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MOLECULAR DETECTION OF HEMOPLASMA INFECTION AMONG CATS FROM SÃO LUÍS ISLAND, MARANHÃO, BRAZIL

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ABSTRACT

Hemoplasmas are bacteria that infect erythrocytes, attaching to the red blood cell. There is a need for more reports of hemoplasma infection prevalence and molecular characterization among cats in Brazil since there are only few published reports. The present work aimed to detect and molecularly characterize the presence of hemotrophic mycoplasmas in domestic cats with outdoor access from São Luís, Maranhão, Brazil. Twenty cats (10%) were positive for *Candidatus* M. haemominutum, five (2.5%) for *M. haemofelis*, and four (2.%) for *M. turicensis* based on 16S rRNA gene PCRs. Five cats (2.5%) were co-positive for *Candidatus* M. haemominutum and *M. haemofelis*. PCR diagnosis was confirmed by sequencing; and phylogenetic analysis was based on 16S rRNA and rnpb genes.

Key words: cats, *Mycoplasma haemofelis*, *Candidatus* Mycoplasma haemominutum, *Candidatus* Mycoplasma turicensis

INTRODUCTION

The hemotropic mycoplasmas (hemoplasmas) are bacteria of small size and genomes, fastidious growth requirements, that lacks cell wall, and infect erythrocytes, attaching to the red blood cytoplasmic membrane (37). Cats can be infected by a range of hemoplasmas: *Mycoplasma haemofelis*, *Candidatus* Mycoplasma haemominutum, *M. turicensis* and *Candidatus* M. haematoparvum. *Mycoplasma haemofelis* is often associated with haemolytic anaemia during acute infection; and infection by *Candidatus* M. haemominutum and *Candidatus* M. turicensis may result in a fall in erythrocytic parameters, but anaemia is not normally found unless concurrent disease is present (37). Common signs exhibited by acutely ill cats include pallor, lethargy, anorexia, weigh loss, depression, dehydration, and intermittent pyrexia (37).

Recently, *M. haemofelis* was detected using PCR in a human immunodeficiency virus-infected human from Brazil that was co-infected with *B. henselae*, suggesting that infection by *M. haemofelis* may be a zoonosis (9).

There are only few reports of hemoplasma infection prevalence among cats in Brazil (27, 33). Besides, molecular characterization of hemoplasmas in domestic cats from Brazil has not been conducted yet, to our knowledge. The present work aimed to detect and molecularly characterize the presence of hemotrophic mycoplasmas in domestic cats from São Luís,

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MATERIALS AND METHODS

Between October 2008 and January 2009, whole blood samples were collected from 200 domestic cats with outdoor access from São Luís, Maranhão, Brazil. All animals were clinically healthy at the time of samples collection. The blood samples were collected in EDTA and stored at -20°C until DNA extraction.

DNA was extracted from 200 µL of whole blood using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, California, USA) according to the manufacturer's instructions.

To amplify a 393pb partial sequence of *M. haemofelis* 16S rRNA gene, the PCR was performed with 5 µL of template DNA in 25 µL reaction mixtures containing 10X PCR buffer, 1.0 mM $MgCl_2$, 0,2 mM deoxynucleotide triphosphate (dNTPs) mixture, 1.5 U Taq DNA Polymerase (Invitrogen, Carlsbad, California, USA) and 0.2 mM of primers (H. felis-F1 – 5'- GA CTTTGGTTTCGGCCAAGG-3'; H. felisR3 -5'- CGAAGTAC TATCATAATTAT CCCTC- 3') described elsewhere (3). The cycling conditions consisted of an initial denaturation of 10 min at 94ºC for 45 s, 54 ºC for 45 s, 72 ºC for 1 min and a final elongation step for 7 min. For *Candidatus* M. haemominutum, the PCR was performed using the primers 1183F (5'- GCATAATGTGTCGCAATC-3') and 1290R (5'- GTTTCAA CTAGTACTTTCTCC C-3') that amplify a 130bp of 16S rRNA gene (12). The cycling conditions consisted an initial denaturation of 4 min at 94ºC, followed by 35 cycles of 94ºC for 30 s, 53ºC for 1 min, 72 ºC for 45 s and a final elongation step for 5 min. Positive samples were submitted to a PCR that amplify a 1457 bp fragment of the 16S rRNA gene using primers 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT -3') (30), which amplify almost the complete length of the 16S rRNA gene. Cycling conditions were as follows: 95ºC for 10 min, followed by 35 cycles of amplification (1 min at 95ºC, 1 min at 48ºC and 2 min at 72ºC), and a final extension of 5 min at 72ºC.

