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Co-infection with arthropod-borne pathogens in domestic cats

Co-infecção por patógenos transmitidos por artrópodes em gatos domésticos

Marcos Rogério André[1*]; Kilder Dantas Filgueira[2]; Ana Cláudia Calchi[1]; Keyla Carstens Marques de Sousa[1]; Luiz Ricardo Gonçalves[1]; Vitor Brasil Medeiros[2]; Poliana Araújo Ximenes[2]; Ivana Cristina Nunes Gadelha Lelis[2]; Maria Vanuza Nunes de Meireles[2]; Rosangela Zacarias Machado[1]

[1] Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP, Jaboticabal, SP, Brasil

[2] Hospital Veterinário Jerônimo Dix-Huit Rosado, Universidade Federal Rural do Semi-Árido – UFERSA, Mossoró, RN, Brasil

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Abstract

The role of several feline vector-borne pathogens (FVBP) as a cause of disease in cats has not been clearly determined. In fact, with the exception of *Bartonella* spp. and hemoplasmas, FVBP in cats has not been clearly determined in Brazil yet. The present study aimed at identifying, by using molecular methods, the presence of FVBP in three cats showing non-specific clinical signs and inclusions suggestive of hemoparasites in blood smears. *Cytauxzoon felis*, '*Candidatus* Mycoplasma haemominutum', *Ehrlichia* sp. closely related to *Ehrlichia canis*, and *Anaplasma* sp. closely related to *Anaplasma phagocytophilum* were detected in blood samples from two out of three sampled cats. Both cats positive for multiple FVBP did not show hematological and biochemical abnormalities. The present work emphasizes the need for molecular confirmation of co-infection by multiple FVBP in cats presenting non-specific clinical signs and inclusions resembling hemoparasites in blood smears.

Keywords: *Anaplasma, Cytauxzoon, Ehrlichia, Mycoplasma*, feline.

Resumo

O papel de diversos patógenos felinos transmitidos por vetores (PFTV) como causa de enfermidades em gatos não tem sido claramente determinado. De fato, com exceção de *Bartonella* spp. e hemoplasmas, PFTV têm sido bem menos estudados no Brasil. O presente estudo objetivou investigar, utilizando métodos moleculares, a presença de PFTV em três gatos apresentando sinais clínicos inespecíficos e inclusões sugestivas de hemoparasitas em esfregaços sanguíneos. *Cytauxzoon felis*, '*Candidatus* Mycoplasma haemominutum', *Ehrlichia* sp. filogeneticamente relacionada a *Ehrlichia canis*, e *Anaplasma* sp. filogeneticamente relacionado a *Anaplasma phagocytophilum* foram detectados em amostras de sangue de dois dos três gatos amostrados. Os dois gatos positivos para múltiplos PFTV não apresentaram alterações hematológicas e bioquímicas. O presente trabalho enfatiza a necessidade de confirmação molecular da infecção por múltiplos PFTV em gatos apresentando sinais clínicos inespecíficos e inclusões sugestivas de hemoparasitas em esfregaços sanguíneos.

Palavras-chave: *Anaplasma, Cytauxzoon, Ehrlichia, Mycoplasma*, felino.

Vector-borne diseases have wide distribution and raising occurrence, mainly due to climatic and environmental changes and the increase of the mobility of people and animals. Such events may contribute to the multiplication and dispersion of vectors and pathogens, causing morbidity and mortality of animal and humans (BANETH et al., 2012).

Cats, especially those wandering or living in public shelters, are at high risk of acquiring vector-borne pathogens (VBP), probably because they are often not treated with ectoparasiticides. In addition, the general conditions of these animals (eg., poor nutrition) may contribute to the susceptibility to VBP (OTRANTO & DANTAS-TORRES, 2010). Previous studies highlighted the circulation of several pathogens with zoonotic potential in cats in Brazil, emphasizing the need for a molecular approach in the identification of such agents (ANDRÉ et al., 2014, 2015). Since most of the diseases caused by VBP show nonspecific clinical signs, which may vary according to the stage of infection, the molecular diagnosis favors the correct determination of the prognosis and the choice of an effective treatment (GONÇALVES et al., 2014).

The present study aimed at identifying, by using molecular methods, the presence of FVBP (*Anaplasma* spp., *Ehrlichia* spp., *Babesia* spp., *Bartonella* spp., *Mycoplasma* spp., *Cytauxzoon* spp.

