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RESEARCH ARTICLE

Investigation and Phylogenetic Analysis of *Hemoplasma* Species In Cats In Different Regions of Türkiye By Molecular Methods

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Abstract

Feline haemoplasma species, *Candidatus* Mycoplasma haemominutum (CMhm), *Candidatus* Mycoplasma turicensis, (CMt) and *Mycoplasma haemofelis* (Mhf) are frequent causes of chronic and acute diseases in cats. The current study aims to research the presence and genetic variety of *Mycoplasma* spp. in cats from different parts of Türkiye using molecular methods. In this study, 460 blood samples collected from cats from various parts of Türkiye (Malatya, Istanbul, Denizli, Konya, and Ankara) within the scope of different studies were investigated. Total genomic DNAs were obtained from the samples. The presence and phylogenetic position of feline haemoplasma species of feline hemoplasma species were detected using triplex-polymerase chain reaction (PCR) and sequence analysis of *16S SSU rRNA* gene. Seven (1.52%) of 460 cat blood samples were found to be positive by triplex-PCR. CMhm was identified in six (1.30%) samples, whereas CMt was detected in two (0.43%) samples. Only one sample (0.21%) was infected with both CMhm and CMt. Mhf was not identified in the study. In addition, according to the sequence results, single nucleotide polymorphisms (SNPs) were detected between both CMt and CMhm isolates. The results of the study revealed that haemoplasma infections are circulating in cats from different parts of Türkiye. It is considered to be very important to perform routine screening and develop effective control and prophylactic strategies to minimize infection.

Keywords: Cat, DNA sequences, Mycoplasma, Triplex-PCR, Türkiye

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INTRODUCTION

Haemoplasmas, formerly of the genus *Haemobartonella* and *Eperythrozoon* order Rickettsiales, are small, pleomorphic, without walls, adherent to the red blood cell surface, and uncultured agents (Neimark et al 2001). The analysis of the *16S rRNA* gene from a phylogenetic perspective and an analysis of the morphological features indicated that these are more closely associated with species in the genus *Mycoplasma*. For this reason, these were reclassified as members of the Mollicutes class, within the Mycoplasmataceae family (Neimark et al 2001).

Some types of haemotropic mycoplasma can infect several animal species, such as cattle, dogs, water buffalo, sheep, and cats (Neimark et al 2001, Messick 2004, Willi et al 2005, dos Santos et al 2008, Altay et al 2023, Erol et al 2023). These species are transmitted to hosts via a variety of arthropods, including ticks, mosquitoes, fleas, and sandflies (Neimark et al 2001, Case et al 2006, Eren 2025). The pathogens can cause anything from mild to severe infections in the hosts. In some cases, death can also occur (Messick 2004, Imre et al 2020, Mesquita et al 2021). Recent investigations have revealed that haemoplasma species, such as *Mycoplasma haemofelis* (Mhf), *Mycoplasma ovis, Mycoplasma suis, Candidatus* Mycoplasma haematoparvum, *and Candidatus* Mycoplasma haemohominis may cause infection in humans (Maggi et al 2013, Hattori et al 2020, Fritschi et al 2020).

The studies have shown that the presence of three cat-associated haemoplasma species [*Candidatus*

Mycoplasma turicensis (CMt), *Candidatus* Mycoplasma haemominutum (CMhm), and Mhf] (Willi et al 2005, Willi et al 2007, Barker 2019, Yamakawa et al 2023, Altay et al 2025). Most of the infections, feline haemoplasma species can cause non-specific clinical signs, however, high fever, pale mucous membranes, anemia, drowsiness, anorexia, breathlessness, tachycardia, dehydration, weight loss, and lymphadenopathy can be associated with these species (Barker and Tasker 2013, Barker 2019, Yamakawa et al 2023). Similar to other haemoplasma species, the life cycle of feline haemoplasma species is not fully understood, but blood-sucking arthropods are thought to be important in the transmission of pathogens between hosts. Additionally, these pathogens can be spread between cats by blood transfusions, vertical transmission, social behavior, or fighting, particularly between male cats (Museux et al 2009, Tasker 2010, Díaz-Regañón et al 2018).

