*. Hematological and some serum biochemical factors were evaluated in both of the infected and non-infected cats. There was no association between *C. felis* infection and age, gender, and laboratory findings except for hematocrit (Hct) and concentration of total protein and globulin. Clinical signs such as fever, dehydration, lethargy, and icterus were observed only in 15.78% (3/19) of the infected cats, while 84.22% (16/19) were asymptomatic. Laboratory findings such as non-regenerative anemia, thrombocytopenia, neutrophilic leukocytosis hyperproteinemia, hypoalbuminemia, and hyperbilirubinemia were detected in the clinically infected cats. This study revealed the relatively high frequency of *C. felis* infection in outdoor domestic cats in Mashahd, Iran. The predominance of asymptomatic infection likely indicates that these cats may be infected with low-virulent strains of *C. felis*.

Keywords *Cytauxzoon felis* · Asymptomatic · Outdoor cats · PCR · Clinicopathological findings

Introduction

Cytauxzoonosis is a tick-borne protozoan disease of domestic cats and wild felids (Brown et al. 2008). Tick vectors such as *Dermacentor variabilis* and *Amblyomma americanum* are able to transmit *C. felis* from bobcats as reservoir host to domestic cats (Allen et al. 2019). Life cycle of *C. felis* is complex and involves schizont stage, typically within macrophage, and piroplasms within erythrocytes (Wang et al. 2017).

The first report of *C. felis* infection in domestic cats was documented from Missouri in 1976 (Wagner 1976). Although, *C. felis* was thought to be found only in North America, this infection has been described in cats in the South America and in Europe (Alho et al. 2016; Carli et al. 2014; Carli et al. 2012; Maia et al. 2013; Nentwig et al. 2018). So far, some sporadic cases of *Cytauxzoon* infection have been reported in Iran (Rassouli et al. 2015; Zaeemi et al. 2015), Mongolia (Reichard et al. 2005), and India (Varshney et al. 2009), and to the authors' knowledge, except in Turkey (Karaca et al. 2007) and China (Zou et al. 2019), no information exists about the epidemiology of *C. felis* infection in other Asian countries.

Generally, schizogenous phase with vascular occlusion in vital organs such as lungs, liver, kidney, and spleen, is responsible for occurrence of a rapidly progressive, systemic disease and death within 3 weeks postinfection (Cohn et al. 2020; Wang et al. 2017). Although it was believed for decades that Cytauxzoonosis is an extremely fatal disease in domestic cats, recently an increasing survival rate has been described in the USA (Brown et al. 2008; Haber et al. 2007; Meinkoth et al. 2000; Rizzi et al. 2015) and other geographic regions (Carli et al. 2012; Karaca et al. 2007; Nentwig et al. 2018; Zou et al. 2019) that suggested the existence of *C. felis* strains with inconsistent virulence in different geographic regions (Brown et al. 2009). However, a subsequent study indicated

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there is no detectable difference in virulence based on strain or ITS haplotypes (Brown et al. 2010).

Since there is no information about epidemiology and clinical aspects of *C. felis* infection in Iran, the aim of this study was to investigate on *C. felis* frequency and some clinicopathological findings in infected outdoor cats in Iran.

Materials and methods

Animals and blood collection

This study was conducted in tick activity season between April and September in 2019 and included 100 outdoor domestic shorthair cats referred to veterinary clinics in Mashhad area, in northeastern Iran. The data such as age, gender, and clinical symptoms was recorded. Cats also were inspected for the presence of ticks.

Blood specimens were obtained through jugular vein and collected in plain (WEGO, China) and K3-EDTA (FL medical, Italy) tubes and immediately transferred in cold condition to the laboratory. Blood smears were prepared from EDTA-anticoagulated whole samples and stained with Giemsa solution (10%). Serum was separated by centrifugation (Jouan, C 412, France) of the plain blood specimens at 1800*g* for 10 min and stored at − 70 °C until biochemical analysis.

Microscopic observation

The blood smears were examined with an oil immersion lens in at least 200 microscopic fields (×1000).

