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VETERINARY AND ANIMAL PRODUCTION - SHORT COMMUNICATION

Presence, genetic characterization, geographic distribution and associated risk factors of feline hemoplasmas in Paraguay

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Abstract

Hemotropic mycoplasmas (hemoplasmas) are small, wall-less bacteria that parasitize red blood cells and can induce hemolytic anemia in felines. The three main species known to infect cats worldwide are *Mycoplasma haemofelis* (Mhf), *Candidatus Mycoplasma haemominutum* (CMht) and *Candidatus Mycoplasma turicencis* (CMt). These species differ in their pathogenicity and prevalence, Mhf being the most pathogenic and CMht the most prevalent. The aim of this study was to determine the presence, genetic characterization, associated risk factors and geographical distribution of feline hemoplasmas in Paraguay. DNA was extracted from feline whole blood samples submitted by local veterinarians to the CEDIVEP laboratory for the detection of feline hemoplasmas; Mhf (456 samples), CMht (428 samples), and CMt (359 samples) by polymerase chain reaction (PCR). A total of 76/456 samples (16%) were positive for Mhf, 77/428 (18%) were positive for CMht, and no animals were positive for CMt by PCR. Sequencing, BLAST and phylogenetic analysis were performed to confirm the identity of 16 S rRNA and was supported by the distinct separation of species-specific clades. Positive animals were found in both regions of the country (eastern and western), and the Department with the highest prevalence was Central with 70/76 (92,1%) positive for Mhf and 70/77 (90,9%) positive for CMht. The prevalence of feline hemoplasmas in domestic cats in both regions of Paraguay was determined by PCR. Male sex was a risk factor for Mhf and CMht. Age between 1 and 3 years was a risk factor for CMht and mixed breed and Siamese was a risk factor for Mhf. Feline mycoplasmosis had a greater presence in Central deparment Paraguay and more frequently affected mixed breed and common European cats.

Keywords Feline hemoplasmas · Molecular detection · Prevalence · Paraguay

Introduction

Hemotropic mycoplasmas (hemoplasmas) are small wall-less bacteria that parasitize red blood cells and can induce hemolytic anemia in felines. The three main species known to infect cats worldwide are *Mycoplasma haemofelis* (Mhf), *Candidatus Mycoplasma haemominutum* (CMht) and *Candidatus Mycoplasma turicencis* (CMt), of which Mhf is the most pathogenic and CMht the most prevalent in most studies [1–3].

Infection with hemoplasmas has been linked to several risk factors, including male sex, nonpedigree status, outdoor access, and feline immunodeficiency virus (FIV) infection [4–10]. The natural route of transmission of hemoplasmas has not been determined. Although transmission through the arthropod vector *Ctenocephalides felis* has been suggested [11], a meta-analysis by Moore et al. [12] of previous

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reports revealed a lack of association between hemoplasma infection and *C. felis* epidemiology. Other proposed routes of transmission include biting and scratching and blood transfusion [11–14].

The diagnosis of hemoplasmas used to rely on cytologic detection in blood smears. However, given the low sensitivity reported in studies [7, 15] and the fact that many cases diagnosed on the basis of blood smear have been false-positives because of stain precipitates or Howell–Jolly bodies, and because of slow blood smear drying [16], PCR has become the preferred diagnostic method for hemoplasma infection owing to its greater sensitivity and specificity [17].

The presence of feline hemoplasmas varies geographically and has been identified in South America, with prevalences ranging from 6.5 to 35.5% in Brazil [9, 18–21], 7.8–15.2% in Chile [4, 22], and 2% in Ecuador [23]. In Paraguay, CMht was reported in cats with a presence of 4.7% from the cities of Asunción and Encarnación [24], and Mhf was confirmed by molecular techniques in Asuncion [25]. Hemoplasmas in Paraguay have been primarily reported as isolated cases, and there are no data on their geographical distribution.

This study aimed to analyze data from blood samples of domestic cats received between 2021 and 2024 at the Veterinary Diagnostic Center (CEDIVEP) for the molecular detection of feline hemoplasmas by PCR, and to determine the presence genetic characterization, geographic distribution, and associated risk factors of these pathogens in Paraguay.