Up to now, different identification methods have been utilized to identify different types of feline haemoplasma (Barker and Tasker 2013, Rosenqvist et al 2016, Barker 2019). Microscopic techniques have several disadvantages in the identification of haemoplasma species in cats. Using these techniques, haemoplasmas that cause infection in hosts cannot be identified at the species level. In addition, mixed infections and animals with low parasitemia cannot be detected (Neimark et al 2001, Willi et al 2005, Sykes and Tasker 2014, Rosenqvist et al 2016, Barker 2019). Researchers prefer molecular-based methods, real-time PCR, conventional PCR, and multiplex PCR, to eliminate these disadvantages of microscopic methods and to diagnose feline haemoplasma species more precisely. These methods have been used for the species identification of pathogens causing infections, to detect infections where different pathogens are present, and to determine which hosts are infected over a long period of time (Rosenqvist et al 2016, Barker 2019, Demkin and Kazakov 2021). Furthermore, recently Altay et al (2025) developed a test that can identify and differentiate between different types of feline haemoplasma in one step. This test has been successfully used to identify infected hosts (Altay et al 2025).

The purposes of the present study are as follows: i) to identify and differentiate feline haemoplasma species among domestic cats in different provinces of Türkiye (Malatya, İstanbul, Denizli, Konya, and Ankara) using triplex-PCR, ii) to detect of phylogenetic position of feline haemoplasma species detected in the study.

MATERIAL AND METHODS

All procedures performed in studies involving animals were in accordance with the ethical standards approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approval number: 05.03.2025-65202830-050.04.04-10).

Study area and blood sampling

Türkiye is located on the Eurasian continent and is classified as having a subtropical climate. It can be further delineated into seven distinct geographical areas: Eastern Anatolia, Mediterranean, Aegean, Southeastern Anatolia, Black Sea, Central Anatolia, and Marmara. These regions have different climatic conditions, and therefore, the abundance and distribution of vector species differ in the regions (Inci et al 2016).

The 460 cat blood samples used in the study were collected from various geographic regions of Türkiye. Blood samples were collected from Eastern Anatolia [Malatya (n: 81)], Marmara [Istanbul (n: 173)], Aegean [Denizli (n: 60)] and Central Anatolia [Konya (n: 10) and Ankara (n: 136)] in different studies between April 2022 and August 2024 (Figure 1). Approximately 1-2 mL of blood was collected in a with EDTA (ethylenediamine tetraacetic acid) tube and transferred immediately to a cooling device until it reached the laboratory. Information about the gender and age of the animals was recorded, and the blood samples were stored at -20°C until use molecular assay.

DNA extraction and triplex-PCR assay

Genomic DNA was obtained from feline blood samples in accordance with the protocol outlined by Altay et al (2005). These DNA samples were kept at -20°C until examined in terms of feline hemoplasma species. Triplex-PCR assay using species-specific primers was performed to investigate the presence and prevalence of feline *Mycoplasma* species (Altay et al 2025). The amplicon sizes of the primers for CMt, CMhm, and Mhf s were 607, 456, and 1,022 base pairs, respectively. Further information on primers was detailed in Table 1.

The triplex-PCR assay was carried out in a final volume of 25 µL, and the assay was conducted in accordance with the protocol delineated by Altay et al (2025). The agarose gel

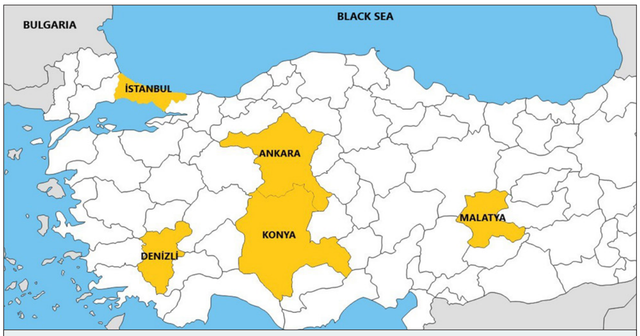

Figure 1. Location of sampling areas in different parts of Türkiye.

Table 1. In the present study, primer pairs were employed as a research tool.

Primer name	Primer (5'→3')	Target gene	Annealing temperature (°C)	References
F2	ACGAAAGTCTGATGGAGCAATA	*16S rRNA*	57	Jensen et al 2001
Mhf R	TCACTCTAGATACAAATGAATGAT	*16S rRNA*	57	Altay et al 2025
CMhm R	TCGCAAAGATTWCAAGCCTC	*16S rRNA*	57	Altay et al 2025
CMt R	CAGGTAAGCTACAACGCCGAA	*16S rRNA*	57	Altay et al 2025

was subjected to electrophoresis at a current of 95 V for a period of 60 min. This was followed by the staining of the gel with ethidium bromide, which was carried out for a duration of 20 min. Subsequently, visualization of agarose gel was done with a UV transilluminator.