Molecular analysis

DNA extraction was performed by using DNA Isolation kits (DENAzist, Iran) based on the manufacturer's instructions. According to the method of Brown et al. (2008), the *C. felis* internal transcribed spacer region 2 (ITS2) plus 5.8S and 28S partial flanking regions were amplified by employing *C. felis F (5-TGA ACG TAT TAG ACA CAC CAC CT-3) and C. felis r (5- TCC TCC CGC TTC ACT CG CCG-3)* primers (Takapouzist, Iran). Amplification was conducted in a 25 μL total reaction volume containing DNA template (1 μL), primer (1 μL), and PCR red master mix (Amplicon, Denmark) using Bio-Rad thermocycler under the following program: denaturation stage (5 min at 95 °C), 34 amplification cycles (denaturation step, 30 s at 94 °C; annealing step, 30 s at 60 °C; extension step, 60 s at 72 °C), and final extension, 10 min at 72 °C. Then PCR products (10-μL) electrophoresed through a 1.7% agarose gel with TAE buffer and visualized by ethidium bromide and UV trans-illuminator. The size of the expected PCR product was approximately 431 bp from genomic *C. felis* DNA that incorporates the 265-bp ITS2 region. Distilled water and DNA isolated from a clinically healthy cat with no history of tick infestation were used in PCR reaction as negative controls. The genome of *C. felis* that was extracted previously from a wild cat (Zaeemi et al. 2015) was used as positive control.

Measurement of clinicopathological parameters

Hematological parameters including hematocrit (Hct), hemoglobin (Hb), erythrocyte count (RBC), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), platelet count (PLT), and total white blood cell count (tWBC) were measured by veterinary hematology autoanalyzer (Nihon Kohden, Japan). Differential cell count was performed manually on Giemsa-stained smears.

Some serum biochemical parameters such as alanine aminotransferase (ALT), aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transferase (GGT), total bilirubin, total protein, albumin, cholesterol, triglyceride, urea, creatinine, calcium, and inorganic phosphorus were measured by an autoanalyzer (Mindray, China) and commercial kits (Pars Azmoon, Iran). The accuracy of results was checked by Randox serum control (Antrim, UK). The serum concentration of total globulin was obtained by subtraction of albumin from the total protein.

Statistical analysis

The obtained data was analyzed by SPSS Software (version 21). The distribution of data was evaluated by *Kolmogorov-Smirnov* test. The association of infection frequency with gender, age and laboratory findings was investigated with *Chi-Square* test. Hematological and biochemical parameters that had significant association with *C. felis* infection were compared between infected and non-infected cats by independent sample *t* test. Results were considered significant at $p < 0.05$.

Results

Based on the microscopic observation (Fig. 1), although the ring-like piroplasms were observed in 5% of the blood smears (parasitemia = 0.01%), only one case of which was confirmed by molecular method. Schizont-laden macrophages were not seen on the blood smears. According to molecular findings, 19% of studied cats were infected with *C. felis* (Fig. 2). Tick infestation was not detected on the cats' body.

Although, the majority of infections was observed in male (16/19, 84.21%) and adult (14/19, 73.68%) cats, this study revealed that infection rate was not different between male and female cats and also between adult cats and kittens (Table 1).

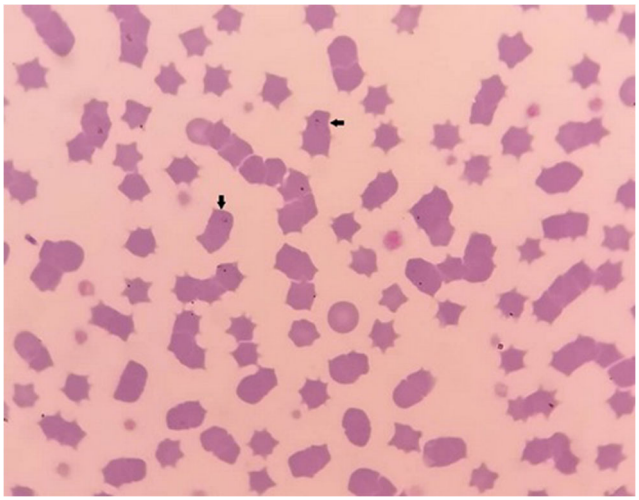

Fig. 1 Giemsa-stained peripheral blood smear from a domestic cat infected with *C. felis*. One ring form *C. felis* piroplasms (arrowheads) present within erythrocytes, 100×