Materials and methods

Study population

Data for this study were retrospectively obtained from the laboratory database CEDIVEP. The records included results from molecular diagnostic by PCR on whole blood samples collected from domestic cats betweenJuly 2021 and April 2024. These samples were submitted by independent veterinary clinicians for the detection of Mhf (456 samples), CMht (428 samples), and CMt (359 samples).

Whole blood was collected and sent in 1mL tubes with k3EDTA, transported under refrigeration and stored at 4 °C until DNA extraction.

The data set included information on the cat's age, breed, sex, department of residence (based on the guardians' address), and PCR results. No additional testing for other pathogens was performed, and clinical data regarding the health status of the cats were not available for this study.

DNA extraction and PCR amplification

DNA was extracted from 200 µL of whole blood using the AccuPrep® Genomic DNA Extraction Kit (Bioneer Corporation, Daejeon, Republic of Korea) following the manufacturer's instructions. DNA quality and concentration were evaluated via a MaestroNano Pro spectrophotometer (Maestrogen INC, Hsinchu, Taiwan), and the 260/280 nm and 260/230 nm absorbance ratios were measured.

Each DNA sample was subjected to PCR assays targeting the 16 S rRNA gene of Mhf, CMht and CMt, according to the veterinary clinician's request, using previously described primers [28, 29]. The sequences of the primers, the amplification conditions, and the sizes of the amplicons are shown in Table 1.

Reactions were performed in a final volume of 20 µL using AccuPower® Taq PCR PreMix (Bioneer Corporation, Daejeon, Republic of Korea). For the amplification of Mhf and CMht, 1.5 µL (0.75 µM) of each primer was combined with in 15 µL of RNAse-free water (Thistle, Rugby, United Kingdom) and 1.5 µL (15–50 ng) of genomic DNA was added. In the case of CMt, 0.5 µL (0.25 µM) of each primer, 18 µL of RNAse-free water, and 1 µL (15–50 ng) of genomic DNA were used.

Amplification was carried out on a GTC96S thermal cycler (Thistle, Rugby, United Kingdom). To ensure accuracy and avoid potential contamination, each PCR included positive controls consisting of Mhf and CMht DNA previously obtained in the laboratory, which were confirmed by sequencing, and negative controls containing only PreMix were also used. No positive controls were available for CMt, as none had been previously detected in the laboratory.

The PCR products were subjected to 2% agarose gel electrophoresis using RunSAFE (Thistle, Rugby, United Kingdom) as the loading dye. Electrophoresis was performed at 90 V for 50 min, bands were visualized using a gelLITE photo documentation system (Thistle, Rugby, United Kingdom).

Table 1 Primers and protocol used for the detection of feline *Mycoplasma spp*

Target gene	Primer sequence (5′-3′)	Product Size (bp)	PCR Conditions	Reference
Mycoplasma spp. 16 S rRNA	F: ATACGGCCCATATTCCTACG R: TGCTCCACCACTTGTTCA	Mhf 595/CMht 618	95 °C 5 min; 40 cycles [94 °C 30 s; 60 °C 30 s; 72 °C 30 s]; 72 °C 7 min	[26]
CMt 16 S rRNA	F: AGAGGCGAAGGCGAAAACT R: CTACAACGCCGAAACACAAA	138		[27]

Sequencing and bioinformatic analysis

Fourteen amplicons of the 16 S rRNA gene were purified and sequenced. The bands observed on the agarose gels were visually intense and, when quantified, had values > 10 ng/µL (Nano Pro quantifier; MaestroGen). Products with the correct band size and required concentration were sent to Macrogen (Korea) for purification and Sanger sequencing. The resulting chromatograms were cleaned removing regions at the ends that were not legible. The obtained sequences were analyzed using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi) to confirm identity with the target genes.

Sequences were edited for homology analysis, and reference sequences were retrieved from GenBank, representing isolates from various countries: KR905451.1(Italy), KU765207.1(Thailand), KR905449.1(Italy), KR905450.1(Italy), KU852586.1(Iran), EU839978.1(Italy), DQ157156.1(Switzerland), AY150977.1(Australia), AY150985.1 (United Kingdom), PP494686.1(Tailand), KM275246.1(Brazil), KY117653.1(Chile), KY117657.1(Chile), EU532066.1(Thailand), EU442629.1(Brazil), KR905460.1(Italy), JQ689950.1 (Taiwan) and DQ464422.1(South Africa). *The Mycoplasma pneumoniae* strain NR113659.1 was used as an outgroup.

