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Hemoplasma prevalence and hematological abnormalities associated with infection in three different cat populations from Southern Brazil

Prevalência da infecção por hemoplasmas e alterações hematológicas associadas à infecção em três diferentes populações de gatos do sul do Brasil

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Abstract

Three hemoplasma species are recognized in domestic cats: *Mycoplasma haemofelis,* '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis'. We report the prevalence and hematological abnormalities of hemoplasma infection in 369 domestic cats from three different populations (blood donors, hospitalized cats and shelter cats) from Southern Brazil. Complete blood counts were performed at the time of blood collection, and DNA was extracted and tested by conventional PCR for each hemoplasma species. A total of 79 samples (21.40%) were positive for at least one species. The most prevalent hemoplasma was '*Candidatus* Mycoplasma haemominutum', with 50/369 (13.55%) positive cats, followed by '*Candidatus* Mycoplasma turicensis', 10/369 (2.71%), and *Mycoplasma haemofelis*, 8/369 (2.16%). *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum' coinfection was observed in 4/369 (1.08%), whereas '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis' in 5/369 (1.35%). Three cats (0.81%) were infected with all three hemoplasmas. There was no association between infection and the different populations. Anemia was associated with *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum', but not with '*Candidatus* Mycoplasma turicensis'. Male cats and cats with outdoor access were more likely to be infected. Although '*Candidatus* Mycoplasma haemominutum' is believed to cause minimal or no hematological alterations, the infected cats studied herein were more likely to be anemic.

Keywords: Hemotropic mycoplasma, hemoplasma, anemia, PCR, cats.

Resumo

Três espécies de hemoplasmas são reconhecidas em gatos domésticos: *Mycoplasma haemofelis*, '*Candidatus* Mycoplasma haemominutum' e '*Candidatus* Mycoplasma turicensis'. A prevalência e alterações hematológicas associadas à infecção por hemoplasmas foi estudada, em 369 gatos domésticos de três populações distintas (doadores de sangue, hospitais e gatos de abrigo) do Sul do Brasil. Foram realizados hemogramas completos no momento da coleta de sangue e as amostras tiveram seu DNA extraído e testado por PCR convencional para cada espécie de hemoplasmas. Setenta e nove amostras (21,40%) foram positivas para pelo menos uma espécie. O mais prevalente foi '*Candidatus* Mycoplasma haemominutum' com 50/369 (13,55%) gatos positivos, seguidos por '*Candidatus* Mycoplasma turicensis' com 10/369 (2,71%) e *Mycoplasma haemofelis* com 8/369 (2,16%). Coinfecção por *Mycoplasma haemofelis* e '*Candidatus* Mycoplasma haemominutum' foi observada em 4/369 (1,08%), enquanto '*Candidatus* Mycoplasma haemominutum'

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e '*Candidatus* Mycoplasma turicensis' coinfectaram 5/369 (1,35%) gatos. Três (0,81%) gatos apresentaram infecção pelos três hemoplasmas. Não houve associação entre a infecção e as diferentes populações. Anemia foi associada com a infecção por *Mycoplasma haemofelis* e '*Candidatus* Mycoplasma haemominutum', mas não com '*Candidatus* Mycoplasma turicensis'. Gatos machos e com acesso à rua apresentaram maior probabilidade de serem infectados. Embora se acredite que '*Candidatus* Mycoplasma haemominutum' possa causar alterações hematológicas mínimas ou ausentes, gatos infectados encontrados neste estudo foram mais propensos à anemia.

Palavras-chave: Mycoplasma hemotrópico, hemoplasma, anemia, PCR, gatos.

Introduction

Hemotropic mycoplasmas (hemoplasmas) are pleomorphic obligate red blood cell parasites that infect a wide range of vertebrates (MESSICK, 2004). These microorganisms were classified within the order Rickettsialles and were known as *Haemobartonella* spp. and *Eperythrozoon* spp. However, phylogenetic analyses of their 16S rRNA gene sequences have shown them to be most closely related to *Mycoplasma* species (RIKIHISA et al., 1997; NEIMARK et al., 2001; MESSICK et al., 2002; NEIMARK et al., 2004; NEIMARK et al., 2005). Thus, they have been transferred to the Mycoplasmataceae family, where they form a unique clade of red blood cell bacteria within the *Mycoplasma* genus.