A significant association ($p < 0.05$) was found between *C. felis* infection and some laboratory findings such as Hct and serum total protein and globulin concentration (Table 1). Comparison of these parameters between infected ($n = 19$) and non-infected ($n = 81$) cats revealed that Hct in the infected cats (24.21 ± 2.03%) was significantly ($p < 0.05$) lower than non-infected cats (29.73 ± 0.93%). While the serum level of total protein (82.0 ± 3.6 g/L) and globulin (49.4 ± 3.4 g/L) in the infected cats also was significantly ($p < 0.05$) higher than those in non-infected cats (total protein 72.7 ± 2.1 g/L and globulin 43.0 ± 2.2 g/L). These dysproteinemia and a non-regenerative normocytic normochromic anemia were detected in the 57.9% (11/19) of the infected cats.

Fig. 2 PCR products (431 bp) of the *C. felis* internal transcribed spacer region 2 (ITS2). Lane M, DNA 100-bp ladder; lane 1, positive control (*C. felis* infected wild cat); lane 2, negative control (DW); lane 3, negative control (clinically healthy cat); lanes 4, 10, and 11, domestic cats that tested positive for *C. felis*; lanes 5–9, domestic cats that tested negative for *C. felis*

Anemia was classified based on the Hct level to mild (20–26%), moderate (14–19%), and severe (10–13%) (Tvedten 2010) that respectively were seen in the 7 (36.8%), 1 (5.26%), and 3 (15.8%) infected cats. None of the infected cats showed very severe anemia (Hct < 10%).

Physical examination revealed clinical symptoms such as anemia, fever, icterus, depression, anorexia, dehydration, and lethargy only in 3 (15.79%) adult male infected cats. Laboratory findings such as non-regenerative anemia, thrombocytopenia, neutrophilic leukocytosis, hyperproteinemia, hypoalbuminemia, and hyperbilirubinemia were detected in the clinically infected cats (Table 2).

In the 3 clinically infected cats, detection of *feline leukemia virus (FeLV)* and *feline immunodeficiency virus (FIV)* antibodies and also infection with *Mycoplasma haemofelis* (Mhf) and *Candidatus Mycoplasma haemominutum* (Cmh) were assessed by a commercial kit (Pet Rapid Test, Quicking Biotech Co Ltd., China) and molecular technique (Torkan et al. 2013), respectively, and results showed that the clinically infected cats were tested positive for Mhf and negative for FIV and FeLV.

Discussion

This study was conducted on outdoor cats that included stray cats as well as owned cats that were allowed to roam. Cats with outdoor access are at higher risk for tick-borne diseases such as cytauxzoonosis (Nagamori and Reichard 2015).

In the present study, 5% of the cats were found positive by microscopic examination, only one of them was confirmed by molecular assay. Karaca et al. (2007) also found piroplasms similar to *C. felis* in 9 out of 120 (7.5%) domestic cats from Turkey. The false positive results are usually related to the low specificity of light microscopy. In microscopic examination, Howell–Jolly bodies, water artifacts, stain precipitate, and even other pathogens specially *Hemoplasma spp*. that is prevalent in this area (unpublished study) could have been mistaken with *C. felis* periplasms (Wang et al. 2017).

According to the molecular results, DNA of *C. felis* was detected in 19 samples. The similar frequency of *C. felis* infection has been reported in domestic cats from Yunnan Province in China and also in Italy (Carli et al. 2012; Zou et al. 2019). The frequency range of *C. felis* among healthy domestic cats in an endemic region including Arkansas, Missouri, and Oklahoma were 3.4–15.3% (Rizzi et al. 2015).

In this study, only 15.79% (3/19) of the infected cats showed clinical signs such as dehydration, lethargy, anemia, and jaundice which previously have been reported by other authors (Alho et al. 2016; Hoover et al. 1994; Maia et al. 2013; Nentwig et al. 2018).

