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Original Article

Cytauxzoon felis in Domestic Cats: A Molecular Study Using Real-Time PCR in Semnan, Iran

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Abstract

Background: *Cytauxzoon felis* is a protozoan parasite transmitted by ticks and affects felids. Acute infection in domestic cats is characterized by symptoms such as lethargy, anorexia, fever, and anemia.

Methods: The present study focuses on diagnosing and molecularly identifying *C. felis* using a real-time PCR method in cats from Semnan, Iran. During the winter and spring of 2024, two hundred cats were randomly selected from veterinary clinics in Semnan. Blood samples were collected from the cats for DNA extraction and molecular analysis. Samples were divided into 40 pooled of 5 samples, each consisting of a combination of 5 blood samples. Then, the genomic DNAs were extracted from blood specimens and screened by real-time PCR (RT-PCR) for the presence of *C. felis* infection by amplifying of ITS2 gene belonging to the *Cytauxzoon* genus.

Results: The results indicated that 6 out of the 200 blood samples were infected (3%).

Conclusion: This study was conducted for the first time in Semnan and shows that the prevalence of *C. felis* in cats is significant.

Introduction

Domestic animals and pets can spread pathogens to humans and local wildlife (1-3). *C. felis* is an apicomplexan parasite transmitted to domestic cats by the American tick *Amblyomma americanum*. Additionally, transmission to domestic cats has

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been reported under experimental conditions via *Dermacentor variabilis*. The infection in cats can be fatal or may resolve with or without treatment. Cats are typically referred to as incidental hosts.

The disease caused by this parasite in its natural host, the bobcat, is usually subclinical with chronic parasitemia. There have also been reports of involvement in other felid families, such as leopards and tigers. This disease has not been confirmed in cheetahs, although suspected cases have been observed (4-7). Transmission is solely possible through ticks.

Symptoms include lethargy, loss of appetite, weight loss, fever, dehydration, and swollen lymph nodes, liver, and spleen. Complications may include pulmonary edema and jaundice. These symptoms can occur from 2 d to 15 d after tick exposure. This parasite can cause death due to symptoms or can improve with or without treatment, leading to chronic parasitemia (8-11).

The first reported *C. felis* infection in domestic cats was documented in Missouri in 1976 (12). Initially, *C. felis* was believed to be restricted to North America, but infections have also been documented in cats in South America and Europe (13-17). Currently, a few sporadic cases of *Cytoxazone* infection have been reported in Iran (8, 18, 19), Mongolia (20), and India (21).

Our knowledge shows limited information on the epidemiology of *C. felis* infection in other Asian countries, aside from reports from Turkey (22) and China (23). One diagnostic method for this disease involves the presence of schizonts in the animal's red blood cells. Additionally, biopsies can be obtained from lymph nodes, spleen, and liver to diagnose schizonts. In later stages, this parasite leads to leukopenia and thrombocytopenia accompanied by anemia (24, 25).

Considering the life cycle of the *C. felis* species, stray cats, particularly in forested areas, are at a higher risk of contact with infected ticks. Stray cats can move freely and transmit the disease by displacing vector ticks or infecting new ticks. In Iran, the tick vectors of *C. felis* remain unknown, and there is no information regarding ticks that could potentially infect cats; moreover, further studies on many cats infected with *C. felis* are necessary to clarify the transmission pathways and epidemiological aspects of the parasite.

There are minimal reports of *Cytauxzoonosis* cases in Iran; however, no epidemiological studies have been conducted to investigate the microscopic or molecular prevalence of *C. felis* in stray and domestic cats in Semnan. The present study for the first time addresses the molecular determination of the prevalence of *C. felis* in cats in the city of Semnan.

Material and Methods

During the winter and spring of 2024, 200 cats from the population attending the Semnan veterinary clinics were randomly selected. A blood sample of 5 ml was taken from each cat and collected in tubes containing EDTA. The sampling was conducted during a routine health check-up program. The blood samples were placed on ice and immediately transported to the Faculty of Veterinary Medicine at Semnan University. The blood samples were divided into 40 groups of 5. In each group, five blood samples were combined. DNA extraction from the blood samples was then performed using a DNA extraction kit (MBST, Iran). The extracted DNA samples were stored at -20°C until molecular assays were conducted. DNA samples extracted from blood were screened for *C. felis* species. This was performed in RT-PCR according to the protocol described by Brown et al (26). The sequences of the primers used for the RT-PCR reaction are given in Table 1. In the second stage, the sample groups that were identified as positive in the first stage were re-examined using the RT-PCR technique to identify individual infected samples. After completing the RT-PCR process, the product's melting temperature was determined

at the end of the reaction, and a melting curve was drawn.

