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HEMOTROPIC MYCOPLASMA IN DOMESTIC CATS FROM THE CENTRAL REGION OF RIO GRANDE DO SUL STATE, BRAZIL

(Micoplasma hemotrópico em felinos domésticos na região central do Rio Grande do Sul, Brasil)

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RESUMO

Os micoplasmas hemotróficos também conhecidos como hemoplasmas são bactérias que se aderem aos eritrócitos e podem levar ao desenvolvimento de uma doença conhecida como anemia infecciosa felina (AIF). A AIF pode ser leve ou grave e pode levar à morte. Os hemoplasmas foram descritos em vários países, incluindo o Brasil, contudo, estudos sobre a frequência de infecção na região central do estado do Rio Grande do Sul, não foram descritos até o momento. O objetivo deste estudo foi verificar a prevalência de hemoplasmas nessa região, bem como avaliar alterações hematológicas, e fatores de risco para infecção, como idade e sexo. Para isso, 192 amostras de sangue de gatos domésticos foram coletadas e analisadas através da reação em cadeia da polimerase (PCR), método de escolha para diagnóstico de infecção. Dos felinos testados, 14,6% apresentaram-se positivos para infecção por hemoplasmas, no entanto, não foram identificadas estruturas compatíveis com micoplasmas na superfície de eritrócitos na avaliação do esfregaço sanguíneo. Em relação à idade, animais com idade ≤3 anos apresentaram maior incidência de infecção do que felinos com mais de 3 anos de idade. Sendo que, 75% das amostras infectadas eram de machos. Não houve diferença nas variáveis hematológicas entre felinos infectados e não infectados. Assim, conclui-se que a prevalência de micoplasmas hemotrópicos na região central do Rio Grande do Sul é de 14,6% e que a infecção não está relacionada à anemia, além de ser mais frequente em animais jovens e machos.

Palavras-chave: Hemoplasma, reação em cadeia da polimerase, felinos.

ABSTRACT

Hemotropic mycoplasmas, also known as hemoplasmas, are bacteria that attach to the red blood cells and are the causative agents of feline infectious anemia (AIF). AIF can be mild or severe and can eventually lead to death. The hemoplasmas have been described in several countries, including Brazil; however, studies on the frequency of infection in central Rio Grande do Sul state is yet unknown. The aim of this study was to report the prevalence of hemoplasmas in this region as well as to evaluate the hematological changes and risk factors such age and sex, associated with infection. For this, 192 blood samples from domestic cats were collected and analyzed by species-specific conventional

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polymerase chain reaction (PCR) assays. Overall, 14,6% cats were infected by at least one hemoplasma, but structures morphologically compatible with hemoplasmas were not identified in blood smears. Concerning age, animals aged ≤3 years had a higher incidence of infection than cats older than 3 years of age with 75% of infected samples being males. There was no statistical difference in hematological variables between infected and uninfected cats. Thus, we conclude that the prevalence of hemotropic mycoplasmas in Central Rio Grande do Sul state is 14.6% and that infection was not correlated with the presence of anemia and young male cats were more likely to be infected.

Key words: Hemoplasma, polymerase chain reaction, cat.

INTRODUCTION

The hemotropic mycoplasmas or hemoplasmas are epi-erythrocyte and highly pleomorphic bacteria that attach to the surface of red blood cells (HARVEY, 2006). These organisms do not synthesize their source of energy and some essential cellular components; therefore, they are considered obligate microorganisms (NEIMARK *et al*., 2001; HARVEY, 2006 SANTOS *et al*., 2011). Hemoplasmas infect many species of mammals (SYKES *et al*., 2008). Species known to infect cats are '*Candidatus* Mycoplasma haemominutum'*, Mycoplasma haemofelis,* and *'Candidatus* Mycoplasma turicensis' (WILLI *et al*., 2007; SANTOS *et al.*, 2009).

