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Molecular detection of hemotrophic mycoplasmas among domiciled and free-roaming cats in Campo Grande, state of Mato Grosso do Sul, Brazil

Detecção molecular de micoplasmas hemotróficos em gatos domiciliados e errantes em Campo Grande, estado do Mato Grosso do Sul, Brasil

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Abstract

Hemoplasmas are bacteria living in feline red blood cells. Feline hemoplasmosis is frequently associated with old male cats that have access to the streets. This study aimed to detect the presence of hemoplasma speciess in domiciled and free-roaming cats in Campo Grande, state of Mato Grosso do Sul (MS), Brazil, using molecular techniques. Between January 2013 and April 2013, EDTA-whole blood samples were collected from 151 domestic cats (65 free-roaming and 86 domiciled cats). Samples were subjected to PCR assays targeting hemoplasmas 16S rRNA, followed by sequencing, BLAST analysis and phylogenetic analysis. Results show an occurrence of 36.4% for hemoplasmas. Twenty-three cats (15.2%) were positive for '*Candidatus* Mycoplasma haemominutum', 17 (11.2%) for *M. haemofelis* and 15 (9.9%) for '*Candidatus* M. turicensis', from PCR. Coinfection by two or three hemoplasmas was found in 25 cats (16.6%). No statistically significant difference between genders or between lifestyles was observed for the presence of hemoplasmas among the cats. Results show different hemoplasma species are present in cat population (Campo Grande, MS, Brazil). It is suggested that a differential diagnosis for feline hemoplasmosis should be made when cats show nonspecific clinical signs of disease with systemic manifestation.

Keywords: Feline hemoplasmosis, hemoplasmas, molecular diagnosis, central-western Brazil.

Resumo

Hemoplasmas são bactérias encontradas aderidas aos eritrócitos de felinos. A hemoplasmose felina está frequentemente associada a gatos velhos machos, sem raça definida e com acesso à rua. O presente estudo objetivou realizar a detecção molecular de espécies de hemoplasmas em gatos domiciliados e errantes em Campo Grande, estado do Mato Grosso do Sul (MS), Brasil. Entre janeiro/2013 e abril/2013, amostras de sangue foram colhidas de 151 gatos domésticos (65 errantes e 86 domiciliados) e avaliadas por PCR frente à presença de sequências do gene do 16S rRNA de hemoplasmas, seguidas de sequenciamento, análise pelo BLAST e análise filogenética. Os resultados deste estudo mostraram uma ocorrência de 36,4%. Vinte e três (15,2%) gatos mostraram-se positivos na PCR para '*Candidatus* Mycoplasma haemominutum', 17 (11,2%) para *Mycoplasma haemofelis*, e 15 (9,9%) para '*Candidatus* Mycoplasma turicensis'. A co-infecção por dois ou três hemoplasmas ocorreu em 25 gatos (16,6%). Não foi observada diferença estatística significativa entre sexo e estilo de vida dos gatos amostrados e a presença de hemoplasmas. O estudo mostrou que diferentes espécies de hemoplasmas circulam na população de gatos (domiciliados e errantes) na cidade de Campo Grande, MS, Brasil. Sugere-se o diagnóstico diferencial para hemoplasmose felina em gatos que apresentam sinais clínicos inespecíficos de doença com manifestação sistêmica.

Palavras-chave: Hemoplasmose felina, hemoplasmas, diagnóstico molecular, centro-oeste brasileiro.

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Introduction

Hemotropic mycoplasmas (hemoplasmas) are bacteria of small size and genome that are found attached to the cytoplasmic membrane of red blood cells (TASKER, 2010). A range of hemoplasmas can infect cats: *Mycoplasma haemofelis*, '*Candidatus* M. haemominutum', '*Candidatus* M. turicensis' and '*Candidatus* M. haematoparvum' (TASKER, 2010; SYKES, 2010). Among feline hemoplasma species, *M. haemofelis* is mainly associated with clinical disease, often during in acute infection (TASKER, 2010). Cats infected by *M. haemofelis* often show prostation, inappetence and dehydration; in some clinical cases, weight loss is also reported. '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' may cause anemia only when concurrent disease is present and/or a condition of immunosuppression. Anemia, which is frequently found in hemoplasma-infected cats, is manifested by weakness, mucosal pallor, tachycardia, tachypnea and, occasionally, syncope or neurological signs (TASKER et al., 2009; SYKES, 2010).

