

# Molecular confirmation of hemotropic mycoplasmas (hemoplasmas) in domestic cats in Romania

**Mirela Imre**

Universitatea de Stiinte Agricole si Medicina Veterinara a Banatului din Timisoara Facultatea de Medicina Veterinara

**Cristina Văduva**

Universitatea de Stiinte Agricole si Medicina Veterinara a Banatului din Timisoara

**Gheorghe Dărăbuș**

Universitatea de Stiinte Agricole si Medicina Veterinara a Banatului din Timisoara

**Sorin Morariu**

Universitatea de Stiinte Agricole si Medicina Veterinara a Banatului din Timisoara

**Tijana Suici**

Universitatea de Stiinte Agricole si Medicina Veterinara a Banatului din Timisoara

**Philippa J.P. Lait**

Langford Veterinary Services

**Kálmán Imre** (✉ [kalman\\_imre27@yahoo.com](mailto:kalman_imre27@yahoo.com))

Universitatea de Stiinte Agricole si Medicina Veterinara a Banatului din Timisoara

<https://orcid.org/0000-0002-6057-882X>

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## Short report

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# Abstract

**Background** The hemotropic mycoplasmas (hemoplasmas) of the genus *Mycoplasma* are recognized as important bacteria that parasitize red blood cells, causing hemolytic anemia in many mammalian species, including cats. No information is available concerning the presence of feline hemoplasma infections in cats in Romania. Thus, the objective of the present study was to provide data on the occurrence and molecular characterization of hemotropic mycoplasmas in client owned cats in Romania.

**Methods** Blood samples from 51 unhealthy cats, originating from Timișoara Municipality, Romania, were screened for the presence of hemoplasmas using conventional polymerase chain reaction (PCR) targeting the 16S rRNA gene and sequencing assays.

**Results** Molecular analysis revealed 11 (21.6%) positive samples, consisting of 8 (72.7%) *Candidatus Mycoplasma haemominutum* and 3 (27.3%) *Mycoplasma haemofelis* confirmed positives. *Candidatus Mycoplasma turicensis* was not detected, and no co-infections were registered. No significant associations ( $p > 0.05$ ) were found between the hemoplasma infection status and age, gender, breed, presence of ectoparasites, FeLV/FIV positivity of cats or the sampling season. However, outdoor access was positively associated ( $p = 0.049$ ) with infection and could be considered a risk factor (OR=4.1) in acquiring feline hemotropic mycoplasmas.

**Conclusions** The findings support the emergence of feline hemoplasma infections in previously uninvestigated territories of Europe, providing useful information for small animal practitioners. To our knowledge, the present survey is the first reported molecular evidence of feline hemoplasma infections in Romania.

## Background

The hemotropic mycoplasmas (hemoplasmas) of the genus *Mycoplasma* within the Mollicutes class are recognized as small, wall-less and uncultivable bacteria that parasitize red blood cells, causing hemolytic anemia in many mammalian species, including cats [1, 2].

Results of several molecular investigations conducted at a worldwide level have shown the involvement of three species in the etiology of hemoplasma infections in cats namely, *Mycoplasma haemofelis* (Mhf), “*Candidatus Mycoplasma haemominutum*” (CMhm) and “*Candidatus Mycoplasma turicensis*” (CMt) (reviewed by [2]). Of these, Mhf has been reported to be the most pathogenic species, being most often associated with clinical disease [3]. Whereas CMhm and CMt usually result in infections in individuals with concurrent retroviral, neoplastic and immune-mediated disease [1].

Hemoplasma infections in cats can vary in severity of symptoms, ranging from complete absence of clinical abnormalities to mild or severe, sometimes fatal acute hemolytic anaemia [1]. Currently, the natural route of transmission of hemoplasmas between cats is unknown, but several modes have been

suggested including: involvement of arthropod vectors (e.g. fleas, ticks) [4–7], direct transmission via blood transfusion [7], aggressive interactions between cats [8] and transplacental transmission from mother to kittens [9].