The infection caused by hemoplasmas can lead to acute or chronic disease, ranging from mild and asymptomatic infection to a fatal form, with extensive destruction of red blood cells, depending on the species involved and the susceptibility of the host (AGUIRRE *et al*., 2009; MESSICK, 2004). *Mycoplasma heamofelis* is mostly associated with the severe form of the disease, whereas infections with '*Candidatus* Mycoplasma haemominutum' is generally asymptomatic. Polymerase chain reaction (PCR) is the method recommended for diagnosis; however, in many laboratories only cytological analysis of blood smears are availables, hindering the diagnosis of infection by being a less sensitive method (BARKER e TASKER, 2013).

Feline hemoplasma infections have been described in several places of the world, including Brazil (BAUER *et al*., 2008; SYKES *et al*., 2008; JENKINS *et al*., 2013; SINEREY, 2013; SANTOS *et al*., 2014; ROSENQVIST *et al*., 2016). However, there is no available data of the infection in the central region of Rio Grande do Sul, therefore, the aim of this study was to describe the occurrence of hemotropic mycoplasma infections in cats from Santa Maria city and surroundings, Rio Grande of Sul, Brazil, as well to investigate the presence of anemia and risk factors such as age and sex of the animals.

MATERIAL AND METHODS

This study was conducted after approval by the Ethics Committee of Animal Use (UFSM) under protocol 5142100515. A total of 192 blood samples from domestic cats presented to the Veterinary Laboratory of Clinical Analyses (Santa Maria, RS, Brazil) for multiple reasons were used in this study (hospital population). Information about animals

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as clinical history, sex, and age were analyzed. All cats had a complete blood count (CBC) performed, as well as the cytological examination of blood smears, in order to verify the presence of inclusions compatible with hemoplasmas.

Erythrocyte count, pack cell volume (PCV) hemoglobin concentration, mean corpuscular volume (MCV), and mean corpuscular hemoglobin concentration (MCHC) were evaluated. PCV and hematimetric index were evaluated according to Feldman *et al*. (2000), whereas erythrocyte count and hemoglobin concentration were determined using an electronic counter (Mindray, BC 2800). Anemia was considered when the PVC was less than 24%.

Molecular diagnosis

DNA extraction was performed on frozen blood samples (EDTA) using a similar protocol described by Rebouças (2008), with a few modifications. The method consists of DNA separation by cell lysis in Sodium Dodecyl Sulfate (SDS), proteinase K, and Tris-HCl, EDTA, NaCl (TEN). Then, extraction was performed with phenol, chloroform, and isoamyl alcohol to separate the DNA from protein followed by DNA precipitation with absolute alcohol. The samples were stored with Tris-HCl, EDTA (TE) and RNAse at -20 °C.

PCR was performed following the protocols described in the literature (SYKES *et al*., 2008; SANTOS *et al*., 2009) with some modifications. The mix used for each reaction was composed of 0.12 µL of AmpliTaq® DNA polymerase (Invitrogen, São Paulo, SP, Brazil), 2.5 µL of 10X Buffer II® (Invitrogen), 2.25 µL of $MgCl_2$, 2.0 µL of 10 mM dNTPs (deoxyribonucleotide triphosphate), 1.0 µL of each primer (Forward and Reverse), 14.63 µL of ultrapure water (Invitrogen), and 1.5 µL of DNA sample, with a total volume of 25 µL.

For amplification of a partial fragment of 393 base pairs (bp) of the 16S rRNA gene of *M. haemofelis*, the following primers were used: *Forward* 5'-GAC TTT GGT TTC GGC CAA GG-3'; and *Reverse* 5'-CGA AGT ACT ATC ATA ATT ATC CCT C-3'. Reaction conditions consisted of an initial denaturation step of 3 min at 94 °C followed by 33 cycles; 94 °C for 45 s (denaturation); 55 °C for 30 s (annealing); 72 °C for 1:30m extension), and in the final stage of extension, 72 °C for 10 min.