Feline hemoplasma infection has been correlated with male gender, non-pedigree status, and access to the outdoors (SYKES, 2010). Although fleas have been incriminated as potential vectors for hemotropic mycoplasmas, experimental attempts to transmit hemoplasmas among cats by these arthropods were inconclusive (WOODS et al., 2006). Direct transmission through biting and fighting is emerging as a possible mode of transmission (SYKES, 2010).

The laboratory diagnosis of feline hemoplasmosis is based on blood smear examinations and PCR assays. False positive diagnoses are frequently found in cytological preparations containing stain precipitate, basophilic stippling and Howell-Jolly bodies. Also, fresh smears should preferentially be examined, because parasites may detach from erythrocytes in the presence of ethylenediaminetetraacetic acid (EDTA). PCR assays are significantly more sensitive than blood smear evaluation (SYKES, 2010).

Hemoplasma infections among cats have been reported in some areas in Brazil over the last decade (MACIEIRA et al., 2008; DE BORTOLI et al., 2012; BRAGA et al., 2012; MICELI et al., 2013). Because of the potential risk to human health (SANTOS et al., 2008), the present study aimed to clarify the epidemiology of hemoplasmas in domiciled and free-roaming cats in Campo Grande, state of Mato Grosso do Sul (MS), Brazil.

Material and Methods

Between January and April 2013, whole blood samples were collected by convenience from 151 cats (54 males, 95 females and two without gender registration) in the city of Campo Grande, which is the capital of the state of Mato Grosso do Sul, Brazil. Free-roaming non-domiciled cats (n = 65) were caught by technical staff from the local zoonosis control center (CCZ). Domiciled cats (n = 86) were sampled during pre-surgical procedures for a castration project at the CCZ; these animals were returned to their homes after surgery. Overall, the domiciled cats were in a better physical condition than the non-domiciled animals. The blood samples were collected in EDTA and stored at –20 °C until DNA extraction.

DNA was extracted from 200 µL of each whole blood sample using the QIAamp DNA blood mini-kit (QIAGEN®, Valencia, California, USA), in accordance with the manufacturer's instructions.

Each DNA sample was subjected to PCR assays targeting the 16S rRNA gene of *M. haemofelis* (393 bp) (BERENT et al., 1998), '*Candidatus* M. haemominutum' (130 bp) (FOLEY et al., 1998) and '*Candidatus* M. turicensis' (488 bp) (SANTOS et al., 2009). Samples that were positive for *M. haemofelis* and '*Candidatus* M. haemominutum' were subjected to another PCR protocol (CRIADO-FORNELIO et al., 2003), in which primers amplify a fragment of 600 bp. *Mycoplasma haemofelis*, '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' DNA obtained from blood samples from naturally infected cats in São Luis, Maranhão, Brazil (BRAGA et al., 2012) were used as positive DNA controls in PCR reactions for hemoplasmas. The reaction products were purified using the Silica Bead DNA gel extraction kit (Fermentas, São Paulo, SP, Brazil). Purified amplified DNA fragments from positive samples were subjected to sequencing for confirmation in an automatic sequencer (ABI Prism 310 genetic analyzer; Applied Biosystems/Perkin-Elmer) and were used for subsequent phylogenetic analysis. The phylogenetic reconstructions were based upon deoxyribonucleic acid. Consensus sequences were obtained through the analysis of the products from sequencing from both forward and reverse olygonucleotides using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py) (ALTSCHUL et al., 1990). Comparisons with sequences deposited in GenBank were made using the basic local alignment search tool (BLAST®). The CLUSTAL W software (THOMPSON et al., 1994) and MEGA software (KUMAR et al., 2004) were used for alignment and phylogenetic analysis, respectively. The maximum likelihood distance method was used to build the phylogenetic tree (SAITOU; NEI, 1987) using the Kimura two-parameter model. The bootstrap test with 1000 replications was repeated in order to estimate the confidence of the branching patterns of the neighbor-joining tree (FELSENSTEIN, 1985).

The variables (origin of the sampled cats [domiciled or free-roaming] and gender) were correlated with the hemoplasma-PCR results using the GraphPad Prism® software, version 5.00.288. For this purpose, we used the two-factor analysis of variance method followed by the Bonferroni test, taking the confidence interval to be 95%.