The majority of infected cats (84.21%) did not display any clinical signs prior to diagnosis. The high frequency of

Table 1 Association of PCR results with age, gender and clinicopathological variables

Variables ($n = 100$)	Non-infected cats	Infected cats	X^2	*P* value
Gender				
Male ($n = 69$)	53 (76.81%)	16 (23.19%)	2.52	0.11
Female ($n = 31$)	28 (90.32%)	3 (9.68%)		
Age ($n = 100$)				
Adult ($n = 83$)	69 (83.13%)	14 (16.87%)	1.44	0.23
Kitten ($n = 17$)	12 (70.59%)	5(29.41%)		
Hct				
Low ($n = 37$)	26 (70.27%)	11 (29.73%)	4.39	0.04
Normal ($n = 63$)	55 (87.30%)	8 (12.70%)		
WBC				
Low ($n = 12$)	11 (91.67%)	1 (8.33%)		
Normal ($n = 57$)	49 (85.97%)	8 (14.03%)	5.34	0.07
High ($n = 31$)	21 (67.74%)	10 (32.26%)		
Platelet				
Low ($n = 52$)	40 (76.92%)	12(23.07%)	1.17	0.28
Normal ($n = 48$)	41 (85.41%)	7(14.58%)		
Total protein				
Low ($n = 6$)	6 (100.0%)	0(0.00%)		
Normal ($n = 60$)	52 (86.67%)	8 (13.33%)	6.59	0.04
High ($n = 34$)	23(67.65%)	11(23.35%)		
Globulin				
Normal ($n = 64$)	56 (87.50%)	8 (12.50%)	4.88	0.03
High ($n = 36$)	25 (69.44%)	11 (30.56%)		
Albumin				
Low ($n = 16$)	9 (56.25%)	5 (43.75%)		
Normal ($n = 77$)	65 (84.41%)	14 (15.59%)	4.26	0.11
High ($n = 7$)	7 (100.0%)	0 (0.00%)		
Creatinine				
Normal ($n = 72$)	61 (84.72%)	11 (15.28%)	2.31	0.12
High ($n = 28$)	20 (71.49%)	8 (28.57%)		
BUN				
Normal ($n = 75$)	62 (82.67%)	13 (17.33%)	0.54	0.46
High ($n = 25$)	19 (76.00%)	6 (24.00%)		
AST				
Normal ($n = 63$)	50 (79.36%)	13 (20.64%)	0.29	0.58
High ($n = 37$)	31(83.78%)	6 (16.22%)		
ALT				
Normal ($n = 71$)	56 (78.87%)	15 (21.13%)	0.72	0.39
High ($n = 29$)	25 (86.21%)	4 (13.79%)		
ALP				
Normal ($n = 57$)	47 (82.46%)	10 (17.54%)	0.18	0.66
High ($n = 43$)	34 (79.07%)	9 (20.93%)		
GGT				
Normal ($n = 90$)	72 (80.00%)	18 (20.00%)	0.58	0.44
High ($n = 10$)	9 (90.00%)	1 (10.00%)		
Total bilirubin				
Normal ($n = 83$)	70 (84.33%)	13 (15.67%)	3.53	0.06
High ($n = 17$)	11 (64.70%)	6 (35.30%)		
Glucose				
Low ($n = 21$)	18 (85.71%)	3 (14.29%)		
Normal ($n = 70$)	56 (80.00%)	14 (20.00%)	0.41	0.81
High ($n = 9$)	7 (77.78%)	2 (22.22%)		
Cholesterol				
Low ($n = 12$)	9 (75.00%)	3 (25.00%)		
Normal ($n = 70$)	60 (85.71%)	10 (14.29%)	3.69	0.15
High ($n = 18$)	12 (66.67%)	6 (33.33%)		
Triglyceride				
Normal ($n = 77$)	62 (80.52%)	15 (19.48%)	0.05	0.82
High ($n = 23$)	19 (82.61%)	4 (17.39%)		
Calcium				
Low ($n = 5$)	5 (100.0%)	0 (0.00%)		
Normal ($n = 66$)	51 (77.27%)	15 (22.72%)	1.92	0.38
High ($n = 26$)	22 (84.61%)	4 (15.38%)		
Phosphorous				
Low ($n = 8$)	6 (75.00%)	2 (25.00%)		
Normal ($n = 78$)	63 (80.77%)	15 (19.23%)	0.39	0.82
High ($n = 14$)	12 (85.71%)	2 (14.29%)		

subclinical infection has also been reported in domestic cats (Brown et al. 2008; Carli et al. 2012; Haber et al. 2007; Karaca et al. 2007; Meinkoth et al. 2000; Nentwig et al. 2018; Rizzi et al. 2015; Zou et al. 2019). This finding contradicts a long-lasting dogma that cytauxzoonosis always is a fatal disease in domestic cats (Birkenheuer et al. 2006) and it supposed that *C. felis* infection described in domestic cats in the USA likely is more virulent than strains that have been reported in other countries (Carli et al. 2012; Rizzi et al. 2015; Zou et al. 2019).

