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Original article

Arthropod-borne pathogens circulating in free-roaming domestic cats in a zoo environment in Brazil

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ABSTRACT

Recently, tick and flea-borne pathogens have been detected in wild carnivores maintained in captivity in Brazilian zoos. Since free-roaming cats are frequently found in Brazilian zoos, they could act as reservoirs for arthropod-borne pathogens, which could be transmitted to endangered wild carnivores maintained in captivity in these institutions. On the other hand, stray cats in zoos may play a role as sentinels to pathogens that circulate among wild animals in captivity. The present work aimed to detect the presence of Anaplasmataceae agents, hemoplasmas, *Bartonella* species, piroplasmas, and *Hepatozoon* sp. DNA in blood samples of 37 free-roaming cats in a Brazilian zoo. Three (8%) cats were positive for *Anaplasma* spp. closed related to *Anaplasma phagocytophilum*; 12 (32%) cats were positive for hemoplasmas [two (5%) for *Mycoplasma haemofelis*, five (13.5%) for *Candidatus* Mycoplasma haemominutum, and five (13.5%) for *Candidatus* Mycoplasma turicensis]; 11 (30%) were positive for *Bartonella* spp., six (16%) were positive *Babesia* vogeli and one (3%) for *Theileria* sp. Coinfection with multiple arthropod-borne agentes was observed in sampled cats. None of sampled cats were positive for *Ehrlichia* spp., *Cytauxzoon* spp., or *Hepatozoon* spp. in PCR. This is the first molecular detection of *Babesia vogeli* and *Theileria* sp. in domestic cats in Brazil. The control of the population of free-roaming cats in these conservation institutions is much needed aiming to prevent the potential transmission to endangered wild animals maintained in captivity, such as wild neotropical wild felids, as well as to human beings visiting zoos.

Introduction

The emergence of new and the reemergence of previously controlled arthropod-borne agents are of major impact in human and veterinary medicine (Dantas-Torres et al., 2012). Both arthropods and arthropod-transmitted pathogens are expanding their zoogeographic range due to climate changes and increased access to niche environments. The expansion of domestic housing and pets into wild environments has resulted in a higher association of wildlife reservoir hosts and invertebrate vectors with humans and pets. Occasionally, domesticated cats prey on wild small mammals, which are also important reservoirs for arthropod-borne pathogens. Additionally, arthropod vectors have now adapted to a peridomestic cycle involving cats (Shaw et al., 2001).

The most prevalent flea-borne bacterial pathogens in cats are represented by hemoplasmas and *Bartonella* species (Chomel et al., 1996; Shaw et al., 2004). Among feline hemotrophic mycoplasmas, *Mycoplasma haemofelis* is considered the most pathogenic species; on the other hand, *Candidatus* Mycoplasma haemominutum and *Candidatus* Mycoplasma turicensis are most often associated to

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subclinical or chronic infections in cats (Tasker, 2010). Cats are the major reservoirs for *Bartonella henselae*, *Bartonella clarridgeiae*, and *Bartonella koehlerae*, showing non-specific or, most often, non-clinical signs of infection. *Bartonella henselae* is frequently associated with cat scratch disease in human beings (Breitschwerdt et al., 2010).

While infection by hemoplasma and *Bartonella* spp. has been documented in cats in Brazil, few reports have been done about the occurrence of Anaplasmataceae agents, piroplasmas, and *Hepatozoon* spp. among this animal species (De Bortoli et al., 2011; Braga et al., 2012). Although it is known that *Ehrlichia* spp., *Anaplasma* spp., and *Babesia* spp. are transmitted by tick bites, the vector species involved in the transmission cycles of feline anaplasmosis, ehrlichiosis, and babesiosis are not identified yet in Brazil. In the USA, *Cytauxzoon felis* is transmitted by *Dermacentor variabilis* (Blouin et al., 1987) and *Amblyomma americanum* (Reichard et al., 2010). *Hepatozoon* sp., incriminated as a low-virulence agent in cats, is transmitted through the ingestion of ticks, their definitive hosts (Baneth et al., 1998).

Recently, tick- and flea-borne pathogens has been detected in wild carnivores maintained in captivity in Brazilian zoos (Willi et al., 2007; André et al., 2009, 2010a, 2010b, 2011a, 2011b, 2012; Guimarães et al., 2010; Filoni et al., 2012). Since free-roaming cats are frequently found in Brazilian zoos, they could act as reservoirs for arthropod-borne pathogens, which could be transmitted to endangered wild carnivores maintained in captivity in these institutions. On the other hand, stray cats in zoos may play a role as sentinels to pathogens that circulate among wild animals in captivity. Additionally, cats can harbor zoonotic pathogens, such as *M. haemofelis* (Dos Santos et al., 2008) and *Bartonella* species, which may be transmitted to visitors in zoological gardens. The present work aimed to detect the presence of Anaplasmataceae agents, hemoplasmas, *Bartonella* species, piroplasmas, and *Hepatozoon* sp. DNA in blood samples of free-roaming cats in a Brazilian zoo.