Results

PCR results showed an overall molecular occurrence of 36.4% (55/151). Twenty-three cats (15.2%) (11 domiciled and 12 free-roaming cats) were positive for '*Candidatus* M. haemominutum', 17 (11.2%) (nine domiciled and eight free-roaming) for *M. haemofelis* and 15 (9.9%) (nine domiciled and six free-roaming) for ´*Candidatus* M. turicensis´. Regarding coinfections by different hemoplasma species, we found that the occurrence rate was 16.6%: nine cats (5.9%) (four domiciled and five free-roaming) were coinfected by *M. haemofelis* and '*Candidatus* M. haemominutum'; six cats (3.9%) (four domiciled and two free-roaming) were coinfected by *M. haemofelis* and '*Candidatus* M. turicensis'; seven cats (4.6%) (four domiciled and three free-

roaming) were coinfected by '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis'; and three cats (1.9%) (one domiciled and two free-roaming) were coinfected by *M. haemofelis,* '*Candidatus* M. haemominutum' and '*Candidatus* M. turicensis' (Table 1). The sequencing followed by the phylogenetic analysis based on 16S rRNA (Table 2; Figure 1) confirmed that the sampled cats were parasitized by *M. haemofelis*, *Candidatus* M. haemominutum and *Candidatus* M. turicensis. No statistically significant difference between genders or lifestyles among the sampled cats was correlated with positivity in PCR assays for hemoplasmas ($P > 0.05$).

Discussion

Although hemoplasma infection has been documented in cats in several regions of Brazil (DE MORAIS et al., 2007; MACIEIRA et al., 2008; SANTOS et al., 2009; BRAGA et al., 2012; DE BORTOLI et al., 2012; MICELI et al., 2013), few reports clarifying actual epidemiology have yet been published (BIONDO et al., 2009). These organisms have been reported in cats in the states of Paraná (three cats showing clinical signs of hemoplasmosis) (DE MORAIS et al., 2007), Rio de Janeiro (cats attended at veterinary clinics) (MACIEIRA et al., 2008), Rio Grande do Sul (non-hospitalized pet cats screened for blood donation and feral cats housed in a local shelter) (SANTOS et al., 2009), Maranhão (domiciled cats with outdoor access) (BRAGA et al., 2012), São Paulo (domiciled cats subjected to a spaying/neutering program) (DE BORTOLI et al., 2012) and Mato Grosso (cats housed in animal shelters) (MICELI et al., 2013). Among wild carnivores, hemotrophic mycoplasmas have been molecularly detected in wild felids maintained in captivity in zoos and institutions in the Federal District (ANDRÉ et al., 2011) and the states of Paraná (GUIMARÃES et al., 2007) and São Paulo (ANDRÉ et al., 2011).

The most prevalent hemoplasma found in the present study was '*Candidatus* M. haemominutum'. In most studies around the world, '*Candidatus* M. haemominutum' has been the most common hemoplasma found, followed by '*Candidatus* M. turicensis' and *M. haemofelis* (SYKES et al., 2007a, b). Herein, the molecular occurrence of *M. haemofelis* was higher than that found for '*Candidatus* M. turicensis'. The rates of occurrence of feline hemoplasmas vary greatly between different populations (healthy vs. sick; feral vs. pet) (BARKER; TASKER, 2013): *M. haemofelis* occurrence has ranged from 0.4 to 46.6% (SYKES et al., 2007a; KAMRANI et al., 2008), '*Candidatus* Mycoplasma haemominutum' from 8.1 to 46.7% (FUJIHARA et al., 2007; ROURA et al., 2010), '*Candidatus* Mycoplasma turicensis' from 0.1 to 26% (WILLI et al., 2006b; KAMRANI et al., 2008) and '*Candidatus* M. haematoparvum'-like from 0 to 0.7% (SYKES et al., 2007a). Since the protocol described by Foley et al. (1998) does not not allow to discriminate between '*Candidatus* Mycoplasma haemominutum' and '*Candidatus* M. haematoparvum', some non-sequenced samples showing positive results in this protocol

Table 1. Number of cats with single or multiple hemoplasma infections by PCR according to origin and gender in Campo Grande, State of Mato Grosso do Sul, Brazil.