DNA sequencing and phylogenetic analyses

Positive samples were selected at random for the purpose of nucleotide sequence analysis. Sequence analysis was performed using primers listed in Table 1 in both directions with BigDye Terminator v3.1 Cycle Sequencing Kit, and ABI 3730XL analyzer (Applied Biosystems, Foster City, CA) and in accordance with the standard procedures suggested by the manufacturer. Before nucleotide sequence, the purification of all PCR products was accomplished by means of the HighPrep™ PCR Clean-Up System (Cat. No.: AC-60005, MagBio), in accordance with the instructions provided by the manufacturer.

The consensus sequences were then prepared with the MUSCLE algorithm of the MEGA-11 (Tamura et al 2021) software, and the obtained sequences were subsequently subject to comparison for similarity with those available in the GenBank database. This was achieved by employing the BLAST. All consensus sequences have been uploaded to GenBank, and accession numbers have been obtained.

Phylogenetic trees were constituted using a maximum likelihood (ML) analysis implemented in the MEGA-11 software (Tamura et al 2021), with the resulting trees evaluated to determine the phylogenetic relationships. Prior to the construction of the phylogenetic tree, the optimal algorithm for use in the phylogenetic tree of related pathogens was ascertained by means of the Find Best-Fit Substitution Model in MEGA-11. This model indicated that the optimal algorithm for the purposes at hand was TN93 (Tamura and Nei 1993). Consequently, this algorithm was utilized in the construction of the phylogenetic tree. The reliability of the tree was determined by means of a bootstrap analysis conducted over 1,000 iterations.

RESULTS

In the current study, a triplex-PCR assay was utilized to research the presence of feline haemoplasma species in a total of 460 samples of cat blood. Of the 460 blood samples analysed in total, seven (1.5%) were identified as positive for haemoplasma. (Figure 2). The presence of CMhm was detected in six (1.30%) feline samples, whereas the presence of CMt was identified in two (0.43%) samples. Five cat samples (1.08%) were detected to be infected with only CMhm and one sample (0.21%) with only CMt, whereas CMt and CMhm were detected in one sample (0.21%). Mhf was not identified in cat samples. For a comprehensive overview of the prevalence of feline haemoplasma, please refer to Table 2.

The *16S rRNA* gene sequence of CMt and CMhm were uploaded to the GenBank database, where they were assigned accession numbers PQ846675 and PQ876676, respectively.

The 99.12-100% nucleotide identities observed between CMhm isolates identified in this study and those present in the GenBank database indicated a high degree of genetic similarity. Furthermore, the CMhm isolates obtained in this study exhibited 100% nucleotide identity with CMhm isolates previously determined from cats in the Switzerland (Accession numbers: DQ157146), Türkiye (Accession numbers: PP265260), Iran (Accession numbers: (MW555989), Italy (Accession numbers: EU839981), USA (Accession numbers: KF743738), Hungary (Accession numbers: EU128752), and Brazil (Accession numbers: KM275254), from dog in Thailand and Cuba (Accession numbers: KU765207, PQ604639 respectively), from wild cat in Germany (Accession numbers: ON202710), and from human in Mexico (Accession numbers: OR225696).

CMt isolate showed 96.88-100% nucleotide similarity with CMt isolates in GenBank database. However, the CMt isolates obtained in this study demonstrated 100% nucleotide identity with previously identified CMt isolates from cats in Brazil (Accession numbers: KM275258), Italy (Accession numbers: KR905459), South Africa (Accession numbers: DQ464424), India (Accession numbers: MN240801) and Thailand (Accession numbers: EU789558).

The study identified CMhm and CMt isolates that were found to be clustered in the same branch. These isolates clustered in the same cluster with isolates detected in different parts of the world (Figure 3). Additionally, in