All sequences were aligned with ClustalW (v.7.1.3.0; BioEdit), and a phylogenetic tree was constructed using the maximum-likelihood with the GTR + F + i + G model implemented in MEGA 7 (https://www.megasoftware.net/) and FIG TREE (https://github.com/rambaut/figtree) software. Accession numbers of the sequences analyzed in this study were submitted to the GenBank database.

Statistical analysis

Age, breed, sex, department of residence of the guardians and PCR results were analyzed to determine the presence of cats infected with Mhf, CMht and CMt.

Fisher's exact test was used to determine the associations between two categorical variables, other than hemoplasma infection. Potential risk factors for infection positivity in cats was considered for the multivariable analysis. We perform two models, were constructed, one for each infection: Model 1 for Mhf and Model 2 for CMht. The variable age was grouped in < 1 year, 1–3 years and > 3 years. Breeds were grouped as others (Russian blue, British Shorthair, Bengal and no data of breed).

Multicollinearity among breed, sex, age and department was assessed using the variance inflation factor (VIF) < 2. We then conducted a logistic regression analyses (generalized linear model with binomial link funtions) were conducted, including all non-colliner variables.

All statistical analyses were conducted using R Studio software (v 4.4.1, 2024-06-14) and the car package was used to assess the multicollinearity and to perform logistic regressions. In addition, maps of Paraguay were created to represent the geographical distribution of cases using the sf package.

Results

Seventy-six out of 456 cats tested positive (16%) for Mhf, and 77/428 cats (18%) tested positive for CMht. No animals tested positive for CMt was by PCR.

The presence of hemoplasmas were detected in both the eastern and western regions of the country.

The highest concentration of positive animals was observed in the Central Department, where Mhf and CMht cases represented 92.1% (70/76) and 90.9% (70/77) of total positives, respectively. Mhf was also to a lesser extent, in the departments of Cordillera, Boquerón and Caaguazú, while CMht was detected in Cordillera, Caazapá, Amambay, and Pte. Hayes (Figs. 1 and 2).

With respect to age and sex, males cats showed a higher frequency of infection than females for both Mhf 67.1% (51/76) and CMht 63.6% (49/77) The age at which the highest number of positive cases occurred in cats was of 1–3 years, with 31/76 (40,8%) for Mhf and 36/77 (46,8%) for CMht. (Table 2).

The presence of CMht DNA was significantly higher in male cats aged 1–3 years, and Mhf was also significantly more frequent in males ($p < 0,05$) (Table 3). No statistically significant association were found between infection status and breed or departments. Likewise, Fisher's exact test revealed no association between location and breed.

Sequencing and BLAST analysis confirmed the identity of 16 S rRNA amplicons for CMht in 9 samples (CDV111, CDV133, CDV127, CDV114, CDV128, CDV118, CDV113, CDV115, and CDV6), MHf in 4 samples (CDV7, CDV179, CDV134, and CDV50) and CMt in one sample (CDV112). Although, this sample tested positive for the detection of Mhf using the *Mycoplasma* spp. 16 S rRNA primer, BLAST analysis revealed a 98.56% nucleotide identity with CMt.

BLAST analysis of the consensus sequences for the detected pathogens showed 98.45–99.83% nucleotide identity with CMht and 98.83–99.45% identity with MHf sequences available in the GenBank database. Molecular identification was further supported by phylogenetic analysis, which showed distinct clustering of species-specific clades (Fig. 3). Accession numbers submitted to the Genbank of the analyzed sequences are: PV446375, PV446376, PV446377, PV446378, PV446379, PV446380, PV446381, PV446382, PV446383, PV446384.