Hemoplasma	Domiciled cats		Free-roaming cats		Total (%)
	Male	Female	Male	Female	
Mf	5	4	2	6	17 (11.3)
CMhm	8	3	5	7	23 (15.2)
CMt	4	5	4	2	15 (9.9)
					55 (36.4)
Co-infection:					
Mh + CMhm	3	1	1	4	9 (6)
Mh + CMt	1	3	1	1	6 (4)
CMhm + CMt	3	1	2	1	7 (4.6)
Mf +CMhm + CMt	0	1	1	1	3 (2)
					25 (16.6)

Mf = Mycoplasma haemofelis; CMhm= *'Candidatus* Mycoplasma haemominutum'; CMt= *'Candidatus* Mycoplasma turicensis'.

Table 2. Sequence analysis for the 16S rRNA gene of hemoplasmas from domiciled and free-roaming cats in Campo Grande, State of Mato Grosso do Sul, Brazil,compared with sequences deposited in the GenBank database.

Hemoplasmas	Number of positive animals	Number of analysed sequences	Closest Genbank entry (by BLAST®) % identity. – 16S rRNA gene	GenBank accession numbers
Mycoplasma haemofelis	17	8	*Mycoplasma haemofelis* - KC331019– 99%	KJ135313-KJ135320
'Candidatus Mycoplasma haemominutum'	23	8	*'Candidatus* M. haemominutum' – KC331022 – 99%	KJ135321-KJ135328
'Candidatus Mycoplasma turicensis'	15	14	*'Candidatus* M. turicensis' – EU839977 – 99%	KJ095684-KJ095697

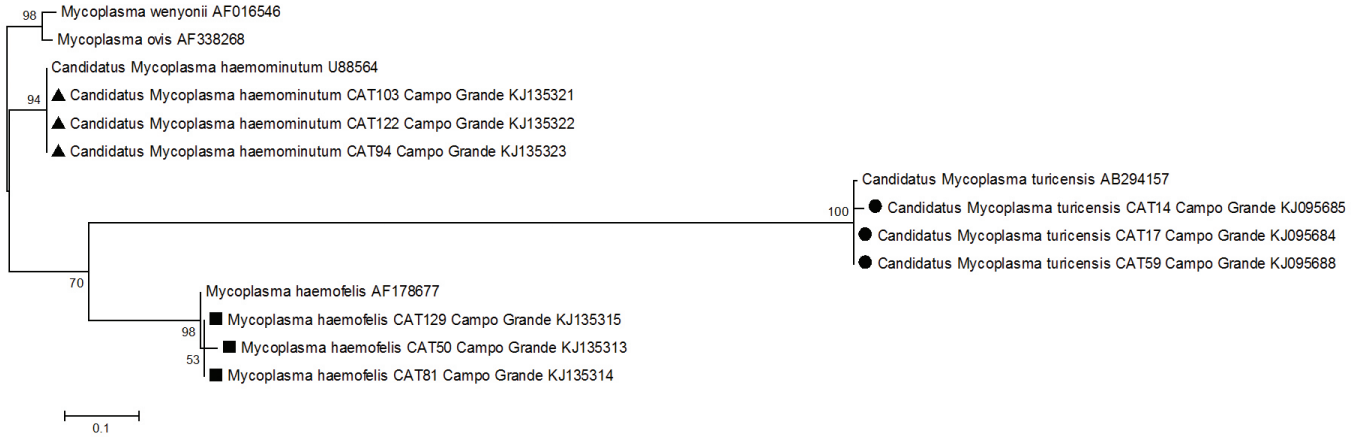

Figure 1. Phylogenetic comparisons of hemoplasmas detected in domestic cats from Campo Grande, MS, Brazil, and sequences deposited in the Genbank database, based on 16S rRNA gene sequences (480bp). The tree was constructed using the Maximum Likelihood Method and kimura-2 parameters model. The numbers on the tree indicate bootstrap values for the branch points. GenBank accession numbers are shown.

could be one of these two organisms, although the occurrence of '*Candidatus* M. haematoparvum' among cats is considered too low.

