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Original article

Tick-borne agents in domesticated and stray cats from the city of Campo Grande, state of Mato Grosso do Sul, midwestern Brazil

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ABSTRACT

Anaplasmataceae agents, piroplasmids and *Hepatozoon* spp. have emerged as important pathogens among domestic and wild felines. The present work aimed to detect the presence of species belonging to the Anaplasmataceae family, piroplasmas and *Hepatozoon* spp. DNA in blood samples of domesticated and stray cats in the city of Campo Grande, state of Mato Grosso do Sul, midwestern Brazil. Between January and April 2013, whole blood samples were collected from 151 cats (54 males, 95 females and two without gender registration) in the city of Campo Grande, state of Mato Grosso do Sul, Brazil. DNA extracted from cat blood samples was submitted to conventional PCR assays for *Theileria*/*Babesia*/*Cytauxzoon* spp. (*18S rRNA*, ITS-1), *Ehrlichia* spp. (*16S rRNA*, *dsb*, *groESL*), *Anaplasma* spp. (*16S rRNA*, *groESL*) and *Hepatozoon* spp. (*18S rRNA*) followed by phylogenetic reconstructions. Out of 151 sampled cats, 13 (8.5%) were positive for *Ehrlichia* spp. closely related to *Ehrlichia canis*, 1 (0.66%) for *Hepatozoon* spp. closely related to *Hepatozoon americanum* and *Hepatozoon* spp. isolate from a wild felid, 1 (0.66%) for *Cytauxzoon* sp. closely related do *Cytauxzoon felis*, and 18 (11.9%) for *Babesia*/*Theileria* (one sequence was closely related to *Babesia bigemina*, eight for *Babesia vogeli*, five to *Theileria* spp. from ruminants [*Theileria ovis*, *Theileria lestoquardi*] and four to *Theileria* sp. recently detected in a cat). The present study showed that *Ehrlichia* spp., piroplasmids (*B. vogeli*, *Theileria* spp. and *Cytauxzoon* spp.) and, more rarely, *Hepatozoon* spp. circulate among stray and domesticated cats in the city of Campo Grande, state of Mato Grosso do Sul, midwestern Brazil.

1. Introduction

Recently, ticks and tick-borne pathogens are expanding their zoo-geographic range due to climate and environmental changes. Additionally, arthropod vectors have now adapted to a peridomiciliary cycle involving cats (Shaw et al., 2001). In this context, species belonging to the Anaplasmataceae family, piroplasmids and *Hepatozoon* spp. have emerged as important pathogens among wild and domestic carnivores.

Piroplasmids (*Babesia* spp., *Theileria* spp., and *Cytauxzoon* spp.) parasitize cats' blood cells, causing sporadic cases of disease, and rarely outbreaks (Carli et al., 2012; Criado-Fornelio, 2012a). These protozoa have a complex life-cycle which includes Ixodid ticks as definitive hosts and felines as intermediate hosts (Criado-Fornelio, 2012a).

On the other hand, vectors of feline ehrlichiosis and anaplasmosis, caused by *Ehrlichia canis* and *Anaplasma phagocytophilum*, respectively, are still unknown (Almosny et al., 1998; Almosny and Massard, 1999; Stubbs et al., 2000; Bjoersdorff et al., 1999; Tarello, 2005). Exposure to arthropods (ticks and fleas) and ingestion of rodents are suggestive routes of transmission of this disease among cats (Beaufils et al., 1999).

Hepatozoonosis, an emergent disease in felines, is transmitted by ingestion of an infected invertebrate definitive host (Criado-Fornelio et al., 2003). Even though hepatozoonosis in felines is normally subclinical, pathogenic effects may be exacerbated in stressed, immunocompromised animals or in concomintant infections. The ticks species involved in transmission cycles are still unknown (Criado-Fornelio, 2012b).

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New *Ehrlichia* spp. and *Anaplasma* spp. genotypes have been detected in wild felines maintained in captivity in the state of São Paulo (André et al., 2010a, 2012). *Ehrlichia* sp. closely related to *E. canis* has been detected in domestic cats sampled in the cities of Viçosa (state of Minas Gerais, southeastern Brazil) (Oliveira et al., 2009), São Luís (state of Maranhão, northeastern Brazil) (Braga et al., 2012) and Cuiabá (state of Mato Grosso, central-midwestern Brazil) (Braga et al., 2014). Recently, *Anaplasma* sp. closely related to *A. phagocytophilum*, *Babesia vogeli* and *Theileria* sp. closely related to *Theileria equi* have been detected in stray cats in a zoo in the city of São Paulo, southeastern Brazil (André et al., 2014). While new genotypes of *Hepatozoon* spp. were reported in wild felids maintained in captivity in zoos in the cities of Jundiaí, Ilha Solteira (state of São Paulo) and Cuiabá (state of Mato Grosso) (André et al., 2010b), *Hepatozoon* spp. closely related to *Hepatozoon canis* (Rubini et al., 2006) and *Hepatozoon felis* (De Bortoli et al., 2011) have been detected in domestic cats in the states of São Paulo and Maranhão (northeastern Brazil), respectively. Although *Cytauxzoon* sp. has been detected in neotropical wild felids and lions maintained in zoos in cities located in the states of São Paulo (André et al., 2009), Rio de Janeiro (Peixoto et al., 2007) and Distrito Federal (André et al., 2009), there is only a report of this parasite in domestic cats in Brazil, in the state of Rio de Janeiro (Maia et al., 2013). The present work aimed to detect the presence of species belonging to the Anaplasmataceae family, piroplasmas and *Hepatozoon* spp. DNA in blood samples of domesticated and stray cats in the city of Campo Grande, state of Mato Grosso do Sul, midwestern Brazil.

