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Short communication

Molecular detection of tick-borne bacterial agents in Brazilian and exotic captive carnivores

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ABSTRACT

The present study aims to detect and characterize by molecular techniques, the presence of tick-borne pathogens in wild captive carnivore blood samples from Brazil. Blood was collected from 76 Brazilian felids, 23 exotic felids, 3 European wolves (*Canis lupus*), and 97 Brazilian canids maintained in captivity in zoos located in São Paulo and Mato Grosso states, Brazil. DNA of each sample was used in PCR reactions for *Ehrlichia*, *Anaplasma*, and *Rickettsia* identification. The blood from 10/100 (10%) of canids (1 European wolf, 3 bush dogs, and 6 crab-eating foxes) and from 21/99 (21%) felids (4 pumas, 6 little spotted cats, 4 ocelots, 3 jaguarundis, 1 tiger, and 3 lions) contained fragments of 16S rRNA gene of *Ehrlichia* spp. Fragments of *Anaplasma* spp. *groESL* and 16S rRNA genes were detected in the blood of 1/100 (1%) canids (1 bush dog) and in 4/99 (3%) felids (4 little spotted cats), respectively. *Rickettsia* species infections were not identified. The present work showed that new strains of *Ehrlichia* and *Anaplasma* spp. circulate among wild carnivores in Brazil.

Introduction

Arthropod-borne diseases are of major global importance to human and animal health. The epidemiology of these diseases involves several infectious agents, hosts, and vectors (Harrus and Baneth, 2005). Both arthropods and arthropod-transmitted infections are expanding their zoogeographic range due to climatic, ecological, and environmental changes. The presence of domestic animals in wildlife environments has resulted in an increased association between wildlife reservoirs and vector species with human and pet activities (Shaw et al., 2001). Although globally important, human tick-borne diseases remain poorly studied in Brazil, where confirmed human Brazilian spotted fever cases have been reported in 13 states (De Sá del Fiol et al., 2010). In Minas Gerais state, antibodies to *E. chaffeensis* were identified among healthy humans and in patients with clinical signs compatible with tick-borne diseases (Calic et al., 2004; Costa et al., 2005, 2006).

While the occurrence of tick-borne agents in domestic animals in Brazil, mainly in dogs, has been extensively evaluated, there are only few reports concerning the identification of tick-borne agents in Brazilian free-ranging and captive wild carnivores. The molecular detection of *E. chaffeensis* in Brazilian marsh deer (*Blastocerus dichotomus*) (Machado et al., 2006) raised the importance of investigating wildlife reservoirs for tick-borne zoonotic agents. Antibodies to *Ehrlichia* spp. have been detected in free-ranging (Filoni et al., 2006) and captive neotropical felids (André et al., 2010; Filoni et al., 2012). In addition, *Ehrlichia* spp. DNA has been detected in 5 Brazilian wild felid species (André et al., 2010; Widmer et al., 2011).

In a previous molecular survey, we found 11 (15%) out of 72 neotropical captive wild felids contained *Ehrlichia* spp. DNA in blood (André et al., 2010). Six of the 7 existing species of Brazilian wild felids, including ocelots (*Leopardus pardalis*), little spotted cats (*Leopardus tigrinus*), margays (*Leopardus wiedii*), pampas cats (*Oncifelis colocolo*), jaguars (*Panthera onca*), and pumas (*Puma concolor*), and 2 of the 4 existing species of Brazilian wild canids, namely bush dogs (*Speothos venaticus*) and maned wolves (*Chrysocyon brachyurus*) are endangered (www.ibama.gov.br). Thus, we aimed to detect and characterize, using molecular techniques, the presence of tick-borne pathogens in additional neotropical and exotic wild felids and canids maintained in captivity in various locations throughout Brazil.

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Table 1
Number, species, and zoo location of sampled wild canids and felids.