***Corresponding author:** Marcos Rogério André. Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista Júlio de Mesquita Filho – UNESP, Via de Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP 14884-900, Jaboticabal, SP, Brasil. e-mail: marcos_andre@fcav.unesp.br

and *Hepatozoon* spp.) in cats showing non-specific clinical signs and inclusions suggestive of hemoparasites in blood smears.

In March, 2016, EDTA-whole blood samples were collected from three cats showing non-specific clinical signs and attended at Veterinary Hospital of University Federal Rural do Semi Árido (UFERSA), Mossoró, Rio Grande do Norte, Brazil (5° 12' 10" S, 37° 19' 32" O).

A non-castrated male cat, 1 year and 2 months old **(# Cat 1**), arrived at the veterinary hospital with signs of pruritus, diarrhea and hyporexia. Clinical examination showed cephalic alopecia in the parietal, retroauricular and tail extremities, melena and thickening of the intestinal loops at abdominal palpation. The animal had access to the street with a history of fights with other cats. In the anamnesis, contact with birds, rabbits and dogs, was reported. The owner informed that while vaccination (triple viral against panleukopenia, rhinotracheitis and calicivirus, and anti-rabies) was updated, deworming was out of date. Although hematological and biochemical alterations were not observed, erythrocytes inclusions inside erythrocytes suggestive of piroplasmids were identified (Figure 1).

In addition, a 3-year-old castrated female **(#Cat2**) presented anorexia, emesis, asthenia, accelerated weight loss, icteric mucosa, hypothermia, opaque fur, dehydration, submandibular lymphadenomegaly, and a firm bulging abdomen. It was an indoor cat living with two other cats. The owner informed that both vaccination and deworm were updated. Hematological examination revealed neutrophilia (15410/mm^3), thrombocytopenia (90 mil/mm^3), anisocytosis, hypochromia, moderate polychromasia, metarrubycytes, presence of Howell-Jolly corpuscles and presence of inclusions inside erythrocytes, suggestive of piroplasmids. In the biochemical analysis, while urea (19 mg/dL) and albumin (1.7 g/dL) were below the reference values, alkaline phosphatase (566 U/L), cholesterol (239 mg/dL) and triglycerides (1146 mg/dL) were above the reference values.

Finally, a 2 years and 2 months old, non-castrated male cat (# **Cat 3**), arrived at the veterinary hospital due to dehiscence of a surgical wound after an osteosynthesis of an exposed fracture in the right thoracic limb. The animal presented asthenia, claudication, edema, ulcer and purulent exudate with exposure of the musculature and bone tissue in the affected limb. The owner reported that the animal had no access to the street and no contact with other animals. Both vaccination (only anti-rabies) and deworm were updated. Despite the lack of hematological and biochemical alterations, inclusions inside erythrocytes suggestive of piroplasmids were found in Panotic-blood smears.

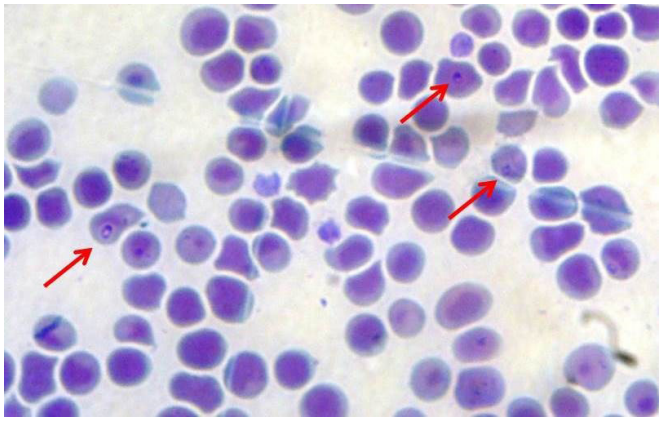

Figure 1. Piroplasmid-suggestive forms found in Panoptic-stained blood smears found within erythrocytes from a domestic cat from Mossoró, Rio Grande do Norte, Brazil.