Materials and methods

From September 2011 to March 2013, EDTA-blood samples were collected from 37 stray cats (15 males, 18 females, and 4 without sex registration) in Fundação Parque Zoológico de São Paulo (FPZSP), state of São Paulo, Brazil. The project was approved by the university's Ethics Committee under the protocol number 004986/13.

DNA was extracted from 200 μL of whole-blood samples using the QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions. Microtubes containing ultra-pure sterile water were intercaled between each series of 5 cat 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). *Ehrlichia canis* and *Anaplasma* sp. DNA-positive controls were obtained from naturally infected dogs from Campo Grande, MS, Brazil (Dagnone, 2009).

Partial sequences of *M. haemofelis*, *Candidatus* M. haemominutum and *Candidatus* M. turicensis 16S rRNA gene were amplified by PCR as previously described (Criado-Fornelio, 2003; Santos et al., 2009). *Mycoplasma haemofelis*, *Candidatus* M. haemominutum and *Candidatus* M. turicensis DNA obtained from naturally infected cat blood samples from Jaboticabal, SP, Brazil (de Bortoli et al., 2012) were used as DNA-positive controls in PCR reactions for hemoplasmas.

Bartonella genus screening was performed by PCR targeting the intergenic transcribed spacer (ITS), as described previously (Maggi and Breitschwerdt, 2005a; Diniz et al., 2007). For further molecular characterization and species differentiation, positive samples in ITS amplification were tested for other genes: the riboflavin synthase gene (*ribC*) (Johnson et al., 2003), the citrate synthase gene (*gltA*) (Norman et al., 1995; Winoto et al., 2005), the bacteriophage-associated heme-binding protein gene (*pap31*) (Maggi and Breitschwerdt, 2005b), and the RNA polymerase beta subunit gene (*rpoB*) (Diniz et al., 2007). *Bartonella henselae* DNA, obtained from a cat's blood sample from São Luís, Maranhão, Brazil (Braga et al., 2012), was used as positive control in *Bartonella* PCR assays.

Previously described PCR protocols based on 18S rRNA gene were used for *Babesia* spp. (Jefferies et al., 2007), *Cytauxzoon felis* (Birkenheuer et al., 2006), and *Hepatozoon* spp. (Criado-Fornelio et al., 2006) DNA amplification. *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 a negative control in all PCR assays described above. In each set of reactions, 5 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 546 bp for *Anaplasma* spp., 400 bp for *Bartonella* sp. *gltA* gene, 300 bp for *Bartonella* sp. ITS region, 600 bp for *M. haemofelis* and *Candidatus* Mycoplasma haemominutum, and 500 bp for *Candidatus* Mycoplasma turicensis, 800 bp for *Babesia* 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 CLUSTAL W (Thompson et al., 1994) and MEGA-4 (Kumar et al., 2004) programs were used for alignment and phylogenetic analysis, respectively. The distance neighbor-joining method was used to build the phylogenetic tree (Saitou and Nei, 1987) using Kimura 2-parameters model. The bootstrap test with 1000 replications was applied to estimate the confidence of branching patterns of the neighbor-joining tree (Felsenstein, 1985).

Results

Out of 37 sampled cats, 3 (8%) were positive for *Anaplasma* spp. (Table 1). The analysis on 3 sequenced products based on the 16S rRNA region (GenBank accession numbers KF964049, KF964050, and KF964051) showed 99% identity with *A. phagocytophilum* (GenBank accession number CP006618). The fragments of *Anaplasma* spp. 16S rRNA gene found in free-roaming cats in FPZSP were in a clade with *A. phagocytophilum* and *A. platys*. Although *A. platys* could be reasonably distinguished from *A. phagocytophilum* (bootstrap values 48/100), the *Anaplasma* spp. detected in sampled cats more consistently grouped with *A. phagocytophilum* (bootstrap values 88/100) (Fig. 1).

Twelve (32%) cats were positive for hemoplasmas: 2 (5%) for *M. haemofelis*, 5 (13.5%) for *Candidatus* M. haemominutum, and 5 (13.5%) for *Candidatus* M. turicensis. Three cats (8%) were positive for both *Candidatus* M. haemominutum and *Candidatus* M. turicensis, and one cat (3%) for all 3 hemoplasma species (Table 1). Eleven sequenced products out of 12 positive samples showed 2 sequences 100% identity with 16S rRNA *M. haemofelis* (CP002808), 4 sequences 99% identity with 16S rRNA *Candidatus* M. haemominutum (KC331022), and 5 sequences 99% identity with 16S rRNA