Species	Common name	Location (*N*)	Total
Felis colocolo	Pampas cat	So (1)	1
Leopardus pardalis	Ocelot	Am (2), IS (2), So (1), Bau (3), Ita (2), Mg (1), Pir (4)	15
Leopardus tigrinus	Little spotted cat	Am (1), IS (2), So (14), SC (1) Ita (1), Pir (3), Cat (1), NO (2)	25
Leopardus wiedii	Margay	Am (2)	2
Panthera onca	Jaguar	AMC (1), Am (1), Bau (1), SP (3)	6
Puma concolor	Puma	Rp (1), Am (2), So (3), Cat (2)	8
Puma yagouaroundi	Jaguarondi	Am (2), IS (4), So (4), SC (2), Bau (3), Ita (3), Pir (1)	19
Panthera tigris[a]	Tiger	So (2), Ita (2), SP (4)	8
Caracal caracal[a]	Caracal	SP (1)	1
Leptailurus serval[a]	Serval	SP (1)	1
Panthera leo[a]	Lion	Rp (1), Am (1), So (2), Bau (3), SP (5)	12
Prionailurus viverrinus[a]	Fishing cat	SP (1)	1
Speothos venaticus	Bush dog	Am (8), IS (1), Pi (6), SP (8), NO (1), Cui (3)	27
Cerdocyon thous	Crab-eating fox	Rp (9), Am (3), Ara (6), IS (3), So (2), SC (4), Bau (1), Ita (1), Pir (3), Cat (6), Le (1)	39
Cerdocyon brachyurus	Maned wolf	Rp (2), Am (1), Ara (1), IS (2), So (2), Ita (3), Mg (3), SP (3), Cat (1), NO (3), Bau (2)	23
Pseudalopex vetulus	Hoary fox	Rp (1), Am (2), Ara (3), IS (1), So (1),	8
Canis lupus[a]	European wolf	SP (3)	3

AMC, Association Mata Ciliar, Jundiaí, São Paulo; SC, São Carlos Zoo, São Paulo; NO, Nova Odessa Zoo, São Paulo; Ped, Pedreira Zoo, São Paulo; Le, Leme Zoo, São Paulo; Cps, Campinas Zoo, São Paulo; Bau, Bauru Zoo, São Paulo; Cui, Cuiabá Zoo, Mato Grosso; Cat, Catanduva Zoo, São Paulo; Rp, Ribeirão Preto, São Paulo; Ita, Itatiba Zoo, São Paulo; Am, Americana Zoo, São Paulo; Mg, Mogi Mirim Zoo, São Paulo; Ara, Araçatuba Zoo, São Paulo; Pir, Piracicaba Zoo, São Paulo; IS, Ilha Solteira Zoo, São Paulo; SP, São Paulo, São Paulo.

[a] Exotic carnivores.

Materials and methods

Five mL of blood was collected from each of 76 Brazilian wild captive felids, 23 exotic captive felids, 3 captive European wolves (*Canis lupus*), and 97 Brazilian wild canids maintained in zoos located in São Paulo and Mato Grosso states (Table 1). All samples were collected under the Brazilian Institute of Environment and Renewable Natural Resources license numbers #S02027.002943/2005 and #15901-1. Animals were immobilized with a mixture of ketamine (Francotar®, Virbac, Carros Cedex, France) (10 mg/kg) and xylazine (Francotar®, Virbac, Carros Cedex, France) (1 mg/kg). Physical examination of each animal did not reveal any clinical signs of disease, and no ticks were found on any of the sampled animals. DNA was extracted from 200 μL of whole blood using the QIAamp DNA Blood Mini kit (QIAGEN, Valencia, CA, USA) according to the manufacturer's instructions.