DNA was extracted from 200 µL of each cat whole blood using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. DNA concentration and quality were measured using $260/280_{nm}$ absorbance ratio (Nanodrop®, Thermo Fisher Scientific, Waltham, MA, USA). In order to verify the presence of amplifiable DNA in the samples, an internal control PCR assay targeting a fragment of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH) (BIRKENHEUER et al., 2003) was performed. Conventional PCR (cPCR) assays targeting 16S rRNA gene of *Ehrlichia* spp. (MURPHY et al., 1998), *Anaplasma* spp. (MASSUNG et al., 1998), *Mycoplasma* spp. (16S rRNA) (MAGGI et al., 2013), and 18S rRNA of *Cytauxzoon felis* (18S rRNA) (BIRKENHEUER et al., 2006), *Hepatozoon* spp. (18S rRNA) (PERKINS & KELLER, 2001; UJVARI et al., 2004), *Babesia* spp. (18S rRNA) (JEFFERIES et al., 2007) were performed. Additionally, a qPCR targeting a *nuoG* gene fragment of *Bartonella* spp. was also performed as previously described (ANDRÉ et al., 2015). *Ehrlichia chaffeensis* and *Anaplasma phagocytophilum* DNA samples, kindly supplied by Prof. John Stephen Dumler (Uniformed Services University of the Health Sciences, Bethesda, Maryland, USA), were used as positive controls in cPCR assays. The Jaboticabal strains of *Ehrlichia canis* and *Babesia vogeli* were obtained from experimentally infected dogs (CASTRO et al., 2004; FURUTA et al., 2009). Finally, *Hepatozoon* spp., *Cytauxzoon* spp., *Bartonella* spp. and *Mycoplasma* spp. DNA positive controls were obtained from naturally infected animals (ANDRÉ et al., 2009, 2010, 2015). Ultra-pure water (Nuclease-FreeWater Promega®, Wisconsin, EUA) was used as negative control in all PCR assays. The gels were imaged under ultraviolet light using the Image Lab Software version 4.1 (Bio-Rad®).

The reaction products of positive samples in the PCR protocols above mentioned were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific®, Waltham, MA, USA). Sanger sequencing was performed of both strands using the BigDye® Terminator v3.1Cycle Sequencing Kit (Thermo Fisher Scientific®, Waltham, MA, USA) and ABI PRISM 310DNA Analyzer (Applied Biosystems®, Foster City, CA, EUA) (SANGER et al., 1977). The sequences were aligned with sequences published in GenBank using MAFFT software, version 7) (KATOH & STANDLEY, 2013). Phylogenetic inference was based on Bayesian Inference (BI). The Bayesian inference (BI) analysis was performed with MrBayes 3.1.2 (RONQUIST & HUELSENBECK, 2003). 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 best model of evolution was selected by the program jModelTest2 (version 2.1.6) on XSEDE (DARRIBA et al., 2012), under the Akaike Information Criterion (AIC) (POSADA & BUCKLEY, 2004). All phylogenetic analyses were performed using CIPRES Science Gateway (MILLER et al., 2011). The trees were examined in Treegraph 2.0.56-381 beta (STOVER & MULLER, 2010).

Cytauxzoon spp., *Mycoplasma* spp., *Ehrlichia* spp. and *Anaplasma* spp. DNA was detected in blood samples from two cats that presented non-specific clinical signs and structures suggestive of piroplasmids in Panoptic-stained blood smears (Table 1). The animal **(#Cat 2**) showing hematological and biochemical alterations were negative for all studied VBP. *Babesia* spp., *Bartonella* spp., and *Hepatozoon* spp. DNA were not detected in sampled cats' blood samples.

Concerning to phylogenetic analysis, the *Anaplasma* 16S rRNA sequences (MF288941-MF288942) detected in two cats were grouped with distinct *Anaplasma* sequences identified in a cat (KF964051) and in a Falconidae specimen (*Caracara plancus* - southern crested caracara) (JN217096) from Brazil; additionally, the sequence showed to be closely related to an *A. phagocytophilum* sequence (GU236670) detected in a dog from Austria, supported by a high posteriori probability (100%) in Bayesian inference (Figure 2). The *Cytauxzoon* sequence amplified in the present study was positioned nearest to *C. felis* sequence (L19080) supported by 88% index (Figure 3). The *Ehrlichia* 16S rRNA sequences amplified in the present study (KY883189 and KY883190) were

Table 1. Vector-borne pathogens molecularly detected in blood samples from three cats. Mossoró, Rio Grande do Norte, northeastern Brazil, 2016.