Three hemoplasmas have been described infecting domestic and wild cats: *Mycoplasma haemofelis* (formerly known as *Haemobartonella felis* – Ohio organism or large form, Mhf) (MESSICK et al., 1998), '*Candidatus* Mycoplasma haemominutum' (formerly known as *Haemobartonella felis* – California organism or small form, CMhm) (FOLEY et al., 1998), and '*Candidatus* Mycoplasma turicensis' (CMtc) (WILLI et al., 2005). All have been detected in Brazilian domestic cats by molecular methods (GUIMARÃES et al., 2005; de MORAIS et al., 2007; MACIEIRA et al., 2008; SANTOS et al., 2009; de BORTOLI et al., 2012).

Acute Mhf infection is associated with severe hemolytic anemia and, if left untreated, may result in death. Despite an intense immune response and/or antibiotic therapy, it is well recognized that cats may remain asymptomatic carriers (MESSICK, 2004). On the other hand, CMhm causes minimal clinical signs and is not associated with mortality. Likewise, the hematological abnormalities are minor, and anemia, if present, can develop particularly with concurrent diseases or coinfections (FOLEY et al., 1998). The newest species, CMtc, might be a potential causative agent of anemia (WILLI et al., 2005).

Diagnosis of infection has historically relied on microscopic identification of bacteria attached to the red blood cells. This method is neither sensitive nor specific. Thus, polymerase chain reaction (PCR)-based assays are the diagnostic method of choice. Several conventional and quantitative PCR assays for each of the three feline hemoplasmas have been developed (FOLEY et al., 1998; BERENT et al., 1998; MESSICK et al., 1998; JENSEN et al., 2001; TASKER et al., 2003a; WILLI et al., 2006a; FUJIHARA et al., 2007; SYKES et al., 2007; PETERS et al., 2008; SANTOS et al., 2009; TASKER et al., 2010).

Feline hemoplasmas are distributed worldwide (JENSEN et al., 2001; CRIADO-FORNELIO et al., 2003; TASKER et al., 2003b; WATANABE et al., 2003; TASKER et al., 2004; WILLI et al., 2006a; WILLI et al., 2006b; FUJIHARA et al., 2007; JUST & PFISTER, 2007; SYKES et al., 2008; SANTOS et al., 2009; BIONDO et al., 2009). Nevertheless, no comprehensive studies in Southern Brazil have been conducted. Therefore, the aim of this study was to evaluate the prevalence and hematological abnormalities associated with hemoplasma infection in three different populations of cats from Southern Brazil.

Materials and Methods

A total of 369 domestic cats were included in this study. Blood samples were obtained from cats registered as blood donors at the Veterinary Hospital of the Federal University of Rio Grande do Sul, Porto Alegre, Rio Grande do Sul, Brazil (n=118); cats admitted to this same hospital during a six-month period (n = 231); and cats housed in a local shelter (n = 20). Data regarding the animals' gender, age and outdoor access were collected. All the shelter animals were considered to have outdoor access. Cats undergoing antimicrobial therapy were excluded from the study. All procedures were approved by the Veterinary Research Committee under project number 11234.

Red blood cell (RBC) count, packed cell volume (PCV), hemoglobin concentration (Hg), total leukocyte count (WBC) and total plasma protein concentration (TPP) were determined using standard methods at the Veterinary Clinical Pathology Laboratory (LACVet) at the Federal University of Rio Grande do Sul, Porto Alegre, Brazil.

DNA was extracted from whole EDTA-blood using a silica-based protocol (BOOM et al., 1990) and was stored at -20 °C until PCR was performed. Ultrapure water was used as negative control in each batch of ten samples. A conventional PCR for the detection of the feline 28S rRNA gene (SANTOS et al., 2009) was used to confirm the presence of amplifiable DNA in the extracted samples.