Each sample of DNA was used as template in conventional PCR assays with genus- and species-specific *rrs* (16S rRNA gene) primers for *Ehrlichia canis* (Murphy et al., 1998), *E. chaffeensis* (Kocan et al., 2000), *E. ewingii* (Persing et al., 1996), *Anaplasma* spp. (Massung et al., 1998), and *A. platys* (Inokuma et al., 2001b). PCR assays for *Ehrlichia*/*Anaplasma* spp. based on *dsb* (Doyle et al., 2005), *groESL* (Sumner et al., 1997; Lotric-Furlan et al., 1997; Nicholson et al., 1999), *ftsz* (Lee et al., 2003), *rpoB* (Taillardt-Bisch et al., 2003), *omp-1* (Inayoshi et al., 2004), and *gltA* (Inokuma et al., 2001a) genes were performed for additional molecular characterization. *Ehrlichia* spp. (*E. canis*, *E. chaffeensis*) and *Anaplasma* spp. (*A. phagocytophilum*, *A. platys*) DNA-positive controls were included in all PCR assays. Negative domestic cat and dog blood samples and ultra-pure water (Promega) were used as negative and no-template controls, respectively. The PCR amplifications were performed in a Gradient Cycler (Perkin-Elmer™ model PT-200). Specific real-time 5′ nuclease PCR reactions for *A. phagocytophilum* msp-2 and *E. chaffeensis* vlpt (Reller et al., 2009) were performed on samples containing 16S RNA genes of *Ehrlichia*/*Anaplasma* spp. in conventional PCR assays. Similarly, a 5′ nuclease real-time PCR was performed for spotted fever (*ompA*) and typhus group *Rickettsia* (17-kDa lipoprotein gene) as previously described (Prakash et al., 2009). These assays were conducted using a BioRad CFX384 real-time PCR analyzer and 1 μL blood DNA in duplicate. The sensitivities of these real-time assays based upon amplification of dilutions of amplicons cloned into plasmids were 1–10 copies/μL blood for *A. phagocytophilum, E. chaffeensis*, and *Rickettsia* spp.

For positive PCRs, purified amplicons (QIAquick Gel Extraction kit, QIAGEN, Valencia, CA, USA) were ligated into the pGEM-T Easy vector (Promega) and transformed into competent JM109 Escherichia coli (Promega; Madison, Wisconsin, USA). Clones were selected by blue/white colony screening; plasmid DNA was isolated using QIAprep Miniprep Kit (QIAGEN, Valencia, CA, USA) and submitted for sequence determination (ABI Prism 310 Genetic Analyser – Applied Biosystem/Perkin Elmer [Foster City, CA, USA]). Consensus DNA sequences were obtained through the analysis of sense and antisense sequences using the CAP3 program (http://mobyle.pasteur.fr/cgi-bin/MobylePortal/portal.py). Comparisons with sequences deposited in GenBank were performed using BLAST (Altschul et al., 1990). BioEdit, CLUSTAL X (Thompson et al., 1994), and TreeView programs were used for alignment and phylogenetic analysis, respectively. The neighbor-joining distance method was used to build the phylogenetic tree (Saitou and Nei, 1987). Bootstrap analysis with 1000 replications was used to estimate the confidence of branching patterns of the trees (Felsenstein, 1985).

Results

The blood of 10/100 (10%) canids (1 European wolf, 3 bush dogs, and 6 crab-eating foxes) and 21/99 (21%) felids (4 pumas, 6 little spotted cats, 4 ocelots, 3 jaguarundis, 1 tiger, and 3 lions) contained fragments of 16S rRNA gene of *Ehrlichia* spp. Fifteen wild carnivores (11 felids and 4 canids) contained ehrlichial DNA closely related to *E. chaffeensis* by BLAST analysis (Table 2). In addition, 16 of the wild carnivores (10 felids and 6 canids) also contained ehrlichial DNA closely related to *E. canis* by BLAST analysis (Table 2).

With reduced confidence (bootstrap value of 348/1000 iterations), the phylogenetic analysis of the amplified 350-bp 16S rRNA gene fragment positioned the ehrlichial DNA in a clade including *E. canis* and *E. chaffeensis* (Fig. 1). However, no samples contained *E. chaffeensis vlpt* genes when subjected to specific real-time PCR.

Anaplasma spp. DNA was detected in the blood of 1/100 (1%) canids (1 bush dog) and in 4/99 (4%) felids (4 little spotted cats). Based on 16S rRNA gene PCR amplification, the *Anaplasma* sp. found in wild felids was closely related to *A. phagocytophilum* by BLAST analysis (Table 2). Using *groESL* PCR amplification, the *Anaplasma* sp. found in one wild canid was closely related to *A. phagocytophilum* by BLAST analysis (Table 2).