Over the last decade the problem of continuous expansion and emergence of pathogens in novel geographical areas has attracted significant attention from researchers. In this regard, providing new data on the occurrence of unreported pathogens in an endemic area represents a priority for veterinary practitioners and the scientific community. Hemoplasma infections in cats have been molecularly confirmed in several South-Western [10–14], Southern [15–17], South-Eastern [18, 19], Central [7, 20, 21] and Northern [22] European Countries. However, to date no data on the presence of feline hemoplasma infections has been reported in Romania. The present study was undertaken to address this gap by investigating the occurrence of hemoplasmas in cats from western Romania and performing the molecular characterization of the species present.

## Methods

From April 2017 to February 2019, a total of 51 client-owned cats originating from Timișoara Municipality were presented at the Veterinary Clinics of the Faculty of Veterinary Medicine Timisoara, Romania, for medical consultation showing one or more suggestive clinical signs (e.g. pallor of the mucous membranes, intermittent pyrexia, weight loss, lethargy, dehydration or weakness) for feline hemoplasma infections. On the day of presentation, beside routine physical examination, all cats were screened for feline leukemia virus (FeLV) and feline immunodeficiency virus (FIV) status using a commercial kit (IDEXX Laboratories, Inc., Westbrook, Maine, USA). In addition, blood was collected from each cat into sterile vacuum tubes with EDTA from the antebraial cephalic vein, in order to be tested for the presence of hemoplasma DNA. During anamnesis owners provided information about the cat including age, gender, breed, outdoor access and any history of previous ectoparasites (i.e. tick and/or flea) infestation were collected during anamnesis.

As PCR assays provide a far more specific and sensitive approach to hemoplasma identification than cytology of blood -smears [2], all biological samples were directly subjected to molecular processing. Genomic DNA was isolated using a PureLink™ Genomic DNA mini kit (Invitrogen™, Carlsbad, CA, USA) according to the manufacturer's instructions. Detection of feline hemotropic mycoplasmas was carried out using conventional polymerase chain reaction (PCR) based on the amplification of a partial sequence of the 16S rRNA gene. The specific forward (5'-ACGAAAGTCTGATGGAGCAATA-3') and reverse (5'-ACGCCAATAAATCCGRATAAT-3') primers and cycling parameters were used as previously described by [23]. These primers produce a 193 bp amplified fragment in CMhm and 170 bp in Mhf or CMt. Next, differentiation between Mhf and CMt in PCR-positive samples was carried out using a second PCR protocol with the CMt specific forward (5'-AGAGGCGAAGGCGAAACT-3') and reverse (5'-CTACAACGCCGAAACACAAA-3') primers and cycling conditions according to [24]. Subsequently, in order to obtain a longer Mycoplasma sequence, and for a better molecular assessment of infections in cats, a third conventional PCR was achieved for the previously obtained PCR-positive results, using the universal

HBT- forward (5'-ATACGGCCCATATTCCTACG-3') and HBT-reverse (5'-TGCTCCACCACTTGTTCA-3') primer set and cycling conditions designed by [10]. The positive PCR reactions produce a 595 bp and 618 bp long amplicons for Mhf and CMhm, respectively. Positive (DNA of *Mycoplasma* spp. from naturally infected cats) and negative (sterile demonized water) controls were included in each PCR run. The PCR amplified 16S rRNA gene amplicons, showing positive results were purified (Isolate II PCR and Gel Kit, Bioline™) and sequenced (performed by MacroGen™ Europe, Amsterdam, the Netherlands). The resulting sequences were subjected to BLAST (Basic Local Alignment Search Tool) analysis in order to compare them to those available in the GenBank™ dataset. Two representative sequences were deposited into GenBank™ as follows: Mhf: MH223461; and CMhm: MH223462. Statistical analyses were performed using the SPSS 21.0 software. The possible association between the hemoplasma infection status of cats and the recorded epidemiological parameters was assessed with the nonparametric Pearson's Chi – square ( $\chi^2$ ) test. Differences were established as statistically significant when  $p$  value  $\leq 0.05$ . Also, the risk factors were evaluated through calculation of odds ratios (ORs) with 95% confidence intervals (CIs), by including each variable in the binary Logit model of the multivariable regression analysis.

