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Prevalence and phylogenetic analysis of haemoplasmas from cats infected with multiple species

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ABSTRACT

Mycoplasma haemofelis (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt) are agents of feline haemoplasmosis and can induce anaemia in cats. This study aimed to determine the prevalence and phylogeny of haemoplasma species in cats from Brazil's capital and surrounding areas, and whether correlation with haematological abnormalities existed. Feline haemoplasmas were found in 13.8% of 432 cats. CMhm was the most prevalent species (in 13.8% of cats), followed by Mhf (11.1%) and CMt (4.4%). Over 80% of haemoplasma-infected cats harboured two or more feline haemoplasma species: 7.1% of cats were co-infected with Mhf/CMhm, 0.4% with CMhm/CMt and 3.9% with Mhf/CMhm/CMt. Male gender was significantly associated with haemoplasma infections. No association was found between qPCR haemoplasma status and haematological variables, however CMhm relative copy numbers were correlated with red blood cell (RBC) numbers and packed cell volume (PCV). Haemoplasma 16S rRNA gene sequences (>1 Kb) were derived from co-infected cats using novel haemoplasma species-specific primers. This allowed 16S rRNA gene sequences to be obtained despite the high level of co-infection, which precluded the use of universal 16S rRNA gene primers. Within each species, the Mhf, CMhm and CMt sequences showed >99.8%, >98.5% and >98.8% identity, respectively. The Mhf, CMhm and CMt sequences showed >99.2%, >98.4% and >97.8% identity, respectively, with GenBank sequences. Phylogenetic analysis showed all Mhf sequences to reside in a single clade, whereas the CMhm and CMt sequences each grouped into three distinct subclades. These phylogeny findings suggest the existence of different CMhm and CMt strains.

1. Introduction

Haemoplasmas are haemotropic mycoplasmas lacking a cell wall that attach and grow on the surface of red blood cells and can cause infectious anaemia in different mammalian species. Although some of their basic characteristics are known (Messick, 2004), they have not yet been successfully cultivated in vitro. Three main haemoplasma species can infect cats: *Mycoplasma haemofelis* (Mhf), '*Candidatus* Mycoplasma haemominutum' (CMhm) and '*Candidatus* Mycoplasma turicensis' (CMt). Mhf is the most pathogenic, often leading to haemolytic anaemia during acute infection. In contrast, CMhm and CMt are less pathogenic, but when combined with Mhf or retrovirus infection may also induce anaemia (Tasker et al., 2009).

Haemoplasmas are found throughout the world and have previously been identified in cats (Biondo et al., 2009a), dogs (Biondo et al., 2009a), cattle (Girotto et al., 2012), capybaras (Vieira et al., 2009), lions (Guimaraes et al., 2007) and deer (Grazziotin et al., 2011) in Brazil. Recently, the zoonotic potential of haemoplasmas has been reported after the molecular identification of haemoplasmas in immunosuppressed humans and professionals, some with frequent exposure to haemoplasma infected animals (dos Santos et al., 2008; Steer et al., 2011; Sykes et al., 2010). Moreover, domestic cats may act as a source of haemoplasma infection for wild animals (André et al., 2014).

Previous studies have reported the prevalence of haemoplasmas in domestic cats from different Brazilian states such as Mato Grosso do Sul (MS) (36.4%) (Santis et al., 2014), Rio de Janeiro (RJ) (12%) (Macieira et al., 2008), Rio Grande do Sul (RS) (21.3%) (Santos et al., 2009), Maranhão (MA) (12%) (Braga et al., 2012), São Paulo (SP) (32% and 6.5%) (André et al., 2014; de Bortoli et al., 2012) and Mato Grosso (MT) (8.4%) (Miceli et al., 2013). However these studies have not consistently reported haematological findings or phylogenetic analysis. In those that have evaluated phylogeny, only short (less than 600 bp) 16S rRNA gene sequences have been used (André et al., 2014; Miceli et al., 2013; Santis et al., 2014) or sequences were not submitted to GenBank (Braga et al., 2012). Additionally, few positive cats and no

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more than two reference sequences for each haemoplasma species were used in phylogenetic analysis. Further work on the phylogeny of Brazilian feline haemoplasmas is required, using (near) complete 16S rRNA and other genes if possible. The aim of this study was to assess the prevalence and phylogeny of haemoplasmas from naturally infected cats in Brazil's capital, Brasília, and surrounding areas, and to determine whether any correlation existed between haemoplasma infection and haematological abnormalities.

2. Materials and methods

2.1. Sample collection

From 2009 to 2013, EDTA-anticoagulated feline blood samples were obtained from the following groups in order to acquire as large and diverse a population of samples as possible: i) cats from Brazil's capital, Brasília, and surrounding areas that attended the local veterinary teaching hospital or private clinics, ii) owned cats from 7 cities in the surrounding areas seen during the 2012 and 2013 anti-rabies vaccination campaign, iii) owned cats from one city village outside Brasília sampled during a leishmaniasis surveillance programme conducted by the public health service, and iv) feral cats from a shelter located in Brasília's surrounding area.

2.2. Ethics Approval

Data regarding gender were available but not health status or age. The project was approved by the University of Brasília (UnB) ethics committee under the protocol number UnBDOC no. 43938/2012.

2.3. Haematological analysis

Haemoglobin concentration and red blood cell (RBC) numbers were determined using a semi-automatic veterinary blood cell counter (ABC Vet-Horiba® ABX diagnostics, Brazil) and the packed cell volume (PCV) was determined by microhaematocrit centrifugation. Mean corpuscular volume (MCV) and mean corpuscular haemoglobin concentration (MCHC) were calculated from haemoglobin, PCV and red blood cell count. Anaemia was defined as a PCV <24%.