Animals	VBP						
	***Anaplasma* spp.**	***Babesia* spp.**	***Bartonella* spp.**	***Cytauxzoon* spp.**	***Ehrlichia* spp.**	***Hepatozoon* spp.**	***Mycoplasma* spp.**
Cat # 1	+	-	-	+	+	-	+
Cat # 2	-	-	-	-	-	-	-
Cat # 3	+	-	-	+	+	-	+

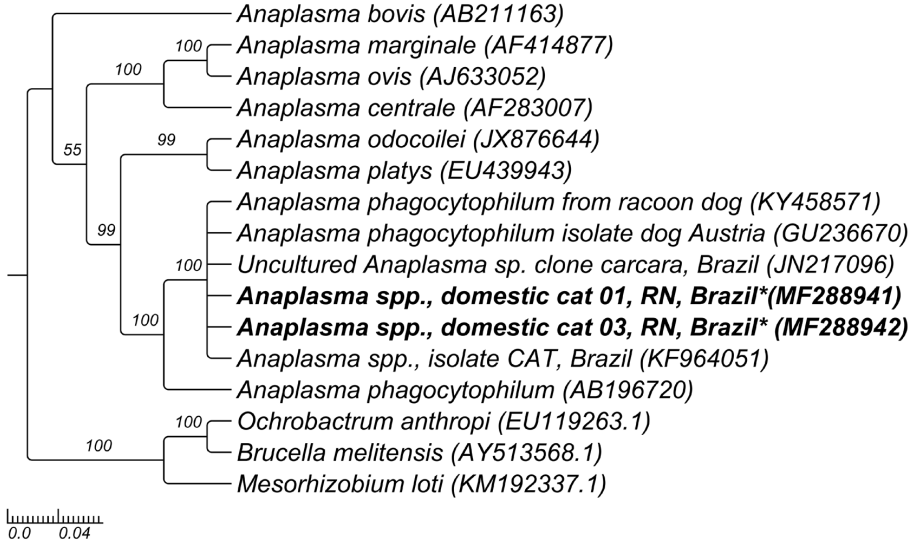

Figure 2. Phylogenetic tree constructed with 600 pb *Anaplasma* spp.-16SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

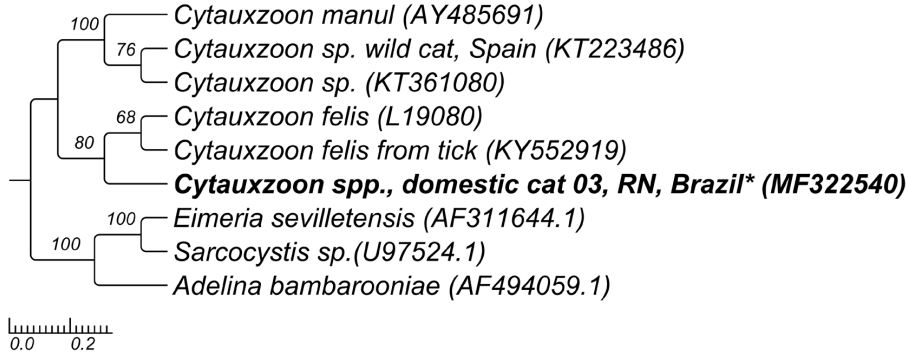

Figure 3. Phylogenetic tree constructed with 320 pb of *Cytauxzoon*-18SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Eimeria seviletensis* (AF311644), *Sarcocystis* sp. (U97524), and *Adelina bambarooniae* (AF494059) as outgroups.

positioned near to *E. canis* (JX437966; EF195135; KC109445) identified in dogs sampled from different Brazilian regions, as well as *Ehrlichia* sp. (JN123433) detected in a cat from São Luís, Maranhão state, and supported by a high posteriori probability (100%) in Bayesian analysis (Figure 4). Lastly, the *Mycoplasma* sequence amplified in our study was positioned closely to 'Candidatus Mycoplasma haemominutum' and supported by a high posteriori probability (100%) in Bayesian analysis (Figure 5).

The role of several FVBP as a cause of disease in cats has not been clearly determined. In fact, with the exception of *Bartonella* spp. and hemotrophic mycoplasmas (ANDRÉ et al., 2014, 2015), FVBP have been less frequently studied in Brazil