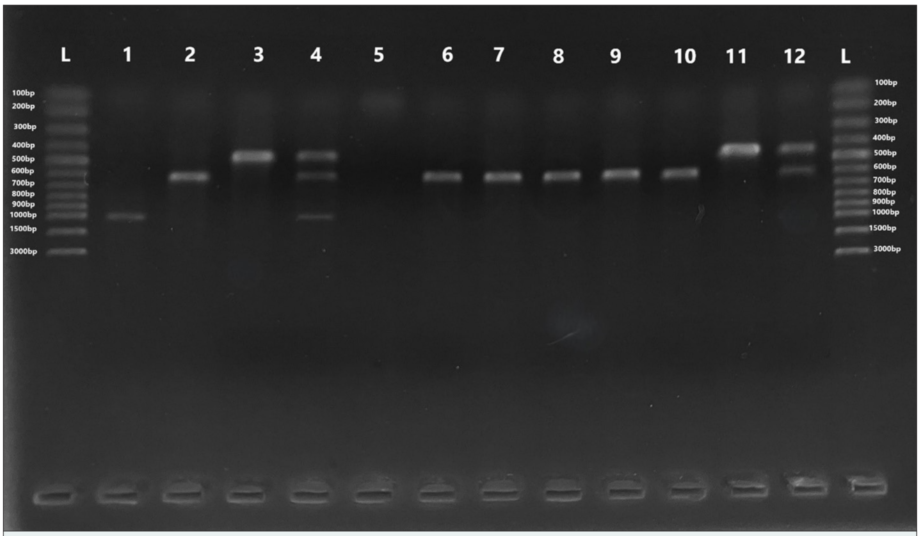

Figure 2. PCR products of feline haemoplasma species. L. Ladder, 1. *Mycoplasma haemofelis* positive control, 2. *Candidatus* Mycoplasma haemominutum positive control, 3. *Candidatus* Mycoplasma turicensis positive control, 4. Positive control of three feline haemoplasma species, 5. Negative control, 6-10. *Candidatus* Mycoplasma haemominutum positive cat samples, 11. *Candidatus* Mycoplasma turicensis positive cat sample, 12. Mixed infected cat sample with *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis.

the current study, phylogenetic analyses of *16S rRNA* gene sequence of CMt and CMhm and revealed that single nucleotide polymorphism (SNPs) were determined between both CMt and CMhm isolates, respectively. SNPs were detected at nucleotides 588th (T→G) and 822th (C→T) for CMhm. In the study, the 588th and 822th nucleotides were thymine and cytosine, respectively, while in some other sequences, these nucleotides were guanine and thymine, respectively. In CMt sequences, SNPs were observed at nucleotides 379th (C→T), 564th (A→G), 738th (C→T), and 742th (A→T), respectively. In the study, these nucleotides were cytosine, adenine, cytosine, and adenine, while in the other sequences, these nucleotides were thymine, guanine, thymine and thymine, respectively.

Discussion

Some pathogens of the *Mycoplasma* genus (including feline haemoplasma species) transmitted by vectors can cause mild to severe infections in humans. In addition, some species can cause serious cases of the disease in cats (Hattori et al 2020, Alcorn et al 2021). Therefore, as in other hosts, rapid and reliable detection of haemoplasma species in cats is very important. Since microscopic examination cannot detect low-level infections and does not allow species distinction, molecular diagnostic methods with higher sensitivity and specificity have been used in recent years (Barker 2019, Altay et al 2025, Dik and Simsek 2021). PCR-based methods are highly effective in identifying infectious hosts even in the presence of low parasitemia and in diagnosing haemoplasma infections that are difficult to diagnose microscopically (Tasker et al 2018). The *16S rRNA* gene region has been triumphantly utilized in the identification of numerous haemoplasma species (Altay et al 2023, Altay et al 2025, Erol et al 2023). In the present study, the triplex-PCR method was utilized to identify feline haemoplasma species among cats obtained

Table 2. Information on the prevalence of feline haemoplasma species in terms of location.

		Total no. of cats	No. of feline haemoplasma positive (%)	Mhf (only) (%)	CMhm (only) (%)	CMt (only) (%)	Mixed-Infection*
Location	Malatya	81	-	-	-	-	
	İstanbul	173	4 (2.31)	-	4 (2.31)	-	-
	Denizli	60	3 (5)	-	1 (1.66)	1 (1.66)	1 (1.66)
	Konya	10	-	-	-	-	-
	Ankara	136	-	-	-	-	-
Sub-Total		460	7 (1.52)	-	5 (1.08)	5 (1.08)	1 (0.21)

*Mixed-Infection with *Candidatus* Mycoplasma haemominutum + *Candidatus* Mycoplasma turicensis