Previously described PCR assays for the detection of Mhf, CMhm, and CMtc were performed (BERENT et al., 1998; FOLEY et al., 1998; SANTOS et al., 2009). The PCR detection limit was defined as the smallest number of copies of recombinant plasmids containing the nearly entire 16SrRNA gene of each feline hemoplasma. Plasmid controls were provided by the Hemoplasma Laboratory at Purdue University, West Lafayette, IN, USA. Standard curves were constructed by means of tenfold serial dilutions (10^9 to 1 copy of plasmid/reaction) for each plasmid diluted in 1 × TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0) combined with 30 mg mL^{-1} of herring sperm DNA (Sigma-Aldrich Corp., St. Louis, MO, USA). For each reaction, positive controls included DNA extracted from the blood of cats naturally infected with each hemoplasma. Negative controls included DNA extracted from a non-infected cat and autoclaved ultrapure water. The amplification products were subjected to electrophoresis in 1.5%

agarose gel for one hour at 80 V, followed by ethidium bromide staining (1 mg/mL) and visualized under UV light. The gels were subsequently photographed using Epi Chemi II Darkroom® (UVP Inc., Upland, CA, USA). A 100 bp DNA ladder (Invitrogen, Carlsbad, CA, USA) was used to compare product sizes.

To confirm the identity of positive samples, the nearly entire sequence of the 16S rRNA gene was obtained using newly designed specific primers for Mhf (DEAMHF F1 5' - ATG CAA GTC GAA CGG ATC TT - 3' and DEAMHF R2 5' - TCC AAT CAG AAT GTT CAC TC - 3'); CMtc (DEAMTC F1 5' - CTG TCC AAA AGG CAG TTA GC - 3' and DEAMTC R1 5' - TGC CCC TTC CTC TCA TAG TTT - 3'), and the forward primer for CMhm DEAMHM F1 (5' - ATG CAA GTC GAA CGA AGA GG - 3') in combination with the primer CALI R2 designed by Foley et al. (1998). The reactions consisted of a PCR mixture of 1X Green GoTaq®Flexi buffer (Promega, Madison, WI, USA), 2.0 mM of $MgCl_2$, deoxynucleoside triphosphates (dNTPs) at a concentration of 200 µM, primers at a concentration of 0.4 µM, 1.25 U of GoTaq® Flexi DNA Polymerase, template DNA (5 ml), and autoclaved ultrapure water to a total volume of 25 µL per reaction. The PCRs were carried out in an Eppendorf® Mastercycler® gradient thermocycler (Eppendorf Scientific, Inc., Westbury, NY, USA), and consisted of one cycle of 95 °C for 2 min; 35 cycles of 94 °C for 1 min, 53 °C for 45 s, and 72 °C for 1.5 min; and one final cycle of 72 °C for 5 min. The purified amplicons (Zymoclean Gel DNA Recovery Kit, Zymo Research, Orange, CA, USA) were cloned into the pGEM®-T EasyVector (Promega, Madison, WI, USA). Plasmids were purified using a QIAprep Spin Miniprep kit (QIAGEN, Valencia, CA, USA) and submitted to sequencing at the Purdue Genomics Core Facility, Purdue University, West Lafayette, IN, USA. A representative sequence of each hemoplasma was submitted to the GenBank database (BENSON et al., 1998).

Comparisons of frequencies between groups (shelter, blood donors, and hospital patients) were performed using Fisher's exact test. For the hematological analyses, distribution normality was evaluated through the Kolmogorov-Smirnov test. The three groups were pooled together and the non-parametric Kruskal-Wallis and Mann-Whitney U tests were used to evaluate the association between hematological findings (PCV, RBC, TPP, WBC and Hg) and hemoplasma infection. The analyses of the associations between infection and gender, age (groups: 0-5, 6-10 and > 10 years old) and outdoor access were conducted using the chi-square test. The results were considered statistically significant when $P \leq 0.05$. Gender was known for 317/369 (85.91%) of the cats, age for 107 (29%) and outdoor/indoor status for 99 (27%). The data analysis for this paper was generated using SAS software, Version 8 (SAS Institute, 2011). SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.