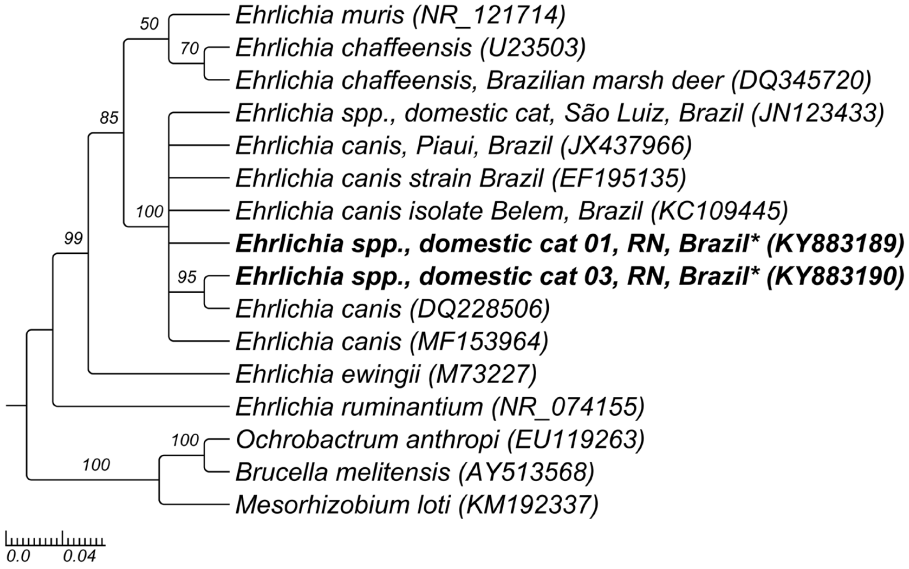

Figure 4. Phylogenetic tree constructed with 860 pb *Ehrlichia* spp.-16SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

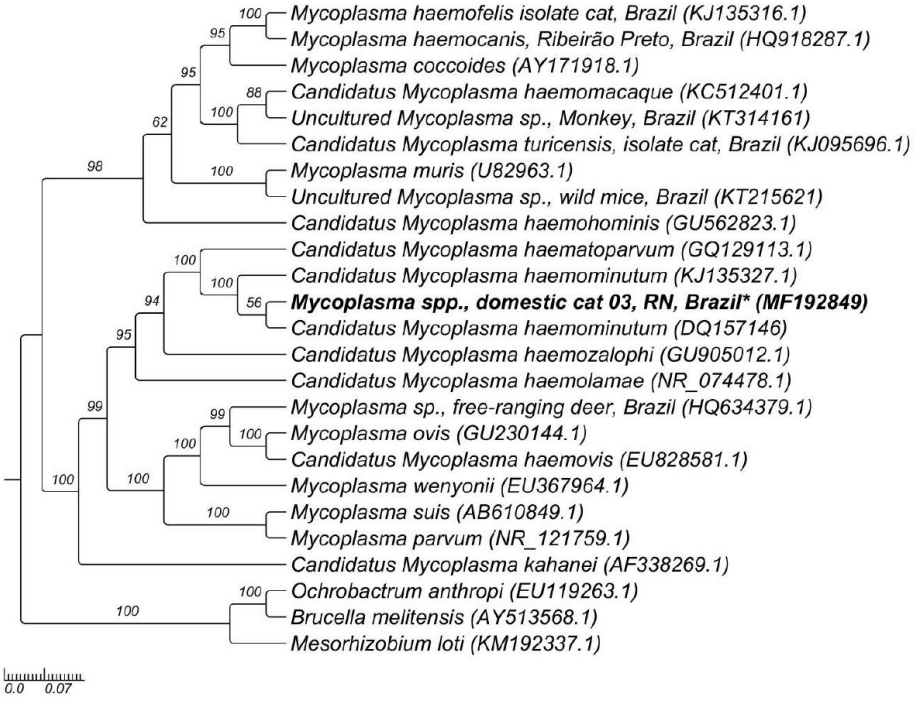

Figure 5. Phylogenetic tree constructed with 800pb *Mycoplasma* spp.16SrRNA sequences, using Bayesian method and GTR+G+I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Mesorhizobium loti* (KM192337), *Brucella melitensis* (AY513568) and *Ochrobactrum anthropi* (EU119263) as outgroups.

and are generally under-estimated as a clinical entity in cats, as compared to dogs or people.