## Results

Overall, a total of 11 out of 51 domestic cats (21.6%, 95% CI 11.8–35.7) were tested positive for hemoplasma by PCR amplification of the 16S rRNA gene. Two species were identified, CMhm (8/11; 72.7%) was the dominant species, and Mhf (3/11; 27.3%) was also present in a lower percentage of cats. No co-infections were registered and CMT was not detected in any of the cats. Sequencing of PCR products was successfully performed in all positive samples. The sequences were highly similar amongst each other and showed > 99% similarity to other GenBank deposited CMhm (Accession no. KR905451) and Mhf (Accession no. KR905465) sequences from Italy isolated from domestic cats. Detailed distribution of the feline hemotropic mycoplasmas in accordance with the registered epidemiological data are shown in Table 1. No correlation was found ( $p > 0.05$ ) between hemoplasma infections and the age, gender, breed, presence of ectoparasites, FeLV/FIV positivity status of cats, or the sampling season. However, cats with outdoor access (36.8%, 95% CI 17.2–61.4) were found to be more susceptible to hemoplasma infections than those without outdoor access (12.5%, 95% CI 4.1–29.9,  $p = 0.049$ ). These results suggest that outdoor access could be considered a risk factor (OR = 4.1, 95% CI 1.0-16.6) in the acquisition of hemotropic mycoplasmas in cats.

Table 1

Distribution of hemoplasma infection in cats according to epidemiological data

Epidemiological data	Total no. of hemoplasma infected/tested cats (%) (95% CI)	Species identified (number)		Odds ratios (OR) (95% CI)	p value
		CMhm	Mhf		
Age					
Young ( $\leq$ 1-year old)	2/13 (15.4) (2.7–46.3)	0	2	1.7 (0.31–9.17)	0.533
Adult ( $>$ 1-year old)	9/38 (23.7) (12.0–40.6)	8	1		
Gender					
Female	4/28 (14.3) (4.7–33.6)	3	1	2.62 (0.7–10.5)	0.171
Male	7/23 (30.4) (14.1–53.0)	5	2		
Breed					
European	7/35 (20.0) (9.1–37.5)	4	3	1.33 (0.3–5.4)	0.687
Non-European	4/16 (25.0) (8.3–52.6)	4	0		
Sampling season					
Warm	7/31 (22.6) (10.3–41.5)	6	1	1.16 (0.3–4.6)	0.826
Cold	4/20 (20) (6.6–44.3)	2	2		
Outdoor access					
Yes	7/19 (36.8) (17.2–61.4)	5	2	4.1 (1.0–16.6)	0.049
No	4/32 (12.5) (4.1–29.9)	3	1		
Presence of ectoparasites					
Yes	3/11 (27.3) (7.3–60.7)	3	0	0.66 (0.1–3.1)	0.605
No	8/40 (20.0) (9.6–36.1)	5	3		
FeLV status					

Epidemiological data	Total no. of hemoplasma infected/tested cats (%) (95% CI)	Species identified (number)		Odds ratios (OR) (95% CI)	p value
		CMhm	Mhf		
Positive	1/5 (20.0) (1.1–70.1)	0	1	1.11 (0.1–11.1)	0.928
Negative	10/46 (21.7) (11.5–36.8)	8	2		
FIV status					
Positive	2/6 (33.3) (6.0–75.9)	2	0	2.00 (0.3–12.7)	0.462
Negative	9/45 (20.0) (10.1–35.1)	6	3		
Total	11/51 (21.6) (11.8–35.7)				

## Discussion

To the authors' knowledge, this is the first molecular study documenting the occurrence of hemotropic mycoplasma infections in cats from Romania, expanding the current knowledge of feline hemoplasmas at mainland Europe.

Comparing to several other European countries, the hemoplasma prevalence (21.6%) was higher than that reported in Spain (12.0%, [11]; 10.6%, [14]), Italy (18.9% [15]; 13.2%, [17]), Germany (15.6%, [20]), Denmark (16.4%, [22]) and Serbia (17.2%, [19]), similar to that described in Greece (20.6%, [18]), but lower than that reported in Portugal (27.1%, [13]; 43.4%, [12]) or northern Italy (33.1%; [16]). It is important to mention that comparing the results of different studies should be interpreted with caution, because differences in study design (e.g. sample size, sampling strategy), epidemiological parameters of the sampled population (e.g. health status of cats, cat's living environment) and the molecular diagnostic techniques used (e.g. conventional PCR, real-time PCR or a combination of both) in processing the blood samples in different studies could be considered sources of variation for the recorded hemoplasma prevalence. In this regard, it is important to highlight that in our study there was a bias towards the elevation of the infection prevalence, because all the investigated animals were clinically ill at presentation and sampling with suggestive signs for hemoplasma infections.