For amplification of the 191 bp fragment of the 16SrDNA gene of '*Candidatus* M. haemominutum', the following primers were used: *Forward* 5'-GCA TAA TGT GTC GCA ATC-3'; and *Reverse* 5'-GTT TCA ACT AGT ACT TTC TCC C-3'. Reaction conditions consisted of an initial denaturation step of 5 min at 94 °C followed by 35 cycles; 94 °C for 45 s, 55 °C for 30 s, 72 °C for 60 s, and in the final stage of extension at 72 °C for 10 min.

For '*Candidatus* M. turicensis' 488 bp of the16SrDNA gene was amplified using primers: *Forward* 5'-CGA ATT GTC GAA AGA CAA TTA GC-3'; *Reverse* 5'-AGA AGT TTC ATT CTT GAC ACA ATT TAA-3'. Reaction conditions consisted of an initial denaturation step of 5 min at 95 °C followed by 31 cycles; 95 °C for 30 s, 60 °C for 30 s, 72 °C for 50 s, and a final extension step at 72 °C for 10 min. For each reaction, positive

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controls were included, kindly supplied by Purdue University, USA; and ultrapure water was used as a negative controls.

After amplification, 2 µL of GelRed® (Unisciense, São Paulo, SP, Brazil) and 4 µL of buffer (composed of 0.25% bromophenol blue, 30% glycerol, and water) were added to 9 µL of each amplified sample, subjected to agarose gel electrophoresis using a 1.5% gel concentration, and ran for 1 h and 10 min (70 Volts). The result was visualized under ultraviolet light using a transilluminator. A molecular marker of 100 bp (Invitrogen) was used to check the size of the amplified products.

Statistical analysis

Infection's frequency was calculated using the formula of prevalence for the three species of *Mycoplasma* in the studied population. The sex and age were analyzed through simple and relative frequencies, and a binomial test was used to determine the influence of age in hemoplasma infection. Fisher's test was used to check the correlation between sex and infection. Analysis of variance was used to compare the hematological parameters between groups, followed by the Student test (t-test).

RESULTS AND DISCUSSION

Hemotropic mycoplasma infection in cats has been described in several regions of Brazil, including Porto Alegre (SANTOS *et al*., 2014), Belém (SINEREY *et al*., 2013), Rio de Janeiro (MACIEIRA *et al*., 2008) with occurrences of 21.4%, 19.9%, and 16.1%, respectively, whith similar results as this study, in which of the 192 animals, 28 were positive for at least one species of hemoplasma; with a prevalence of 14.6%. The most prevalent species was *'Candidatus* M. haemominutum' (7.83%), followed by *Mycoplasma haemofelis* (4.17%) and '*Candidatus* M. turicensis' (1.56%). Co-infection by two species of hemotropic mycoplasmas were found in two cats: '*Candidatus* M. haemominutum' and *Mycoplasma haemofelis* (0.52%); and '*Candidatus* M. turicensis' and '*Candidatus* M. haemominutum' (0.52%).

Three species of hemoplasmas in cats were diagnosed in this study, however, comparisons to other studies must be taken with caution, given the differences in populations studied and diagnostic techniques. Similar for this study, these used species-specific PCR. In the study in Rio de Janeiro (MACIEIRA *et al*., 2008), the population of cats was like the study herein (hospital population). In the studies from Belem (SINEREY *et al*., 2013), and Porto Alegre (SANTOS *et al*., 2014), the prevalence of hemoplasmas in a hospital population only was 46/231 (19.91%), in Belem 10/38 (26.3.7%). The higher prevalence of the study in Belem could be due to geographic differences or due to the low sample size. Nevertheless, the prevalence of hemoplasmas affecting cats seems to vary minimally between studies with similar population and methods.