2.4. DNA extraction

DNA extraction from 100 μL of blood was performed using a commercial kit (Illustra blood genomicPrep Mini Spin Kit, GE Healthcare, UK) according to the manufacturer's instructions. The DNA was eluted with 100 μL of elution buffer provided with the commercial kit and stored at −20 °C prior to analysis.

2.5. Control and generic haemoplasma conventional PCR assays

In order to check for the presence of PCR inhibitors and evaluate the DNA quality, all samples were subjected to a control conventional PCR to amplify a fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene using primers described by Birkenheuer et al. (2003), as part of another previous study assessing DNA extraction efficiency. The protocol for this PCR included the following: 1 μL of DNA template was amplified using 0.5 μM of each primer, 1.25 U *Taq* DNA Polymerase recombinant, 0.2 mM dNTPs, 1× PCR buffer and 1.5 mM $MgCl_2$ (all Invitrogen™), with water to 25 μL. Amplification was performed in a thermal-cycler (FTGene5D, Techgene, UK) with an initial denaturation step of 94 °C for 5 min, followed by 40 cycles of denaturation (94 °C; 30 s), annealing (52 °C; 1 min), extension (72 °C; 1 min) and a final extension at 72 °C for 5 min. Water was used as a negative control and DNA extracted from non-infected cat blood as a positive control. Samples giving bands of 400 bp on a 1.5% agarose gel stained with ethidium bromide were considered positive and, therefore, had DNA of sufficient quantity and quality for further molecular analysis.

Following GAPDH conventional PCR, DNA samples were then screened for haemoplasma infection using a generic haemoplasma conventional PCR adapted from Criado-Fornelio et al. (2003) to amplify 595 bp fragments of the 16S rRNA gene. In the reaction, 2 μL of DNA template was amplified using 0.5 μM of each primer, 1.0 U *Taq* DNA Polymerase recombinant, 0.25 mM dNTPs, 1× PCR buffer and 2.5 mM $MgCl_2$ (all Invitrogen™), with water to 25 μL. Amplification was performed in a thermal-cycler (FTGene5D, Techgene, UK) with an initial denaturation step of 94 °C for 10 min, followed by 40 cycles of denaturation (94 °C; 30 s), annealing (50 °C; 30 s), extension (72 °C; 30 s) and a final extension at 72 °C for 10 min. Water was used as negative control and DNA from a naturally infected haemoplasma cat previously diagnosed by cytology and PCR (species not defined) was used as a positive control. PCR products underwent electrophoresis in a 1.5% agarose gel stained with ethidium bromide, and samples with fragments of 595 bp were identified as positive.

2.6. Haemoplasma species-specific real-time quantitative PCR assays and relative copy number determination

All of the DNA samples generating positive results by the conventional generic PCR were shipped to the University of Bristol, UK for subsequent real-time quantitative PCR (qPCR) as previously described (Peters et al., 2008b) to identify the haemoplasma species (Mhf, CMhm and/or CMt) present and determine relative copy numbers. All qPCR reactions were duplexed with an internal control (feline 28S rRNA gene) assay to demonstrate the presence of amplifiable DNA and the absence of PCR inhibitors; a threshold cycle (Ct) value of 22 was used as a cut off. In each run, DNA from known haemoplasma positive cats was used as a positive control and water as a negative control.

Relative copy numbers were calculated using the $E^{\Delta Ct}$ equation, with the assumption that the highest Ct in the sample set equalled 1 haemoplasma copy/reaction (ΔCt = highest Ct − sample Ct) and taking into account the Mhf, CMhm and CMt qPCR reaction efficiencies (E) previously determined (Peters et al., 2008b) from standard curves.

2.7. 16S rRNA gene sequencing

The 11 CMt, 10 Mhf and 10 CMhm positive samples representing different geographical locations with the lowest Ct values were chosen for 16S rRNA gene amplification and sequencing. However, in order to sequence the 16S rRNA gene of individual haemoplasma species from the many cats with haemoplasma co-infections, species-specific 16S rRNA gene primers were designed to amplify around 1200 bp of the 16S rRNA gene through a conventional PCR. Briefly, complete 16S rRNA gene sequences from feline haemoplasma species available on GenBank (accession numbers AY831867, DQ157150, DQ157151, DQ464417, DQ464418, DQ464419, DQ464420, DQ464421, DQ464422, DQ464423, DQ464424, DQ464425, AF271154, AY150974, AY150978, AY150979, AY150980, AY150981, U88564, AF178677, AF548631, AY069948, AY150972, AY150976, AY150977, AY150984, AY150985, U88563, U95297) were downloaded and aligned using Clustal-W on MacVector ver 13.0.3. The consensus sequence generated for each of the three feline haemoplasma species was used to manually identify the most specific regions of the 16S rRNA gene to which species specific primer pairs could be designed. Short sequences ranging from 25 to 30 bp were selected as forward and reverse primers and tested using Primer3 (web version 4.0.0; http://primer3.ut.ee/) against the consensus sequence to obtain primer pairs with suitable melting temperatures (Tm), self-complementarity, 3′ self-complementarity and hairpin formation.

Two primer pairs for each feline haemoplasma species were selected for evaluation based on their predicted product size, Tm and minimal pair complementarity (Table 1). The primer specificities were first