2. Material and methods

Between January and April 2013, whole blood samples were collected 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. Stray non-domesticated cats ($n = 65$) were caught by technical staff from the local zoonosis control center (CCZ). Domesticated 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 domesticated cats were in a better physical condition than the non-domesticated animals (Santis et al., 2014). The blood samples were collected in EDTA and stored at −20 °C until DNA extraction. The project was approved by the university's Ethics Committee under the protocol number 004987/13.

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. DNA concentration and quality was measured using absorbance ratio between 260/280 nm (Nanodrop, Term Scientific, USA). In order to confirm the presence of amplifiable DNA in samples, an internal control PCR targeting *28S rRNA* feline gene was used (Helps et al., 2003) (Table 1). Microtubes containing ultra-pure sterile water were intercaled between each series of five cats' blood samples and submitted to DNA extraction.

Each sample of extracted DNA was used as a template in *16S rRNA*-based nested PCR assays for *Ehrlichia* spp. (*16S rRNA* gene) (Murphy et al., 1998) and *Anaplasma* spp. (*16S rRNA* gene) (Massung et al., 1998) (Table 1). *E. canis* and *Anaplasma platys* DNA positive controls were obtained from naturally infected dogs from Campo Grande, MS, Brazil (Dagnone et al., 2009). Positive samples were submitted to additional molecular characterization using nested PCR protocols based on *omp-1* (Inayoshi et al., 2004), *dsb* (Doyle et al., 2005) and *groESL* (Sumner et al., 1997; Lotric-Furlan et al., 1998; Nicholson et al., 1999) (Table 1).

Previously described PCR protocols based on ITS-1 region for piroplasmids (Shock et al., 2014) and *18S rRNA* gene for *Babesia*/*Theileria* spp. (Jefferies et al., 2007), *Cytauxzoon felis* (Birkenheuer et al., 2006) and *Hepatozoon* spp. (Ujvari et al., 2004) were used for DNA amplification (Table 1). *Babesia* sp. (André et al., 2012), *Cytauxzoon* sp. (André et al., 2009) and *Hepatozoon* sp. (André et al., 2010b) DNA samples obtained from naturally infected wild felids were also used as positive controls. Ultra-pure sterile water was used as negative control in all PCR assays described above. In each set of reactions, five tubes containing ultra-pure water were used as controls. In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated rooms.

The reaction products (fragments of 358 bp for *16S rRNA Ehrlichia* spp., 409 bp for dsb *Ehrlichia* spp., 800 bp for *18S rRNA Babesia* spp./*Theileria* spp., 400 bp for ITS *Cytauxzoon* spp., and 600 bp for *Hepatozoon* spp.) were purified using Silica Bead DNA Gel Extraction Kit (Fermentas, São Paulo, SP, Brazil). Purified amplified DNA fragments from positive samples were submitted to sequence confirmation in an automatic sequencer (ABI Prism 310 Genetic Analyser–Applied Byosystem/Perkin Elmer). Consensus sequences were obtained through the analysis of the sense and antisense sequences 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) (Altschul et al., 1990). The sequences were aligned with sequences published in GenBank using Clustal/W (Thompson et al., 1994) in Bioedit v. 7.0.5.3 (Hall, 1999). Phylogenetic inference was based on Bayesian (BI) and maximum likelihood (ML) inference. The Bayesian inference (BI) analysis was performed with MrBayes 3.1.2 (Ronquist and Huelsenbeck, 2003) on the CIPRES Science Gateway (Miller et al., 2010) using the best models elected by the program MEGA-4 version 4.0 (Kumar et al., 2004) under the Corrected Akaike Information Criterion (AICc). Markov chain Monte Carlo (MCMC) simulations were run for 10^9 generations with a sampling frequency of every 100 generations and a burn-in of 25%. The Maximum-likelihood (ML) phylogenies were inferred with RAxML-HPC BlackBox 7.6.3 (Stamatakis et al., 2008) (which includes an estimation of bootstrap node support) through the CIPRES Science Gateway (Miller et al., 2010), using a GTRGAMMA model of evolution and 1000 bootstrapping replicates. The trees were examined in Treegraph 2.0.56-381 beta (Stover and Muller, 2010).

Logistic regression models were employed to assess the effect of the putative predictor variables (i.e., gender, area of activity and the interaction between them) on the logit of the probability relative to the positive PCR assays for *Ehrlichia* sp. (*16S rRNA*), *Ehrlichia* sp. (*dsb* gene), *Babesia* sp. (*18S rRNA*), *Babesia* sp., (ITS-1 region), *Theileria* sp. (*18S rRNA*), *Cytauxzoon* sp. (ITS region) and *Hepatozoon* sp. (*18S rRNA*). All analyses were carried out using R 3.0.2 software (R Core Team, 2013).

3. Results

All DNA samples amplified the predicted product for feline *28S rDNA*, which indicates a successful DNA extraction. Out of 151 sampled cats, 13 (8.5%) were positive for *Ehrlichia* spp. *16SrRNA* (eight [5.29%] females (three [1.98%] domesticated and five [3.31%] stray) and four (2.64%) males (three [1.98%] domesticated and one [0.66%] stray) and one cat (0.66%) without gender and area of activity records. The analysis on 13 sequenced products based on the *16S rRNA* region (GenBank accession numbers KP65973, KP659738, KP659739, KP659740, KP659741, KP659742, KP659743, KP659744, KP659745, KP659746, KP659747, KP659748, and KP659749) showed 100% sequence identity with *E. canis* (GenBank accession number KJ995844). The fragments of *Ehrlichia* spp. *16S rRNA* gene found in sampled cats were in the same clade as other