For *Candidatus* M. turicensis, the PCR was performed using the primers Mt1Fw (5'-GTATCCTCCATCAGACAGA A-3') and Mt2Rv–(5'-CGCTCCATATTTAATTCCAA-3') that amplify a 488pb fragment of 16S rRNA (33). The cycling conditions consisted an initial denaturation of 2 min at 95ºC, followed by 35 cycles of 94ºC for 1 min, 55ºC for 45 s, 72 ºC for 45 s and a final elongation step for 5 min. Fragments of expected size from *M. haemofelis, Candidatus* M. haemominutum and *Candidatus* M. turicensis cloned into pGEM T-Easy Vector System II (Promega, Madison, Wisconsin, USA) were used as positive controls. Ultra-pure sterile water was used as negative control.

Samples that were posiive to the above described PCRs were submitted to a PCR based on RNAase P gene (rnpb), using the primers 80F1 (5'-GAGGAAAGTCCRYGCTW GCAC-3') and 290R1 (5'-TCCCYTACCRAAATTTRGGTTT CT-3) (5). The cycling conditions consisted an initial denaturation of 5 min at 95ºC, followed by 45 cycles of 95ºC for 1 min, 45ºC for 1 min, 72 ºC for 1 min and a final elongation step for 5 min.

The reaction products were purified using 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 Analyser – Applied Byosystem/ Perkin Elmer) and used for subsequent phylogenetic analysis. Phylogenetic reconstructions were based upon desoxiribonucleic acid sequences. Consensus sequences were obtained through the analysis of the products from sequencing from both forward and reverse oligonucleotides using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). Comparisons with sequences deposited in GenBank were done using the basic local alignment search tool (BLAST®). The CLUSTAL W (40) and MEGA (21) programs were used for alignment and phylogenetic analysis, respectively. The distance neighbor-

joining method was used to build the phylogenetic tree (32) using the Kimura-2-parameter model. The bootstrap test with 1000 replications was replied to estimate the confidence of branching patterns of the neighbor-joining tree (11).

RESULTS

Twenty cats (10%) were positive for *Candidatus* M. haemominutum, five (2.5%) for *M. haemofelis*, and four (2.%) for *M. turicensis* based on 16S rRNA PCRs. Five cats (2.5%) were co-positive for *Candidatus* M. haemominutum and *M. haemofelis*. Positive cats to *Candidatus* M. turicensis based on 16S rRNA were not positive at PCR based on rnpb gene. The sequencing based on16S rRNA and rnpb genes confirmed that the sampled cats were parasitized by *M. haemofelis*, *Candidatus* M. haemominutum and *Candidatus* M. turicensis (Table 1; Figures 1 and 2).