In this study, no association was found between *C. felis* infection and cats' gender and age. This finding is in agreement with other studies (Carli et al. 2012; Zieman et al. 2017; Zou et al. 2019).

Also, *C. felis* infection was not associated with the most of clinicopathological findings in this study.

The laboratory findings in the clinically infected cats included non-regenerative normocytic normochromic anemia, thrombocytopenia, neutrophilic leukocytosis, left shift, hyperbilirubinemia, hyperproteinemia, and hypoalbuminemia. Although the majority of *C. felis* infected cats in Italy were also asymptomatic, anemia in 30% (8/27) and leukocytosis in 70% (19/27) of the infected cats were observed (Carli et al. 2012). Normocytic normochromic anemia, leukopenia, thrombocytopenia, high serum level of bilirubin, glucose, low level of albumin, and increased serum activity of liver enzymes have been described as pertinent clinicopathological findings in clinically infected domestic cats (Birkenheuer et al. 2006; Hoover et al. 1994). Similar findings also have been described in some case reports of domestic infection with *C. felis* (Alho et al. 2016; Carli et al. 2014; Legroux et al. 2017; Maia et al. 2013; Nentwig et al. 2018).

Although a non-regenerative extravascular hemolytic anemia typically occurs in domestic cats with cytauxzoonosis, the bone marrow response has been reported in some animals that survived infection (Harvey et al. 2007; Nentwig et al. 2018; Walker and Cowell 1995). The severity of anemia and also bone marrow response are likely related to the stage and severity of illness (Brown et al. 2008; Sherrill and Cohn 2015).

Variable leukograms has been described in cats with cytauxzoonosis. Neutrophilic leukocytosis along with left shift detected herein indicates an inflammatory leukogram due to the release of pro-inflammatory mediators. While in the terminal stages of disease, crowded bone marrow with schizont-laden macrophages generally results in pancytopenia (Bondy et al. 2005; Harvey et al. 2007).

In the literature, thrombocytopenia often has been attributed to the occurrence of disseminated intravascular coagulation (DIC) as a common complication of feline cytauxzoonosis (Conner et al. 2015). However, this fact should not be overlooked that accurate platelet counts in cats often is difficult because their platelets are prone to clump (Rizzi et al. 2010).

Hyperbilirubinemia is a common biochemical abnormality in cytauxzoonosis as a result of both extravascular hemolysis as well as liver injury due to infiltration of schizont-laden macrophages (Brown et al. 2008; Harvey et al. 2007,). It seems that hyperbilirubinemia in this study has been caused by increased erythrocyte break down because hepatic enzymes activity remained within reference interval.

In this study, dysproteinemia has been detected in 57.9% of the infected cats that most of them were asymptomatic. Hyperproteinemia found herein could be due to dehydration as well as increased production of acute phase protein or immunoglobulins following stimulation of the immune system. Cytauxzoonosis in domestic cats induces a systemic pro-inflammatory cytokine release that is characterized by an acute-phase response as well as local inflammatory response (Frontera-Acevedo et al. 2013). Serum protein electrophoresis in clinically infected cats revealed the elevation of α- and β-globulin concentration and reduction of albumin concentration. It seems cytauxzoonosis has a lingering systemic effect because the similar electrophoretogram has also been obtained in cats that survive the acute infection (Frontera-Acevedo et al. 2013).

Albumin is known as a negative acute-phase protein and its production is inhibited by pro-inflammatory cytokines during acute phase response (Kaneko et al. 2008). However, various processes such as liver dysfunction, vasculitis, and even protein losing nephropathy and enteropath, can contribute in reduction of the serum level of albumin in cytauxzoonosis (Frontera-Acevedo et al. 2013). Although abnormal biochemical and clinical signs related to the liver and kidney dysfunction were not found here, certainly, other causes cannot be excluded.