In accordance with our results, the dominance of CMhm in the screened feline population has been previously confirmed in other molecular surveys [7, 11–22], but others have reported Mhf as the predominant species [10]. It has been hypothesized previously [24], that CMHm has a more efficient replication and infection capacity, but is associated with a lower pathogenic potential compared to other two species of hemoplasma and often result in asymptomatic carriage status in cats. This supports its

dominant occurrence in this study and the other reports. Currently, without hematological examination, the evaluation of the pathogenicity of the recorded mycoplasma species in Romanian cats remains an open question for future investigation.

The carriage of multiple hemoplasma species, as well as the presence of CMt was not detected. Results of other studies have shown that co-infections with different combination of hemoplasmas frequently occur [14, 17, 19], and CMt seems to be the least frequently encountered feline hemotropic mycoplasma species, its prevalence ranging from 0.5 to 6.2% [7, 11, 12, 14, 19]. In other investigations [10, 18, 22], that process a limited number of samples as in the case of our study, the lack of the CMt detection was reported. Therefore, further studies, processing a significantly larger number of samples, are still necessary in order to obtain a more accurate overview and to conclude whether CMt is implicated in Romanian cats' mycoplasma infections.

Outdoor access lifestyle was the only epidemiological measure that was associated with hemoplasma infection in this study. Other studies have described several factors significantly associated with the presence of hemotropic mycoplasmas in cats including adult [14] or older [7, 18, 20–22, 24] age, male gender [7, 14, 17, 19–21, 24], non-pedigree breed [19], collection of blood during warm months [14, 15], and FeLV/FIV positivity status [11–15, 17, 19–21, 24, ]. Similar to our finding, the increased likelihood of cats being infected with hemotropic mycoplasmas with outdoor access has been frequently reported in other studies [7, 11, 14, 19]. This observation can be sustained by the fact that this lifestyle increases, diversifies and perpetuates the relationships between cats, resulting in the possible transmission of mycoplasma from positive to negative animals, via direct (e.g. fighting or biting) or vector borne (fleas or ticks) transmission, as have been previously suggested [4–8, 11, 17, 24]. However, the scientific demonstration of this hypothesis remains, until now, unfulfilled.

## Conclusions

The present study provides data on the occurrence of hemoplasma infections, as well as the molecular evidence of CMHm and Mhf in domestic cats for the first time in Romania. As such, it provides the first indication of the prevalence of hemoplasmas in this previously uninvestigated territory and useful information for small animal practitioners. It implicates the outdoor access lifestyle as a risk factor in the acquisition of disease. The occurrence of feline hemoplasmas in this geographical area, previously thought to be hemoplasma free, opens the opportunity for a larger scale study to be carried out to address some of the limitations of the current survey. In this regard, further studies focusing on the relationship between the hemoplasma species and the resultant hematologic profile, with special emphasis on anemia are recommended.

## Abbreviations

Mhf

"Mycoplasma haemofelis"; CMhm:"Candidatus Mycoplasma haemominutum"; "CMT": "Candidatus Mycoplasma turicensis"; FeLV: Feline leukemia virus; FIV: Feline immunodeficiency virus; DNA: deoxyribonucleic acid; EDTA: Ethylenediaminetetraacetic acid; PCR: Polymerase chain reaction; RNA: Ribonucleic acid; BLAST: Basic Local Alignment Search Tool; OR: Odds ratio; CI: Confidence interval;

## **Declarations**

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### **Availability of data and materials**

The sequences (accession numbers MH223461 and MH223462) used to support the findings of this study have been deposited in the GenBank repository. The datasets generated and analyzed during the current study are included within the article.

### **Authors' contributions**

MI, TS performed laboratory work and participated in data analysis. CV, performed the sampling and clinical evaluation of cats. GD and SM helped the study design and implementation, and data interpretation. PJPL contributed to the Discussion and participated in the writing the manuscript. KI, conceived and designed the study, coordinated the research team and drafted the manuscript. All authors read and approved the final manuscript.