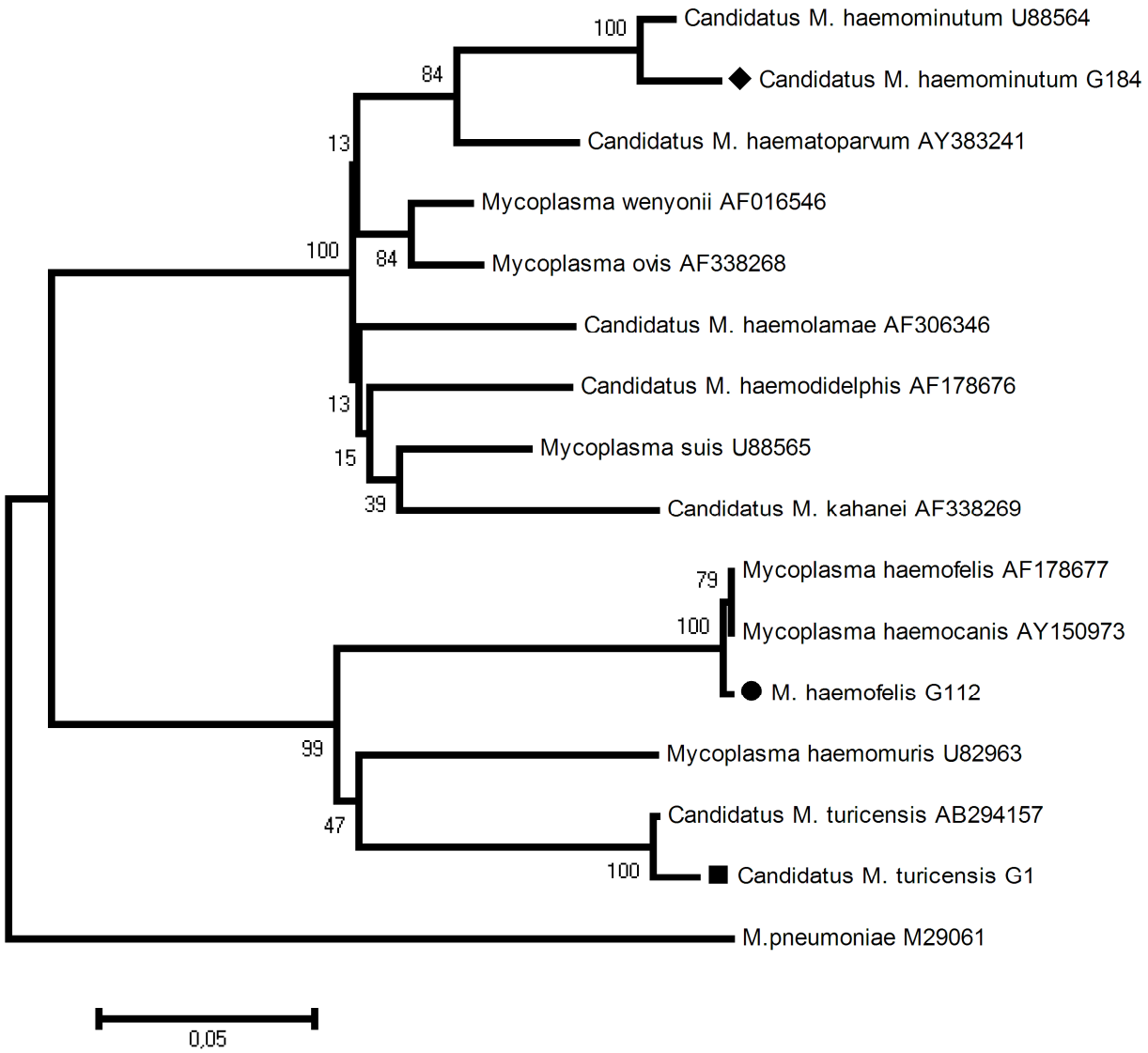

Figure 1. Neighbor-joining Kimura 2-parameters method based phylogenetic position of *Mycoplasma* spp. 16 rRNA gene from cats.