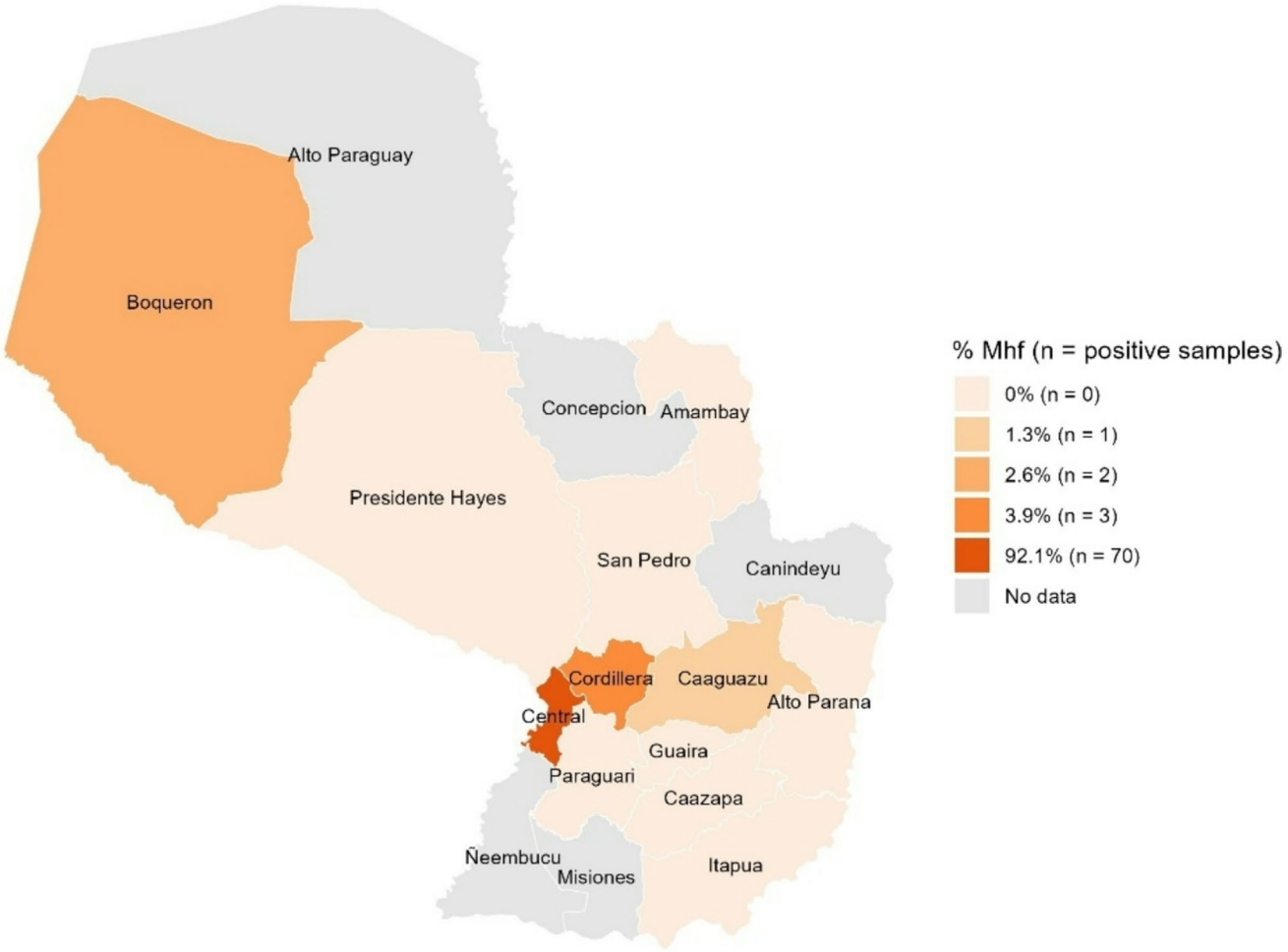

Fig. 1 Geographic distribution of *Mycoplasma haemofelis* samples in cats from Paraguay

The potential risk factors for Mhf and CMht infection included in the multivariable analysis are shown in Table 3. No collinearity was detected among the explanatory variables.

Significant associations were observed for both pathogens. Male cats were at significantly higher risk of being infected with Mhf (OR = 2.23; 95% CI: 1.28–4.00; $p = 0.005$) and CMht (OR = 1.75; 95% CI: 1.03–3.03; $p = 0.041$) compared to female cats. Regarding age, cats aged between 1 and 3 years showed increased odds of CMht infection (OR = 3.05; 95% CI: 1.23–9.26; $p = 0.027$) in comparison with cats under 1 year old. Mixed breed and Siamese were at significantly higher risk of being infected with Mhf more than others breeds (OR = 2.86; 95% CI: 1.62–5.00; $p = 0.00028$ and OR = 3.24; 95% CI: 1.13–8.64; $p = 0.021$) respectively.