### **Ethics approval and consent to participate**

The research protocol was reviewed and approved by the Research Ethics Committee of BUASVM "King Michail I of Romania", Timișoara, Romania, and registration No. 128-06.12.2018. Written consent was

obtained from cat owners for blood samples to be used in research/publication.

### Consent for publication

Not applicable.

### Competing interests

The authors declare that they have no competing interests.

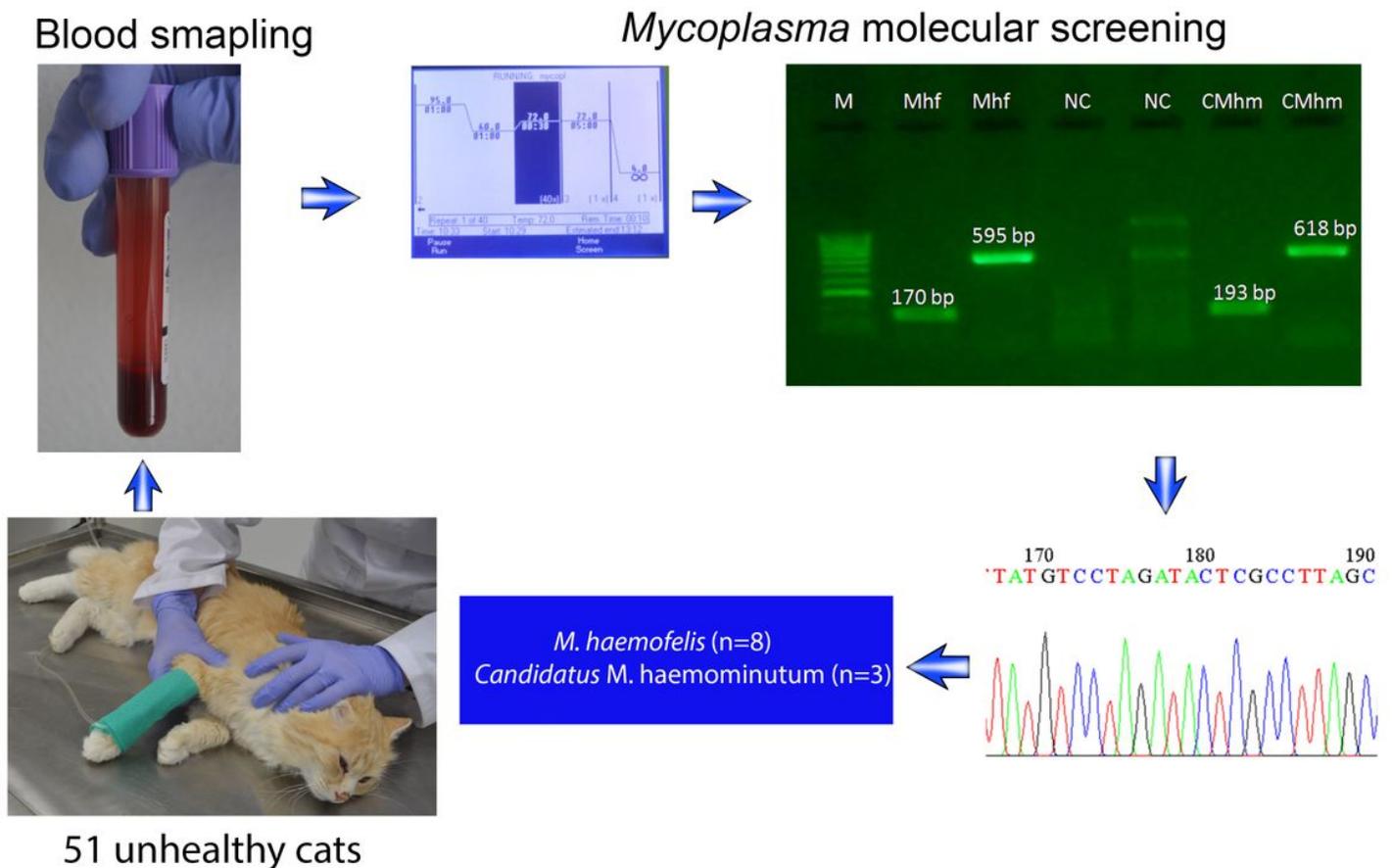
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## Figures



## Figure 2

Legend not available in this version