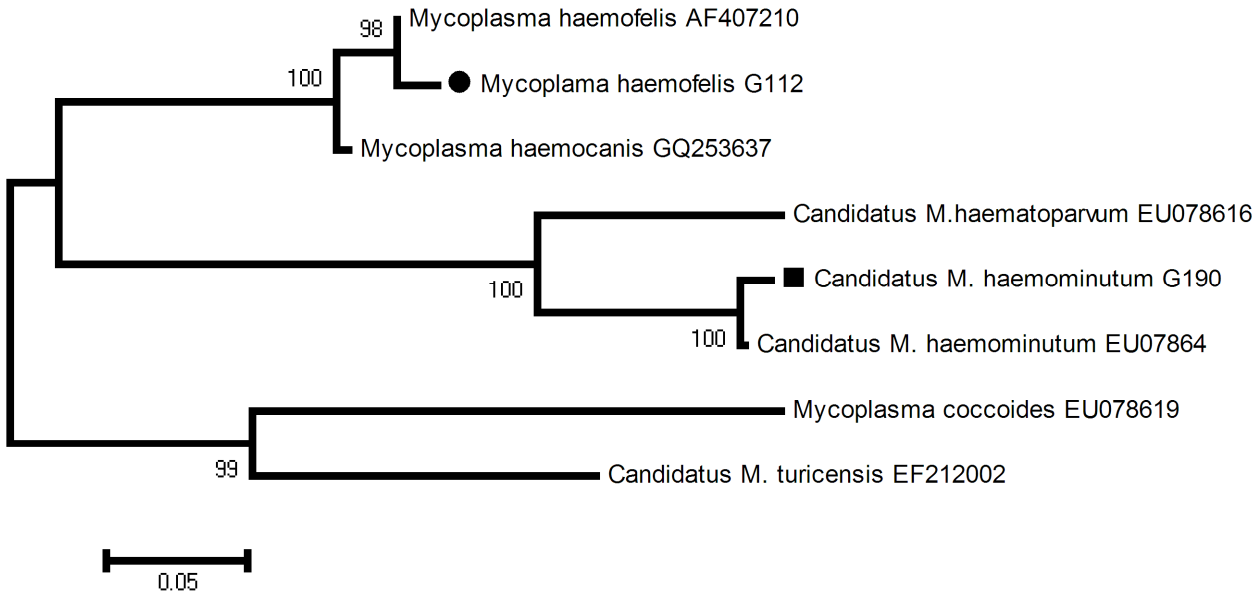

Figure 2. Neighbor-joining Kimura 2-parameters method based phylogenetic position of *Mycoplasma* spp. rnpb gene from cats.

Table 1. Closest Genbank entry for the consensus sequence of 16S RNA and rnpb genes from hemoplasmas in cats from São Luis, Maranhão, Brazil

Hemoplasmas	Number of positive animals	Closest Genbank entry (by BLAST®) % sim. – 16S rRNA gene	Closest Genbank entry (by BLAST®) % sim. – rnpb gene
Mycoplasma haemofelis	5	*Mycoplasma haemofelis* - AF178677 – 99%	*Mycoplasma haemofelis* - AF407210 – 99%
Candidatus Mycoplasma haemominutum	20	*Candidatus* M. haemominutum – EU839983 – 99%	*Candidatus* M. haemominutum - EU078614 - 98%
Candidatus Mycoplasma turicensis	4	*Candidatus* M. turicensis – EU839977 – 99%	-

DISCUSSION

The most prevalent hemoplasma found in the present study was *Candidatus* M. haemominutum. In the same way, in most of the prevalence studies around the world, *Candidatus* M. haemominutum has been the most common hemoplasma found, with lower prevalence of *Candidatus* M. turicensis and *M. haemofelis* detected (36). However, high prevalences of the latter two species have occasionally been reported, such as in a South African study with *Candidatus* M. turicensis (24), and in a Canadian study with *M. haemofelis* (18). The prevalence among domestic cats around the world ranged from 0.4% to 35% for *Candidatus* M. haemominutum, from 0.3% to 6.5% for *Candidatus* M. turicensis, from 0.125% to 10% to *Mycoplasma haemofelis*, and from 0 to 0.7% for *Candidatus* M. haematoparvum-like (1, 2, 10, 13, 14, 16, 17, 18, 19, 20, 22, 23, 24, 26, 27, 28, 31, 36, 38, 39, 41, 45).

Bloodsucking arthropods, such as ticks and fleas, are suspected to be involved in the transmission of feline hemoplasmas between domestic cats (23, 34), but an attempted

experimental transmission between cats via fleas has not been conclusive (43). Ingestion of *Mycoplasma*-infected-*Ctenocephalides felis* or by-products are not important means of transmission for *M. haemofelis* or *Candidatus* M. haemominutum (44). The transmission of hemoplasmas by social contact seems less likely than transmission by aggressive interaction (7, 29).

Although hemoplasmas have been detected in cats from several regions of Brazil (4), few reports have been published yet. Until the present time, *M. haemofelis* and *Candidatus* M. haemominutum have been reported in cats from Paraná (8) and Rio de Janeiro states (27), and in wild felids maintained in captivity in São Paulo state (42). *Candidatus* Mycoplasma haemominutum DNA was detected in a lion from a zoo in Curitiba, Paraná state, Brazil (15). *Candidatus* M. turicensis have only been detected in cats from Rio Grande do Sul state (33) and wild felids maintained in captivity in São Paulo (42).

CONCLUSION

Using molecular tools, the present work showed that *Mycoplasma haemofelis*, *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis circulate among cats in Maranhão state, Brazil. More studies concerning the genetic variability among hemoplasmas isolates infecting domestic and wild animals in Brazil and around the world should be done, aiming to verify the relationship among geographic distribution, genetic diversity and threat to animal and human healthy.

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