No statistically significant associations were found between infection status and breed for either Mhf or CMht, nor for other age categories or cats with undetermined sex.

Discussion

A total of 16% (76/456) of the tested cats were positive for Mhf, and 18% (77/428) tested positive for CMht, based on samples submitted to a private veterinary diagnostic laboratory in Paraguay. This study demonstrated the wide geographical distribution of the pathogens covering both regions of the country (eastern and western). The highest number of positive cases was recorded in the Central Department, which is likely due to its high human population density, greater access to veterinary care, and higher pet ownership. Although the department itself was not identified as a risk factor in this study, peri-urban areas could be a risk factor [19], considering that these types of locations are predominant in Paraguay, as in most departments except Central. Notably, isolated positive cases were also found in the western region. To our knowledge, this is the first study describing the distribution of feline hemoplasmas across both Paraguayan regions.

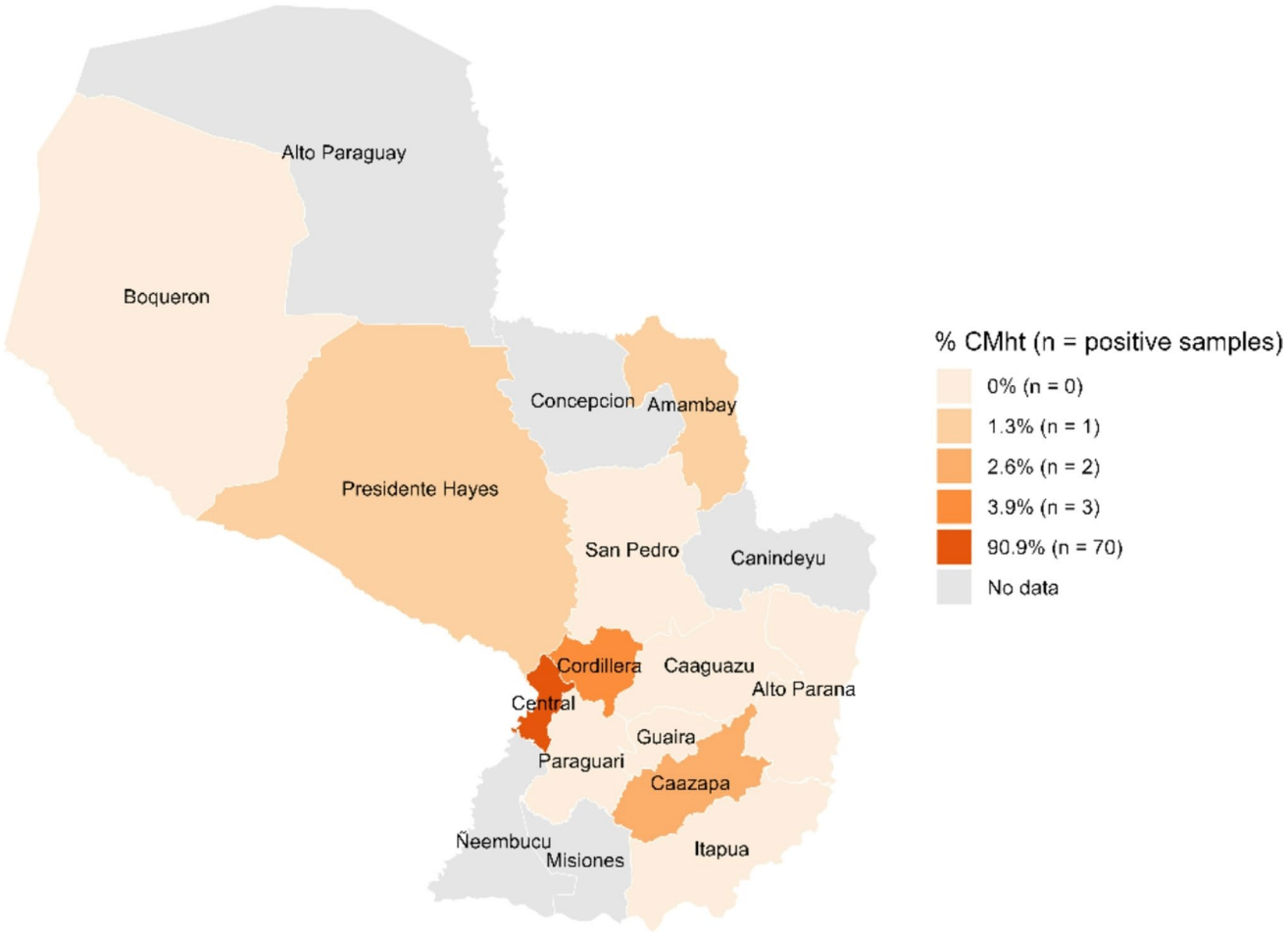

Fig. 2 Geographic distribution of *Candidatus Mycoplasma haemominutum* samples in cats from Paraguay

In relation to the data obtained on breed, sex and age, the most frequently infected cats were of the mixed breed for Mhf and Common European for CMht, males of 1–3 years. These findings align with studies from Paraguay [25] and other countries of the region [4, 19]. Non pedigree breed is associated as a risk factor to intection Mhf and CMht [5, 7]. Male sex was a factor associated with positivity for Mhf and CMht, the association with male sex may be due to their behavior patterns such as roaming, biting, and fighting [4] suporting the hypothesis of horizontal transmission via fighting between cats [28]. Direct transmission in nonpedigree male cats of older age by biting and scratching during fighting has been reported [5, 12], although the presence of ectoparasites cannot be excluded. Independent of the clinical status of cats, some studies suggest that both sick and healthy male cats are at greater risk of infection [7].