Also like this study, other researchers describe '*Candidatus* M. haemominutum' as the most prevalent species in cats (MACIEIRA *et al*., 2008; TANAHARA *et al*., 2010; SANTOS *et al*., 2014; CETINKAYA *et al*., 2016). An explanation for the absence of

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anemia in most infected animals in this research may be that this species, as well as the '*Candidatus* M. turicensis*',* has low pathogenicity and can maintain asymptomatic and chronic infections (BIONDO *et al*., 2009; ROSENQVIST *et al*., 2016). Some authors claim that the absence of anemia may be linked to chronic disease, mainly in animals affected by *M. haemofelis* (more pathogenic species). According to literature, at that stage of disease, the relationship between bacteria-host appears to strike a balance, in which the replication agent is stabilized by phagocytosis and eliminated from the body, causing a low bacteremic load (SANTOS *et al*., 2014; DUARTE *et al*., 2015). Thus, the hematological disorders found in only three cats can be explained by the presence of concomitant diseases, such as chronic renal failure, found in two animals, and liver disease, found in one animal (SINEREY *et al*., 2013; SANTOS *et al*., 2014). Although co-infections are contributing factors to develop anemia, the two animals, one co-infected with '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' and the other with *M. haemofelis* and '*Candidatus* M. haemominutum', showed no clinical and laboratory abnormalities.

In Brazil, the most common method performed for diagnosis of hemoplasma infection performed is through the cytological examination of blood smears, which has low sensitivity and specificity, and is unable to distinguish between the hemoplasma species. In addition, due to its small size, '*Candidatus* M. turicensis' was never diagnosed using this method (TASKER, 2004; WILLI *et al*., 2005). In the present study, it wasn't detected structures morphologically compatible with hemoplasmas in cytological examination of the blood smears, confirming its low sensitivity. The low bacteremia found in the chronic stages of infection and the use of EDTA (ethylenediaminetetraacetic acid) in the routine laboratory, which promotes the detachment of hemoplasma on the surface of red blood cells, likely contributed to the false-negative results by optical microscopy (SYKES, 2010; TASKER, 2010). Information of sex and age in cats infected with hemoplasmas are described in Tab. 01.

Table 01: Number of cats infected by hemotropic mycoplasma, according to sex and age.

	CMhm	MhF	CMt	CMhm/CMt	CMhm/MhF	Total
Infected Cats	15	08	03	01	01	28
SEX						
Male	10	07	02	01	01	21
Female	06	00	01	00	00	07
AGE						
≤3 years	10	06	02	00	01	19
>3 years	05	02	01	01	00	09

Note: *C*Mhm: *'Candidatus* Mycoplasma haemominutum; MhF: *Mycoplasma haemofelis*; *C*Mt: '*Candidatus* Mycoplasma turicensis'.

Among analyzed samples, 51.5% (99/192) were males and 48.5% (93/192) were females where 75% (21/28) of infected samples were males and 25% (7/28) were females,

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indicating a significant difference between sex and infection by hemoplasma in cats (p=0.0036). Males were significantly more affected by hemoplasma than females. The DNA of hemotropic mycoplasma has been found in saliva, salivary glands, oral mucosa, and feces of cats; therefore, it can be transmitted by direct contact with other animals. Furthermore, the development of experimental infection by inoculating a small volume of blood suggests that aggressive contact between cats could play a role in the transmission of these organisms (WILLI *et al*., 2007; DEAN *et al*., 2008; MUSEUX *et al*., 2009; BENNETT *et al*., 2011). Thus, males might become more susceptible to infection because they are usually of more aggressive behavior. Additionally, wounds and abscesses due to biting and scratching are described as risk factors associated with infection (TASKER, 2010).