Table 1: Sequences of primers used for the RT-PCR reaction based on ITS2 gene

Target Genome	Primers	Sequence 5-3
Cytotoxoon felis	Forward	5'- TGA ACG TAT TAG ACA CAC CT-3'
	Reverse	5'- TCC CGC TTC ACT CG CCG-3'

Results

RT-PCR was performed to evaluate the parasite-specific gene. In the first stage, 40 sample groups (each group comprising a combination of 5 cat blood samples) was examined. The Cycle Threshold (CT) parameter indicates the number of cycles required for the definitive detection of an infected sample. This study considered a maximum of 40 cycles for sample identification.

The results from the data analysis indicate infection in groups 3 (CT= 27.83), group 17 (CT= 28.19), group 19 (CT= 28.76), group 21 (CT= 27.87), and group 22 (CT= 31.63). The positive control group was also identified with a CT value 28.63 (Fig. 1). Distilled water and DNA from a clinically healthy cat without a history of tick infestation were used as negative controls in the RT-PCR reactions.

As observed in Fig. 1, the intersection points of the curve corresponding to each sample with the threshold line indicate the definitive detection of the target gene. Therefore, lower CT values indicate a higher parasite load in the sample, resulting in earlier detection of the target gene in the initial cycles. In other words, the lower the CT value, the faster the replication of the target gene occurs in the sample, allowing for its detection in the early cycles of RT-PCR.

The melting of double-stranded gene products in the first stage showed that at temperatures of 80 and 90 °C, the same genes transitioned to a single-stranded state (parasite-specific gene) (Fig. 2). Supplementary tests were conducted to identify infected blood samples within each positive group. According to the data presented in Fig. 3, 6 out of 200 samples (3%) tested positive for the target gene (Fig. 3). The lowest cycle threshold (Ct) value was observed in group 5, indicating that one of the samples in this group had the highest Bacterial load. The target gene amplification in the sample from group 5 was significantly greater than in the other sample.

The melting of double-stranded gene products in the second stage showed that at temperatures of 79 and 88 degrees Celsius, the same genes became single-stranded (specific parasite gene) (Fig. 4).