On the other hand, the greater presence of CMht in older animals may be associated with persistent long-term chronic infection [8]. Persistent carrier status appears to be especially common following CMht infection, although suspected clearance of this infection has been reported with and without antimicrobial treatment [6]. Mhf-infected cats may spontaneously clear infection from peripheral blood after infection without antimicrobial treatment [28]. Some research studies indicated that these older cats have an increased risk of exposure over time, producing a chronic asymptomatic carrier state [4]. Since no data were obtained on the clinical status of the sampled animals, it was not possible to correlate the presence of infection with disease, whether they presented clinical signs, or were carriers of other hemopathogens or coinfections with retroviruses such as feline leukemia virus (FeLV) or feline immunodeficiency virus (FIV), Data of these coinfections were not available in this study.

The elevated odds ratios observed, particularly for CMht in male cats aged 1–3 years (OR = 3.05), may reflect both a real increase in risk and the influence of sample size limitations within subgroups. Wide confidence intervals suggest uncertainty around the estimates, which can be exacerbated by a small number of positive

Table 2 Percentages of positive results for different Hemoplasma species in the studied population

Variable	Hemoplasma Positive Cats	
	Mhf ($n=76$)	CMht ($n=77$)
Sex		
Male	51 (67,1%)	49 (63,6%)
Female	21 (27,6%)	25 (32,5%)
ND[a]	4 (5,3%)	3 (3,9%)
Age		
< 1 year	9 (11,8%)	5 (6,5%)
1–3 years	31 (40,8%)	36 (46,8%)
> 3 years	24 (31,6%)	22 (28,6%)
ND[a]	12 (15,8%)	14 (18,2%)
Breed		
Common European	28 (36,8%)	40 (51.9%)
Mixed breed	35 (46,1%)	24 (31,2%)
Siamese	7 (9,2%)	4 (5,2%)
Persian	0 (0,0%)	3 (3,9%)
Ragdoll	0 (0,0%)	2 (2,6%)
ND[a]	4 (5,3%)	2 (2,6%)
Others	6 (7,8%)	4 (5,1%)
Department		
Central	70 (92,1%)	70 (90,9%)
Boquerón	2 (2,6%)	0 (0,0%)
Caaguazú	1 (1,3%)	0 (0,0%)
Cordillera	3 (3.9%)	3 (3,9%)
Amambay	0 (0,0%)	1 (1,3%)
Caazapá	0 (0,0%)	2 (2,6%)
Presidente Hayes	0 (0,0%)	1 (1,3%)

[a]ND (Not determined)

cases in specific categories. Moreover, residual confounding from unmeasured variables such as access to outdoor environments, interactions with other animals, fighting behavior, or coinfections could lead to an overestimation of the effects attributed to sex or age alone. In future analyses, several statistical adjustments could be considered to improve the robustness [29] of the estimates. Ultimately, expanding future studies with larger and more balanced sample sizes across categories, and incorporating behavioral and environmental data, would improve the explanatory power of multivariable models and reduce the impact of potential omitted variable bias.