Results

Each PCR assay consistently detected as few as 10 copies of plasmid/reaction when using the plasmid standards. A total of 21.40% (79/369) of cats were infected with at least one species of hemoplasma. PCR amplification products of the predicted sizes were obtained for Mhf (393 bp), CMhm (192 bp) and CMtc (488 bp) in 18/369, 50/369, and 10/369 of samples, respectively. Four cats were co-infected with Mhf and CMhm, five with CMhm and CMtc, and three with the three species of hemoplasmas. All the samples were positive for the detection of the feline 28S rRNA gene. Figure 1 shows the prevalence of each hemoplasma species in the three populations evaluated and Table 1 shows the number of infected cats per group.

Although hemoplasma infection seemed to be somewhat higher in shelter cats (30.0%) when compared to blood donors and the hospital population (22.8% and 19.9%, respectively), the prevalence of infection did not vary ($P = 0.5126$). When considering the different mycoplasma species involved, the difference among groups was also not statistically significant ($P = 0.7326$).

The 16S rDNA sequences were submitted to the GenBank database under the accession numbers EU930823 (*M. haemofelis*),

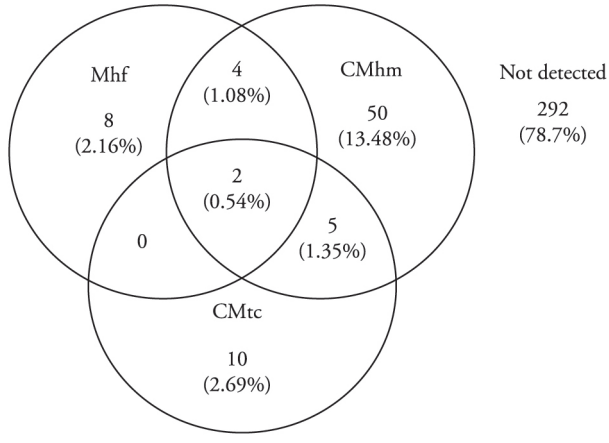

Figure 1. Prevalence of hemoplasma infection in 369 domestic cats from southern Brazil. Mhf = *Mycoplasma haemofelis*, CMhm = '*Candidatus* Mycoplasma haemominutum'; CMtc = '*Candidatus* Mycoplasma turicensis'.

Table 1. Number of hemoplasma infected cats per group (blood donors, hospital cats, shelter cats).

	Infection [number of positive cats (% related to the group)]								
Group	**Mhf**	**CMhm**	**CMtc**	**Mhf/CMhm**	**CMhm/ CMtc**	**Mhf/CMhm/ CMtc**	**Non-infected**	***Infected**	**Total**
Blood donors	3 (2.54%)	18 (15.25%)	4 (3.39%)	1 (0.85%)	1 (0.85%)	0	91 (77.12%)	27 (22.88%)	118
Hospital	4 (1.73%)	28 (12.12%)	6 (2.59%)	3 (1.3%)	3 (1.3%)	2 (0.87%)	185 (80.09%)	46 (19.91%)	231
Shelter	1 (5%)	4 (20%)	0	0	1 (5%)	0	14 (70%)	6 (30%)	20
Total	8 (2.16%)	50 (13.55%)	10 (2.71%)	4 (1.08%)	5 (1.36%)	2 (0.54%)	290 (78.6%)	79 (21.4%)	369

*Mhf and CMtc co-infection was not observed.

FJ004275 ('*Candidatus* M. haemominutum') and EU861063 ('*Candidatus* M. turicensis').

Infected cats showed significantly lower PCV, Hg and RBC values when compared to non-infected cats ($P = 0.006$, $P < 0.004$, $P < 0.0001$, respectively). PCV values are shown in Figure 2. The means ± standard deviations for hematological parameters among the clinically normal cats that were used for reference intervals were: erythrocytes ($x10^6/\mu L$) 8.41 ± 1.37, PCV (%) 38.17 ± 5.67, leukocytes (/μL) 12,719.29 ± 4,359.64, and total plasma proteins (g/L) 73.87 ± 7.44. When animals infected with each hemoplasma species individually were compared with the non-infected animals, we found that cats infected with Mhf or CMhm had significantly lower PCV, RBC and Hg ($P \leq 0.05$). On the other hand, cats infected with CMtc did not show significantly lower PCV, RBC or Hg in relation to non-infected animals. No statistical difference was observed regarding WBC or TPP.