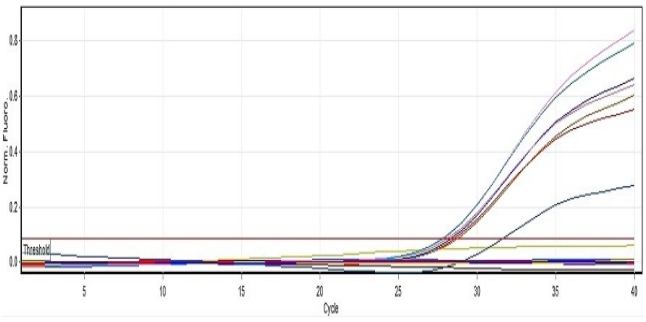

Fig. 1: The cycle of identifying infected samples in the first stage

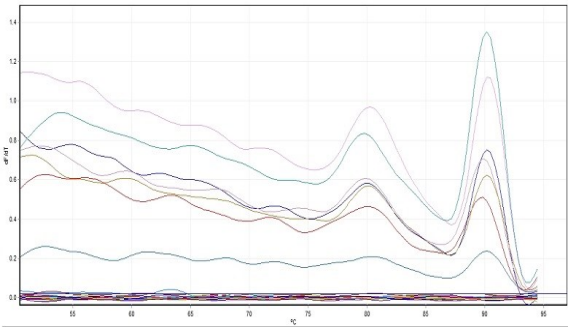

Fig. 2: Melting of double-stranded gene products in the first stage

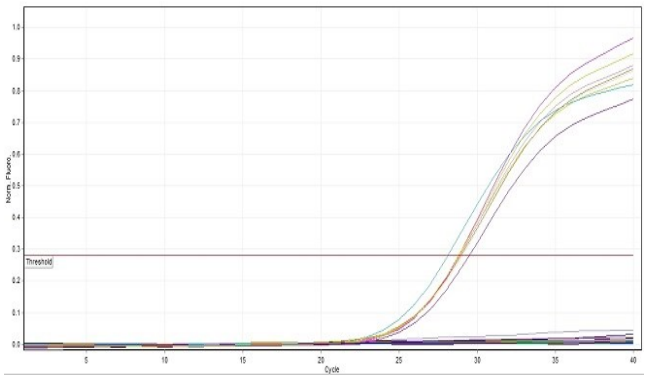

Fig. 3: The cycle of identifying infected samples in the second stage

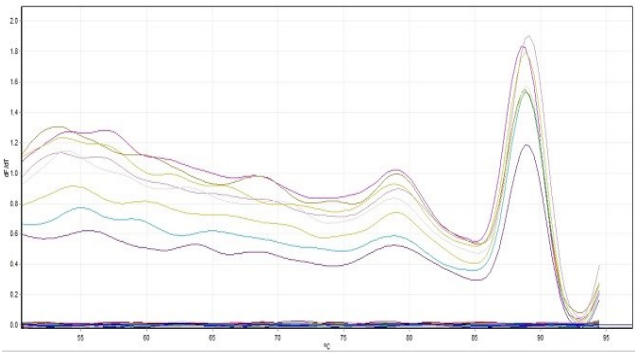

Fig. 4: Melting of double-stranded gene products in the second stage

Discussion

The cats studied in this research include all the cats that have visited veterinary clinics in Semnan. The results showed that 6 out of 200 samples (3%) contained the target gene. Limited cases of *C. felis* infection have been reported in Iran. Molecular testing on 100 outdoor cats in Mashhad (Iran) revealed that 19% of the cats were infected with *C. Felis* (8). The presence of *C. felis* in blood smears and serum from a stray cat in Iran was described during the analysis (18). Zaeemi et al (19) reported that this is the first time a *C. felis* has been discovered in wild Felidae in Iran.

Even though the origin of *C. felis* is reported to be in North America, there are also reports of infections from other parts of the world, including the continent of Asia. The first report of *C. felis* infection in Asia was a case study involving a stray cat in India, identified through microscopic methods (21). In Turkey, a study examined 120 cats aged between 1 and 7 years using microscopic methods. Blood smears from these cats revealed that 7.5% were positive for *C. felis* in their blood. The 120 studied cats did not exhibit clinical signs of the disease, likely due to pre-immunity against the parasite. This phenomenon can occur with many blood parasites and may prevent the manifestation of clinical symptoms (22).

In Greece, to investigate the molecular prevalence of several selected pathogens, blood samples were collected from 50 healthy cats and 50 sick cats. One of the pathogens examined in this study was *C. felis*, undetected in any of the samples. These results contradict our study findings (27).

In the United States, to determine the prevalence of *C. felis* in stray cats, blood samples were collected using PCR and RNA gene sequencing from 380 cats in Oklahoma and 292 cats in Iowa. No positive samples were reported among the 292 cats in Iowa, while three positive samples (0.8%) were found among the 380 cats in Oklahoma (28). The distribution and these states were chosen for their variety of vectors.

Blood and spleen samples were collected from 696 bobtail cats, and PCR was utilized to detect *C. felis* through ITS2. The prevalence of *C. felis* was identified in Missouri (79%, n=39), North Carolina (63%, n=8), Oklahoma (60%, n=20), South Carolina (57%, n=7), Kentucky (55%, 74%), Florida (44%, 45%), and Kansas (27%, 41%) when compared to Georgia (9%, n=159), North Dakota (2.4%, n=124), Ohio (0%, n=19), West Virginia (0%, n=37), California (0%, n=26), and Colorado (0%, n=67). (10).

In the United States, 494 cats from Florida, 392 from North Carolina, and 75 from Ten-

nessee were tested using the PCR method. The prevalence of *C. felis* infection was 0.3%. Two cats from Florida and one from Tennessee tested positive (29).

In a study involving 151 blood samples from cats aimed at identifying multiple pathogens in one of the states in Brazil, DNA extracted from these samples was analyzed using PCR. Only one of the 151 samples studied (0.8%) was found to be infected with the genus *C. felis* (30).