A sample, CDV112, that was positive for MHf by PCR was determined to be infected with CMt via sequencing. The amplicon from this sample had 98.56% identity to CMt via BLAST N, indicating that a cat with CMt was identified. However, this infection was not detected by the CMt-specific PCR primers, and yielded amplicons of similar intensity to true MHf infected samples. Infection with CMt is often associated with infection with other hemoplasma species, particularly CMht [6]. Coinfection among the samples analyzed cannot be ruled out, since it was not possible to determine the presence of more than one pathogen per animal in this study. Detection of coinfection with universal primers rather than species-specific primers may be inhibited by competition for reaction components [17]. We postulate that the sample CDV112 was coinfected with both CMt and MHf. To the best of our knowledge, this is the first study to record the presence of CMt in Paraguay by sequencing.

Sequence and phylogenetic analyses revealed that the three species could be divided into separate clades and

Table 3 Results of multivariable logistic regression analysis identifying significant risk factors for Hemoplasma infections (Mhf and CMht) in domestic cats in paraguay, based on samples submitted to the laboratory between July 2021 and April 2024. Significant *p*-values are highlighted in bold

Model	Variable	OR	95% CI (Lower)	95% CI (Upper)	*p* value
Model 1 (Mhf)	Breed (Common European)	-	-	-	*Reference*
	Breed (Mixed breed)	**2.83**	**1.62**	**5.00**	**0.00028***
	Breed (Persian)	1.80×10^{-7}	-	9.73×10^{19}	0.644
	Breed (Ragdoll)	3.04×10^{-7}	-	1.07×10^{184}	0.98728
	Breed (Others)	1.26	0.44	3.13	0.99572
	Breed (Siamese)	**3.24**	**1.13**	**8.64**	**0.02177***
	Sex (Female)	-	-	-	*Reference*
	Sex (Male)	**2.31**	**1.34**	**4.12**	**0.00338***
	Sex (ND[a])	2.06	0.52	6.90	0.262
Model 2 (CMht)	Sex (Female)	**-**	**-**	**-**	*Reference*
	Sex (Male)	**1.75**	1.03	**3.03**	**0.041***
	Sex (ND[a])	1.46	0.311	5.18	0.583
	Age < 1 year	-	-	-	*Reference*
	Age 1–3 years	**3.05**	**1.23**	**9.26**	**0.027***
	Age > 3 years	2.26	0.867	7.05	0.120
	Age ND[a]	2.72	0.953	8.98	0.075

[a]ND (Not determined), * *p* value (< 0.05)