The infection status was associated with gender, age and outdoor access. Infected cats were more likely to be male ($P = 0.0013$) and 10 years of age or older ($P = 0.018$). In addition, intact males were more likely to be infected than neutered males ($P = 0.0025$), and cats with outdoor access were 3.9 times more likely to be infected ($P = 0.0407$).

Discussion

The present study revealed that 21.40% of the domestic cats tested in Porto Alegre, Southern Brazil are infected with at least one species of hemoplasma. Hemoplasma prevalence comparisons among different studies should be cautiously performed because differences in animal populations and diagnostic techniques are likely to influence the results. In addition, the fact that not all hemoplasmas are evaluated in every study further confounds the ability to compare these results.

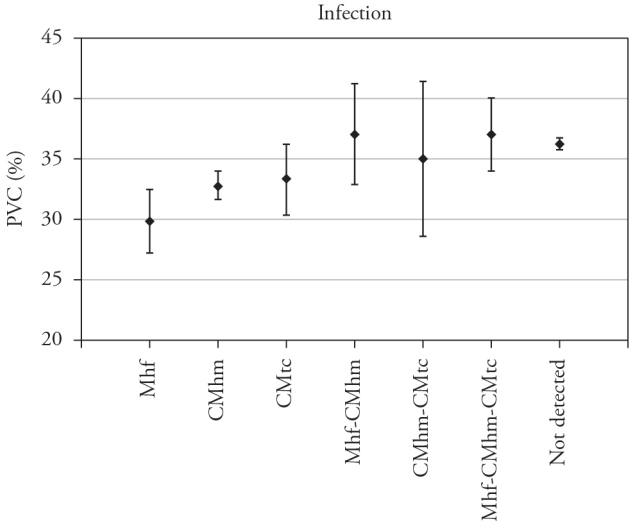

Figure 2. PCV values (Mean ± SE) of cats grouped by hemoplasma infection status. Mhf = *Mycoplasma haemofelis*, CMhm = '*Candidatus* Mycoplasma haemominutum'; CMtc = '*Candidatus* Mycoplasma turicensis', Mhf-CMhm = co-infection with *Mycoplasma haemofelis* and '*Candidatus* Mycoplasma haemominutum', CMhm-CMtc = co-infection with '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* Mycoplasma turicensis', Mhf-CMhm-CMtc = co-infection with the three hemoplasmas, Not detected = cats tested negative for all three hemoplasmas.

Our results are in agreement with studies in the United Kingdom and Germany, with prevalences of 21.12% (WILLI et al., 2006b) and 22.5% (BAUER et al., 2008), respectively. However, the prevalence reported herein was higher than that of blood samples submitted to a diagnostic laboratory in the United Kingdom (14%) (PETERS et al., 2008) and a population of healthy and unhealthy Swiss cats (9.92%) (WILLI et al., 2006a). Geographical variations regarding seasonality of potential vectors in Porto Alegre, which is a subtropical location, might in part explain the higher prevalence observed (WILLI et al., 2006a). On the other hand, the prevalence in our study was lower than reported for cats in Australia (32%), South Africa (52%) (WILLI et al., 2006b), Japan (26%) (TANAHARA et al., 2010) and Portugal (43%) (MARTÍNEZ-DÍAZ et al., 2013). Although conventional PCR is generally less sensitive than quantitative PCR, the sensitivity of the assays performed herein was relatively high and therefore it is unlikely that methodological differences are solely responsible for these disparate findings. A more likely explanation for the higher prevalence in these studies is that the cat populations are very distinct.

The most prevalent species was CMhm (13.55%), in agreement with all of the prevalence studies cited above. This implies that CMhm is the most common species of hemoplasma infecting cats in Brazil, as well as in many other countries. CMtc infection alone was slightly more frequent than Mhf (2.71% and 2.17% respectively).