The first reported case of Cytauxzoonosis in France was the prevalence of piroplasm in carnivores and ruminants, examined using PCR with sequencing of the 18S rRNA gene. Only one of the 116 cats studied (0.8%) was infected with *C. felis* (31).

In Spain, a study involving 644 domestic and stray cats identified eight (1.2%) infected with the genus *C. felis* using the molecular method PCR and sequencing of the 18S rRNA gene. Infections were more common in samples collected during the year's colder months (between January and March) and in samples gathered from rural areas. These findings may relate to chronic infection; however, the possibility of infection during winter cannot be ruled out (32). In contrast, Cytauxzoonosis caused by *C. felis* has been more prevalent in the United States from spring to early autumn (33). In the present study, samples were collected in winter and spring.

In northern Italy, the prevalence of *C. felis* infection was investigated in 260 stray cats using PCR methods. 27.7% of the cats were healthy, while 72.3% were unhealthy and exhibited clinical abnormalities. The prevalence of *C. felis* infection was 0%. The evidence presented in this study suggests that the prevalence of *C. felis* in in the stray cat population of northern Italy is limited (34). In contrast, another study in northeastern Italy examined 118 domestic and stray cats for the first-time regarding infection with the genus *C. felis* using microscopic methods and PCR with 18S rRNA gene sequencing. This study, *C. felis* was reported in 15% of cats using microscopic methods and 23% of 27 using molecular methods (15). The difference between the results obtained from these two studies in Italy can be attributed to the fact that the cats studied in Trieste, located in northeastern Italy, lived in an urban area near wooded forests that are habitats for tick vectors (34). In southwestern China, 311 cats (including 74 stray and 237 domestic cats) were examined using PCR to detect *C. felis*, and ITS2 was detected. Sixty-seven cats tested positive for *C. felis*, resulting in a reported infection rate of 21.5%. The prevalence of Cytauxzoonosis in stray cats was reported at 51.4%, while in domestic cats, it was 12.2% (23).

In a study on domestic and bobtail cats from Arkansas and Georgia, using Real-time PCR targeting the 18s rRNA sequence, 30.3% of domestic cats from Arkansas and Georgia and 25.6% of bobtail cats from Arkansas, Georgia, and Florida were found to be positive (26).

In another molecular study conducted in Oklahoma, Missouri, and Arkansas, 902 blood samples were collected from healthy domestic cats, and the DNA of the parasite was detected in 56 samples. The highest prevalence of infection with *C. felis* (15.5%) was observed in cats from Arkansas, while the infection rates in Missouri and Oklahoma were reported at 12.9% and 3.4%, respectively (35). The study by Nagamori (28) and its comparison with the survey by Rizzi (35) confirm that the prevalence of Cytauxzoonosis in a specific enzootic area (Oklahoma in the United States) can vary based on location, time, and the population of sampled cats. The differences in the prevalence of *C. felis* in Oklahoma may include variations in the virulence of different strains of *C. felis*, differences in the immunological response of cats to infection with *C. felis*, and differences in the transmission of *C. felis* from ticks (28).

Furthermore, blood samples were collected from relatively young cats (28). This may affect the study results, considering that young

cats have been exposed to tick vectors for a shorter period. Since cats become infected with *C. felis* through tick bites, the prevalence of *C. felis* infection in domestic cats is likely influenced by geographic diversity, tick activity, and reservoir host populations (35).

The present study, with a limited sample size in the Semnan region, provided preliminary evidence of *C. felis* in cats; however, the limitations related to sample size and geographic selection restrict the generalizability of the results. Additionally, using diagnostic methods with limited sensitivity and specificity may affect the accuracy of prevalence estimates. Future research should employ a broader sampling strategy with wider geographic coverage and utilize advanced molecular techniques to enhance the accuracy and generalizability of the findings. Furthermore, studying the epidemiological factors influencing parasite transmission could aid in optimizing prevention and control strategies.

Conclusion

Before the current study, there has been no related research on the prevalence of *C. felis* infection in Semnan. The microscopic examination of stained blood smears for diagnosing this parasitic infection is unreliable, and RT-PCR is a method with higher specificity and sensitivity than blood smears. The number of cats examined in this study was limited, the results cannot be generalized to the cat population of Semnan; however, the prevalence of *C. felis* infection in cats in Semnan may be low, although proving this claim requires more comprehensive investigations.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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