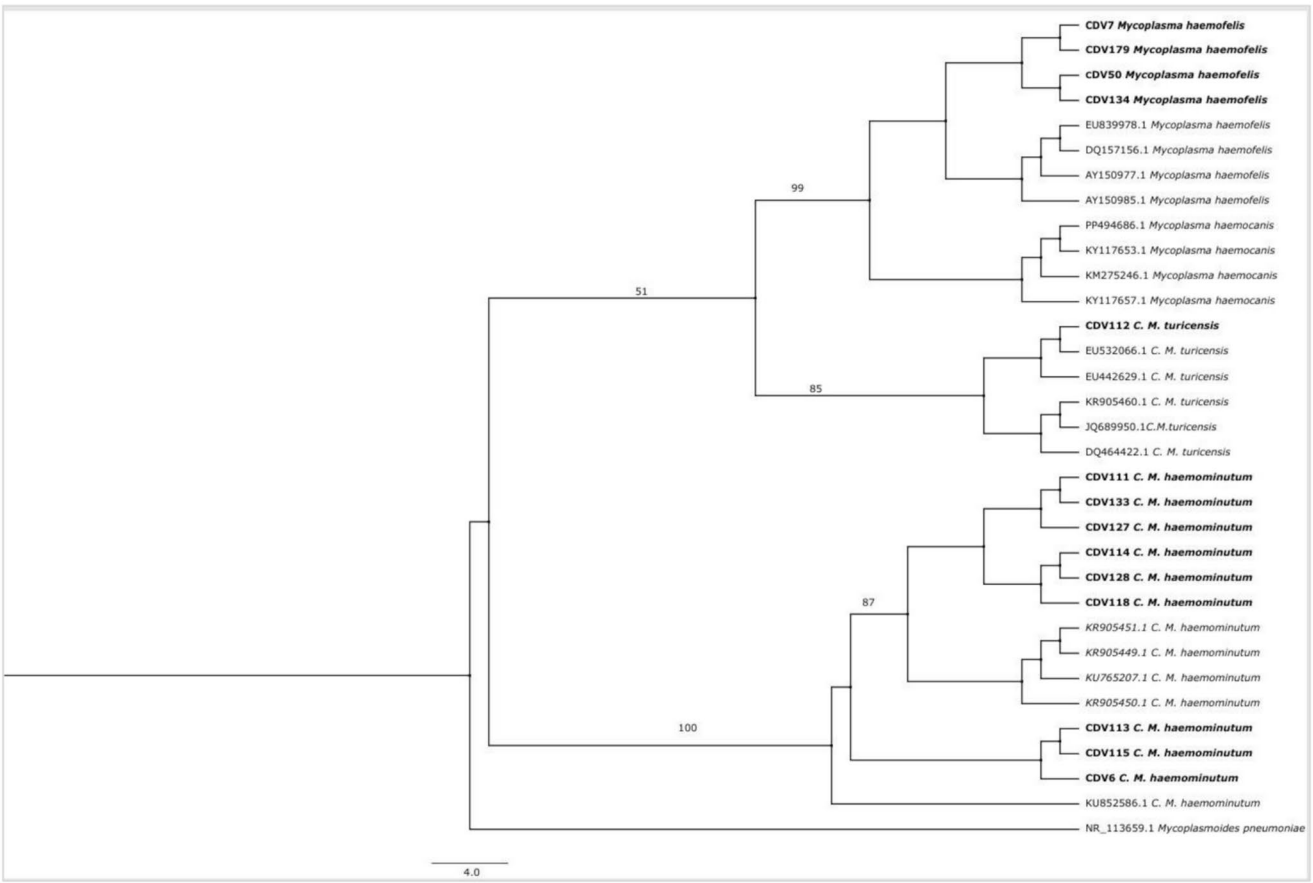

Fig. 3 Phylogenetic tree based on the partial 16 S rRNA gene. Tree was generated using the maximum-likelihood method based on the GTR + F + i + G model, with MEGA software, the numbers on the tree indicate boostrap values for the branch. Paraguayan's strain is marked in bold

that the differences among the species could be determined. The structure of the constructed tree, based on 16SrRNA gene phylogeny, demonstrated that our isolates were grouped in distinct clades, together with other GenBank-deposited Mycoplasma sequences isolated from cats in different countries from various parts of the world. This phenomenon has been previously observed [30]. Although 16 S rRNA sequences have proved to be a very effective tool in determining the phylogeny and taxonomy of the mollicutes, additional phylogenetic markers would be helpful to support the conclusions based on 16 S rRNA gene data [31].

There were several limitations in the present study, such as the lack of positive controls for CMt and the limited number of Sanger-sequenced samples. Moreover, hematological analyses could provide insightful information about pathogenicity and require more information about the clinical status of the animals, and the presence of retroviral coinfections. Understanding transmission dynamics and risk factors in various environmental settings will be essential for improving feline hemoplasma surveillance in Paraguay.

Conclusions

To our knowledge, this is the first large-scale molecular epidemiology study of feline hemoplasmosis in Paraguay. The presence rates of 76/456 (16%) for Mhf and 77/428 (18%) for CMht were determined by PCR in domestic cats in both regions of Paraguay. It is more prevalent in the Central Department and more frequently affects mixed breed and common European cats. Male sex was a risk factor for Mhf and CMht, age between 1 and 3 years was a risk factor for CMht and mixed breed and Siamese was a risk factor for Mhf. The presence of CMt was detected in a single cat by Sanger sequencing. Future studies are needed, especially related to coinfections, transmission routes and clinical signs. This study provides the first indication of the presence of hemoplasmas in the entire country and is useful information for small animal practitioners.

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Data availability The datasets generated during and/or analyzed during the current study are available from the corresponding author upon reasonable request.

Declarations

Conflict of interest The authors declare that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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