Previous studies conducted in Brazil have focused on different cat populations, such as animals maintained in animal shelters (SANTOS et al., 2009; MICELI et al., 2013), domiciled cats with outdoor access (BRAGA et al., 2012), cats subjected to a spaying/neutering program (DE BORTOLI et al., 2012) and cats attended at veterinary clinics (MACIEIRA et al., 2008). The molecular occurrences of *M. haemofelis* (10.5%), '*Candidatus* M. haemominutum' (12.8%) and '*Candidatus* M. turicensis' (10.5%) found in the present study among domiciled cats subjected to a spaying/neutering program in the CCZ of Campo Grande were higher than those found by de Bortoli et al. (2012) (2.2%; 4.3%; 2.2%), who sampled a similar population of cats in the city of Jaboticabal, state of São Paulo.

Furthermore, the molecular occurrences of *M. haemofelis* (12.3%), '*Candidatus* M. haemominutum' (18.5%) and '*Candidatus* M. turicensis' (9.2%) found among free-roaming cats housed in the CCZ of the city of Campo Grande, state of Mato Grosso do Sul, were higher than those found among free-roaming cats maintained in animal shelters in the city of Cuiabá, state of Mato Grosso (2.2; 6.7; 0.5%, respectively) (MICELI et al., 2013). The poor fitness observed among free-roaming cats, in comparison with domiciled cats, can be explained by the poor nutritional support and likely presence of coinfections in this cat population. The good fitness observed among hemoplasma-positive domiciled cats suggests that these animals probably are asymptomatic carriers of these pathogens.

It is noteworthy to state that Criado-Fornelio et al. (2003) PCR protocol was described previously to '*Candidatus* M. turicensis' detection (WILLI et al., 2005), aiming to amplify a 16S rRNA fragment (600 pb) of *M. haemofelis* and '*Candidatus* M. haemominutum'. Besides, Santos et al. (2009) described a conventional PCR aiming to amplify a 16S rRNA fragment of '*Candidatus* M. turicensis'. When we aligned primers designed by Criado-Fornelio et al. (2003) to 16S rRNA '*Candidatus* M. turicensis' sequence, a 595 pb fragment can be amplified, similar to that found for *M. haemofelis* and '*Candidatus* M. haemominutum' (600 pb). We observed that samples positive to only '*Candidatus* M. turicensis' when submitted to Criado-Fornelio et al. (2003) PCR protocol, showed negative results or positive results with bands showing low intensity in gel electrophoresis (data not shown). Another question to point out is that the sensitivity of PCR assays used in the present study was not assessed. This fact associated to the absence of internal controls (such as use of a PCR protocol to amplify the feline 28S rRNA) are limitations of the present work.

Unlike our results, feline hemoplasma infection has reported to be correlated with male gender, non-pedigree status and access to the outdoors (SYKES et al., 2008; WILLI et al., 2006a, b; TASKER et al., 2003; GRINDEM et al., 1990; LURIA et al., 2004). Some studies (NASH; BOBADE, 1986; GRINDEM et al., 1990; HARRUS et al., 2002; MACIEIRA et al., 2008; SYKES et al., 2008; GENTILINI et al., 2009) but not others (WILLI et al., 2006a; DE BORTOLI et al., 2012) have shown an association between retrovirus infection and hemoplasmosis. Herein, 22 cats (14.6%) were coinfected by two hemoplasmas and three (1.9%) by three hemoplasmas. In agreement with our findings, coinfections with two or three feline hemoplasma species have also been reported (SYKES et al., 2008; WILLI et al., 2006a, b; FUJIHARA et al., 2007; PETERS et al., 2008; GENTILINI et al., 2009; TASKER et al., 2003; JENSEN et al., 2001; LOBETTI; TASKER, 2004; LURIA et al., 2004; DE BORTOLI et al., 2012; BRAGA et al., 2012; MICELI et al., 2013). The present study shows that hemoplasmas circulate among domiciled and stray cats in Campo Grande, State of Mato Grosso do Sul, in central-western Brazil. More than one third of the sampled cats living in the city of Campo Grande are found to be infected with hemoplasmas and hemoplasmosis, showing non-specific clinical signs such as pale mucous membranes, lethargy, weakness, tachycardia, dyspnea, tachypnea, hepatosplenomegaly, lymphadenopathy, depression, inappetence, dehydration, pica, pyrexia and weight loss. Moreover, hemoplasmas present a potential threat to public health, Therefore, hemoplasmosis needs to be included as a differential diagnosis whenever cats show manifestations of systemic disease.

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