We also found that 1.08% of the cats were coinfected with Mhf and CMhm, 1.36% with CMhm and CMtc, and 0.54% with the three species of feline hemoplasmas. Concurrent infection with more than one hemoplasma has been previously reported (JENSEN et al., 2001; WESTFALL et al., 2001; TASKER et al., 2004; LOBETTI & TASKER, 2004; LURIA et al., 2004; WILLI et al., 2006a; WILLI et al., 2006b; de MORAIS et al., 2007).

There was no significant difference in hemoplasma prevalence between the three populations of cats. This finding is in line with a similar study on cats in Switzerland (WILLI et al., 2006a) and could imply that the pathogenic potential of the bacteria is low. In addition, the presence of hemoplasmas in the blood of healthy cats could represent chronic infection, which is not usually associated with anemia (BARKER & TASKER, 2013). Shelter cats were presumably more exposed to infection, but we did not find any significant difference in their infection prevalence. However, the small number of cats from shelters included in the study might have been insufficient to demonstrate statistical difference, in accordance with the study by Nibblett et al. (2010).

Several studies have indicated that hemoplasma infection is a predisposing condition for anemia (GRINDEM et al., 1990; JENSEN et al., 2001; HARRUS et al., 2002; TASKER et al., 2003b). However, some other studies did not find this association (WILLI et al., 2006a; MACIEIRA et al., 2008); this can be explained by the presence of chronic carriers, with no RBC abnormalities, among the animals tested.

Significant differences were observed when comparing the RBC, Hg and PCV of the non-infected and infected cats with Mhf and CMhm. The association between anemia and CMhm infection was somewhat surprising, since CMhm alone is not associated with the development of anemia (FOLEY et al., 1998; WESTFALL et al., 2001; SYKES et al., 2007). However, few studies have reported decreases in PCV values in cats infected only with CMhm (TASKER et al., 2006; REYNOLDS & LAPPIN, 2007), thus indicating that this microorganism may have some virulence in experimental and naturally infected cats. According to our findings, we can hypothesize that: a different (more pathogenic) strain may be infecting cats in Southern Brazil, differences in the host-parasite relationship caused by seasonal and/or geographical conditions may be occurring, and/or coinfections with other pathogens or other concurrent diseases not studied herein could be present.

There was no association between anemia and CMtc infection in this study, which was in agreement with the findings from the study by Willi et al. (2006b). However, in another study, CMtc induced mild to marked anemia in two experimentally infected cats (WILLI et al., 2005), thus suggesting that different clinical signs may develop in specific cases, including the high copy numbers of organisms used for experimental studies. Although CMhm and CMtc are not associated with induction of anemia or clinical signs, both of them can be associated with small decreases in PCV, which suggests that they have an effect on RBC parameters (TASKER et al., 2009). Interestingly, cats coinfected with more than one species of hemoplasmas did not show any association with anemia. However, the low number of observations could have affected these results.

Male cats were more likely to be infected, and a significant difference was observed between cats that had outdoor access and those that lived indoors. The higher prevalence among intact male cats with outdoor access supports the hypothesis of horizontal transmission. Although chronic carriers may not develop recurrent bacteremia even if immunocompromised (NOVACCO et al., 2011), detection of hemoplasma DNA in saliva and feces from cats and development of infection following subcutaneous inoculation of contaminated blood suggest that aggressive interactions may play an important role in transmission of these microorganisms (WILLI et al., 2007; MUSEUX et al., 2009). In addition, Grindem et al. (1990) found an association between the presence of abscesses caused by cat bites and hemoplasma infection. Several studies have also reported that older cats are more likely to be infected with hemoplasmas (WILLI et al., 2006a; TANAHARA et al., 2010; BAUER et al., 2008). The association between infection and older age might be a reflection of the chronic state of the disease, in which animals can become infected and not develop the disease, thus remaining subclinical carriers for years. Cats infected with *Mycoplasma haemofelis* may be carriers for this organism and may demonstrate clinical signs indicative of Mhf infection at the time of initial infection.

In conclusion, hemoplasma infection is common in domestic cats in Southern Brazil. CMhm infection shown to be the most prevalent and was associated with anemia. Male gender, older age and outdoor exposure were associated with hemoplasma infection.

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