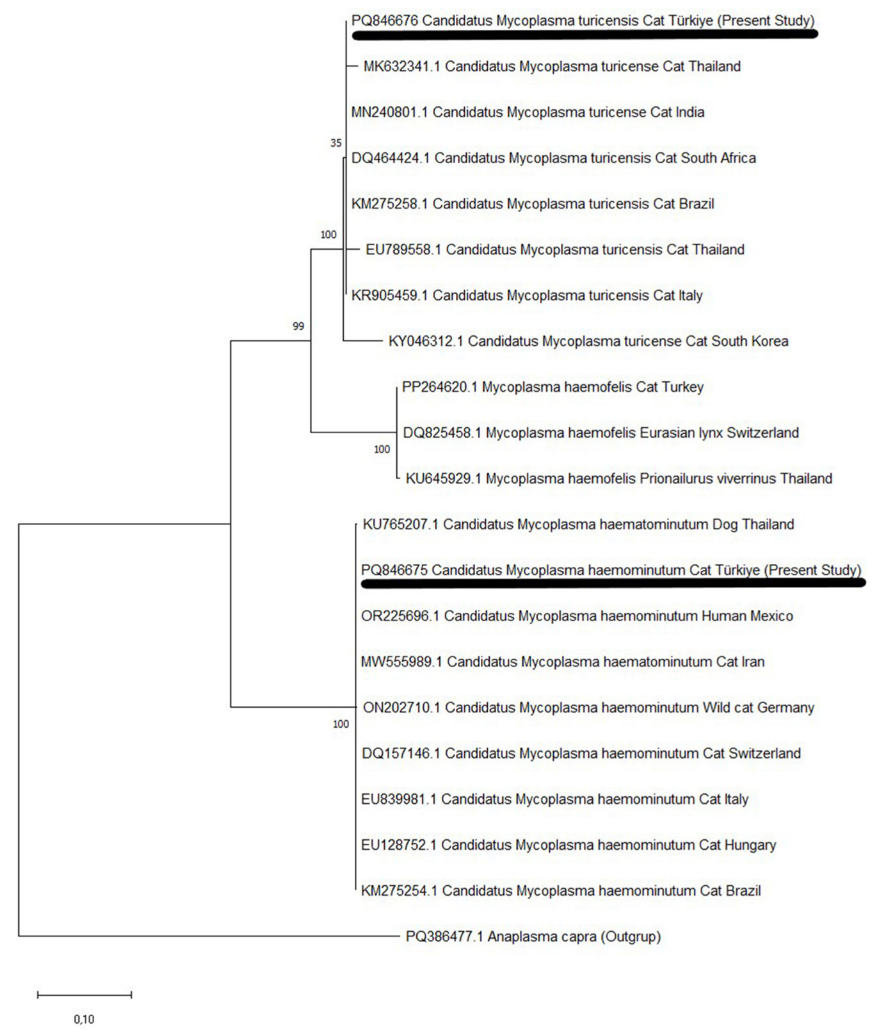

Figure 3. Phylogenetic trees according to *16S rRNA* gene sequences of haemoplasma species. Phylogenetic position of *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis. The figure was created through the utilization of an ML method. The bootstrap values were calculated on the basis of 1,000 replicates. The evolutionary history was investigated by employing the ML method, with the TN93 model being utilized in the construction of both phylogenic trees (Tamura and Nei 1993). Evolutionary analyses were conducted in MEGA-11 (Tamura et al 2021). *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis species obtained in this study are shown underlined in the phylogenetic tree.

from different parts of the Türkiye. Feline haemoplasma species are widespread throughout the world (Ravagnan et al 2017, Barker 2019, Yamakawa et al 2023). In recent years, several of studies have been done on the prevalence of haemoplasma species detected in cats worldwide and it has been reported in Greece (19%) (Kokkinaki et al 2022), India (9.01%) (Malangmei et al 2021), Thailand (38.05%) (Do et al 2020), Germany (12%) (Schäfer et al 2023), Portugal (27.1-43.43%) (Martínez-Díaz et al 2013, Duarte et al 2015), Albania (30.8%) (Silaghi et al 2014), Chile (15.1%) (Vergara et al 2016), Italy (13.2%) (Ravagnan et al 2017), Denmark (16.4%) (Rosenqvist et al 2016), Switzerland (9.9%) (Willi et al 2006), Russia (13.8%) (Demkin and Kazakov 2021), Spain (10.6-22.9%) (Díaz-Regañón et al 2018, Villanueva-Saz et al 2023), Brazil (13.3%) (Yamakawa et al 2023), United Kingdom (18.5%) (Tasker et al 2003), Japan (26.4%) (Tanahara et al 2010), Latvia (17%) (Berzina et al 2021), Romania (21.6%) (Imre et al 2020), and Lithuania (7.2%) (Razgūnaitė et al 2024). Feline haemoplasma species have been detected in different parts of Türkiye with the prevalence between 8.54% and 19.3% (Ural et al 2009, Cetinkaya et al 2016, Celik et al 2021, Ceylan et al 2024, Altay et al 2025). In the present study, feline haemoplasma species were identified in 1.52% of cats collected from diverse regions of Türkiye. This may be related to the different climatic characteristics of the provinces. Therefore, it is thought that the vector population varies. Similarly, the prevalence of haemoplasma infections in hosts (cats) may vary in the same country, in the same city, and even in the same region depending on various parameters (Díaz-Regañón et al 2018, Villanueva-Saz et al 2023). This variation depends on epidemiological factors such as sample size, sampling strategy, study design such as age and sex of animals, presence of pathogens and vector status in the region, and the environment, climatic conditions, and health status of cats (Duarte et al 2015, Díaz-Regañón et al 2018, Do et al 2020).