The overall age of study population ranged from 1 to 18 years (mean of 5.75±5.16 years), being 58.4% (112/192) of animals ≤3 years old and 41.6% (80/192) >3 years old. The total number of positive animals with age ≤3 years old was 67.85% (19/28); and 32.15% (9/28) with age >3 years old; thus, a significant difference concerning age and the presence of infection in cats was also observed (p=0.0285). Presence of infection in younger animals may be associated with immunological immaturity of young animals, with confers a higher susceptibility to infections (SYKES, 2008; TASKER, 2010; DUARTE *et al*., 2015). Besides, it's suggested that transplancetary transmission may cause a higher number of young animals infected (HARBUTT, 1963; HORNOK *et al*., 2011; GIROTTO-SOARES *et al*., 2016). On the other hand, some authors reported that older cats are at higher risk of infection, likely due to the chronic nature of disease combined with more exposure time (SANTOS *et al*., 2014). Santos *et al*. (2014) showed that older male cats (greater than 10-years-old), intact and with outdoor access were more likely to be infected. Outdoor access and neutered status were not investigated herein, which could explain the differences in the age discrepancies. Also, the age grouping was distinct. The hematological variables of infected and uninfected cats are described in Tab. 02.

Table 02: Mean and standard deviation of hematological parameters from the cats infected with hemotropic mycoplasmas.

Variables*	Uninfected cats	Infected cats	RI
Erythrocytes (x10^6/µL)	7.30±1.69	7.53±2.19	5.0 – 10.0
Hemoglobin (g/dL)	10.58±2.60	10.63±2.96	8.0 – 15.0
PVC (%)	32.62±7.76	32.34±9.13	24.0 – 45.0
MCV (fL)	44.78±5.23	43.19±4.37	39.0 – 55.0
MCHC (%)	32.31±1.79	32.87±1.12	31.0 – 35.0

Note: mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC); reference interval (RI). *There was no statistical difference between groups for all variables studied.

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While in Tab. 03 the hematological variables of the 3 infected animals that presented anemia can be observed.

There was no significant difference ($p>0.05$) between uninfected and infected cats regarding hematological variables. As in the present study, many other works describe '*Candidatus* M. haemominutum' as the most prevalent species (MACIEIRA *et al*., 2008; TANAHARA *et al*., 2010; SANTOS *et al*., 2014). This may explain the absence of anemia in most positive animals in this study, as this agent, like '*Candidatus* M. turicensis', has low pathogenicity and is able to maintain infection without leading to the development of serious disease (BIONDO *et al*., 2009). In addition, some authors state that the absence of anemia may be linked to the presence of chronic carriers, especially in animals affected by *M. haemofelis*, a more pathogenic species, since at this stage the parasite-host relationship seems to reach equilibrium, in which replication of the agent is stabilized by phagocytosis and organism elimination, making the parasite burden low (SANTOS *et al*., 2014; DUARTE *et al*., 2015). Thus, the hematological alterations found in only 3 cats can be explained by the presence of concomitant diseases in these animals, since two cats had chronic renal failure and one liver disease, both diseases are capable of leading to the development of anemia (SINEREY *et al*., 2013; SANTOS *et al*., 2014).

Table 03: Hematological variables of the three anemic cats. These cats were infected by *Mycoplasma haemofelis* (MhF), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt)**.**

Variables	CMhm	MhF	CMt	RI
Diagnosis				
Erythrocytes (x10^6/µL)	1.9	4.86	2.59	5.0 – 10.0
Hemoglobin (g/dL)	2.7	5.5	3.7	8.0 – 15.0
PVC (%)	8.1	16.0	11.3	24.0 – 45.0
MCV (fL)	43.3	32.9	43.8	39.0 – 55.0
MCHC (%)	33.3	34.3	32.7	31.0 – 35.0

Note: mean corpuscular volume (MCV); mean corpuscular hemoglobin concentration (MCHC); reference interval (RI); chronic renal failure (CRF); and liver disease (LD).

CONCLUSION

Hemoplasm's infecctions in cats are present in the central region of Rio Grande of Sul, Brazil, with an overall prevalence of 14.6% in the population studied and '*Candidatus* M. haemominutum' is the most prevalent species. Younger and male cats were identified as risk factors for hemoplasma infection. Relationship between mycoplasma infection and anemia was not observed in this population.

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