Table 2 Clinicopathological findings in the three clinically infected cats with *C. felis* in comparison with feline reference intervals (Rizzi et al. 2010)

Variables	Cat 1	Cat 2	Cat 3	Reference interval
Hct (%)	10.80	19.80	10.00	24.00–45.00
Hemoglobin (g/dL)	3.80	8.30	3.20	8.00–15.00
RBC (10^6/μL)	2.25	3.80	2.17	5.00–10.00
MCV (fL)	48.00	52.10	48.10	39.00–55.00
MCH (pg)	16.90	21.80	14.71	12.05–17.50
MCHC (%)	35.20	41.90	32.00	31.00–35.00
Platelet (10^3/μL)	55	189	134	300–800
WBC (10^3/μL)	24.00	31.40	11.31	5.50–19.50
Neutrophil (10^3/μL)	15.23	21.84	7.68	2.50–12.50
Band (10^3/μL)	0.00	2.10	0.13	0.0–0.30
Lymphocyte (10^3/μL)	6.94	6.71	1.69	1.50–7.00
Monocyte (10^3/μL)	1.20	0.75	0.45	0.0–0.85
Eosinophil (10^3/μL)	0.63	0.00	1.36	0.0–1.50
Total protein (g/L)	103	84	120	54–78
Albumin (g/L)	20	24	15	21–33
Globulins (g/L)	83	60	105	26–51
Creatinine (μ mol/L)	79.56	150.41	138.68	70.7–159
BUN (mg/dL)	15.71	25.56	22.10	14.28–21.42
ALT (U/L)	20	33	71	6–83
AST (U/L)	19	39	38	26–43
ALP (U/L)	91	27	35	25–93
GGT (U/L)	1.3	4.5	3.3	1.3–5.1
Total bilirubin (μmol/L)	14	28.2	60.6	2.57–8.55
Glucose (mmol/L)	5.16	7.77	5.93	3.89–6.11
Triglyceride (mmol/L)	0.96	1.129	0.79	0.1–1.3
Cholesterol (mmol/L)	2.87	1.65	3.14	2.46–3.37
Calcium (mmol/L)	1.99	1.77	2.09	1.55–2.55
Phosphate (Pi) (mmol/L)	1.68	0.89	1.94	1.45–2.62

It should be noticed that mentioned clinicopathological abnormalities herein are not specific and pathognomonic for cytauxzoonosis. As previously mentioned, a high prevalence of *Hemoplasma* spp. infection (23%) has been diagnosed in the studied area (unpublished) and unfortunately, coinfection with *Hemoplasma* spp. was not investigated in this study. So, it is not clear whether the observed laboratory findings were a result of *C. felis* or *Hemoplasma* spp. infection. Maybe *Hemoplasma* spp. has contributed to the aggravation of the clinical and clinicopathological alterations.

The blood-feeding of infected ticks such as *Dermacentor variabilis and Amblyomma americanum* harboring parasite sporozoites are known as the natural route of *C. felis* transmission to cats. These species are broadly distributed in North America (Allen et al. 2019). Unfortunately, tick infestation was not detected in this study, and to the authors' knowledge, *Dermacentor variabilis* and *Amblyomma americanum* have

not been reported in Iran. Generally, no effort has been undertaken in Iran to monitor various aspects of *Cytauxzoon* infection such as epidemiology, pathogenesis and also its vectors. Therefore this parasite needs to be more investigated in the future.

Conclusions

This study preliminarily described the frequency and clinicopathological aspects of *C. felis* infection for the first time in domestic cats in Mashhad, Iran. Based on the results of this study, it concluded that there are considerable populations of asymptomatic domestic cats that silently harbor the *C. felis*.

Since cytauxzoonosis is an emerging pathogen in domestic cats especially in non-endemic area, the epidemiological and clinical aspects of this disease needs to be further investigated.

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Compliance with ethical standards

Conflict of interest None of the authors has any conflict of interest to declare.

Ethics approval All animal experiments were performed in strict accordance with the guidelines approved (IR.UM.REC.1398.131) by the Animal Ethics Committee of School of the Veterinary Medicine, Ferdowsi University of Mashhad, Mashhad, Iran.

Consent to participate Not applicable.

Consent for publication Not applicable.

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