In this study, it was determined that CMhm (1.30%) was more prevalent than CMt (0.43%) among the cats. Similar results were seen studies conducted in Italy, Spain, Russia, Germany, and Brazil (Ravagnan et al 2017, Díaz-Regañón et al 2018, Schäfer et al, 2023, Villanueva-Saz et al 2023). Studies have revealed that this condition may be related to various factors. For example, CMhm has a higher replication capacity than other feline haemoplasma species (Tanahara et al 2010). Additionally, Tanahara et al (2010) and Rosenqvist et al (2016) suggested that the greater prevalence of CMhm relative to other feline hemoplasma species might be due to the pathogen's lower virulence, allowing for a more harmonious coexistence with cats. Another reason is that it has been established that the presence of CMhm DNA has been identified in both saliva and salivary glands in felines (Dean et al 2008, Tanahara et al 2010). It is hypothesized that the pathogen may be transmitted from one cat to another during social interactions and confrontations (Tanahara et al 2010).

In recent years, with the advent of more accessible DNA sequence analysis techniques, this approach has been increasingly employed for a variety of purposes, including the confirmation of PCR results, the determination of phylogenetic analysis and genetic diversity of pathogens, and the identification of new species and genotypes (Alkan 2021, Sahin et al 2023, Erol et al 2024). In this study, DNA sequence analyses were used to confirm the triplex-PCR assay. For this purpose, we randomly selected two positive samples, one CMt and one CMhm, was sent for DNA sequence analyses. The consensus sequence revealed that the CMhm isolate identified in the study showed 99.12-100% identity with the CMhm isolates are registered in GenBank, while the CMt isolate identified in the study showed 96.88-100% nucleotide identity with the CMt isolates present in GenBank. In this study, according to the phylogenetic analysis of the *16S rRNA* gene, SNPs were observed in both CMhm isolate (588th and 822th nucleotides) and CMt isolate (379th, 564th, 738th, and 742th nucleotides) between our sequences and the sequences available in GenBank. While this provided insights into genetic similarity with global isolates, it revealed the need for further studies on genetic diversity or population structure. It is evaluated that the genetic data obtained within the scope of the study will contribute to the understanding of the phylogenetic characteristics of CMt and CMhm.

CONCLUSION

In this study, CMhm, CMt, and Mhf were investigated in cats using the triplex-PCR test. CMt and CMhm were detected in cats from different regions of Türkiye. In addition, according to the sequence results, SNPs were detected between both CMhm and CMt isolates. The present study and previous studies have shown that haemoplasma species are circulating in cats in Türkiye. However, phylogenetic analyses of feline haemoplasma species revealed that there are several variants based on nucleotide differences. The increasing public awareness and expanding the frequency of screening is very important for effective control.

DECLARATIONS

Competing Interests

Authors declare that there are no conflicts of interest related to the publication of this article.

Availability of Data and Materials

The data that support the findings of this study are available on request from the corresponding author.

Ethical Statement

All procedures done in this work involving animals were in accordance with the ethical standards approved by the Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Approval number: 65202830-050.04.04-10).

Author Contributions

Motivation/Concept, Design: OFS, UE, KA; Data Collection and Processing: OFS, UE, OFU, MGG, HFS; Analysis and Interpretation: OFS, UE, OFU, MGG, HFS, KA; Control/Supervision: KA; Writing the Article: OFS, UE, KA; Critical Review: OFS, UE, KA

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