Feline infection with pathogens from order Rickettsiales is mostly associated with non-specific clinical signs, such as anorexia, lethargy, dehydration and fever (PENNISI et al., 2017). In Brazil, the occurrence of *Ehrlichia* sp. closely related to *E. canis* has been already reported in cats sampled in the states of Minas Gerais (OLIVEIRA et al., 2009), Maranhão (BRAGA et al., 2012), Mato Grosso (BRAGA et al., 2013, 2014) and Mato Grosso do Sul (ANDRÉ et al., 2015), and in wild felids maintained in captive in zoos in the state of São Paulo and Brasília (ANDRÉ et al., 2010; 2012). Although both cats positive in PCR assays for *Ehrlichia* sp. did not show any hematological alteration, low erythrocyte count, thrombocytopenia, lymphopenia, and monocytosis were previously reported in *Ehrlichia*-PCR positive cats in midwestern Brazil (BRAGA et al., 2013).

Even though the positive cats sampled in the present study for *Anaplasma* sp. did not show any hematological disorders, neutrophilia with left shift, lymphopenia and thrombocytopenia have been found in *A. phagocytophilum*-naturally infected cats (BJÖERSDORFF et al., 1999; LAPPIN et al., 2004). In Brazil, genotypes closely related to *A. phagocytophilum* has been detected in stray cats São Paulo (ANDRÉ et al., 2014) and in wild felids maintained in captivity (ANDRÉ et al., 2012). Once infected, cats seem to remain chronic carriers for rickettsial agents (PENNISI et al., 2017).

In the present study, two cats were infected by '*Candidatus* M. haemominutum', the most frequent hemoplasma species found in cats, albeit showing a low pathogenicity (TASKER, 2010). This hemoplasma species frequently does not induce anemia, unless concurrent disease occurs (TASKER, 2010). In the present study, the two hemoplasma positive-cats were co-infected with other hemoparasites and the erythrocyte count was within the reference values.

Herein, two out of three cats presenting structures similar to piroplasms inside erythrocytes were positive for *Cytauxzoon* spp. The phylogenetic analysis positioned one obtained 18S rRNA sequence near to *C. felis*, corroborating previous studies involving wild felids (ANDRÉ et al., 2009) and domesticated cats (MAIA et al., 2013; ANDRÉ et al., 2015) in Brazil. The occurrence of *Cytauxzoon* sp. seems to be more frequent among wild felids than in domestic cats in Brazil (ANDRÉ et al., 2015). In fact, neotropical wild felids may act as reservoirs for species of family Theileriidae in South America (ANDRÉ et al., 2009). Clinico-pathological findings associated to cytauxzoonosis, such as anemia, thrombocytopenia, leukopenia, hypoproteinemia, and hyperbilirrubinemia (SHERRILL & COHN, 2015) were not found in the two *Cytauxzoon*-PCR positive cats. Therefore, we assumed that both cats might have survived to the schyzogonous phase, the most life-threatening phase of this parasite (WANG et al., 2017). In this case, both cats may act as chronic carriers, playing a role as source of infection for arthropod vectors.

One animal (Cat#2) was negative by molecular testing in spite of showing suggestive intra-erythrocytic piroplasmids structures in blood smears. Although rapid diagnosis can be performed with thin blood smears, false positive results are often observed, since pleomorphic piroplasms may be mistaken as Howell-Jolly bodies, stain precipitate, water artifacts, or even hemoplasmas (WANG et al., 2017), *Babesia vogeli* and *Theileria* spp. (ANDRÉ et al., 2014, 2015). Therefore, the molecular confirmation of inclusions in feline erythrocytes is necessary in order to achieve a correct diagnosis and choose the correct therapy.

In the present study, co-infection by *Ehrlichia, Anaplasma, Cytauxzoon* and *'Candidatus* Mycoplasma haemominutum' were detected in two cats. Previously, co-positivity for *Ehrlichia/Anaplasma* and *Babesia* was reported in cats in Portugal (MAIA et al., 2014). In Brazil, co-positivity for *B. vogeli* and '*Candidatus* Mycoplasma haemominutum' was reported among stray cats in São Paulo (ANDRÉ et al., 2014). A cat co-infected by and *Cytauxzoon* sp. and '*Candidatus* Mycoplasma haemominutum' died in Rio de Janeiro (MAIA et al., 2013). It is already known that co-infections with different canine vector-borne pathogens are quite frequent in dogs living in geographic areas where the presence of competent vectors overlaps (OTRANTO et al., 2009). It seems like that this pattern is also valid for feline vector-borne pathogens. The effect of multiple arthropod-borne hemoparasites in the pathogenesis of feline vector-borne diseases warrants further investigation. Therefore, veterinarians should keep in mind that cats co-infected by several FVBP may present a non-characteristic, and even severe, clinical outcome, which will further complicate the diagnosis, treatment and prognosis (MAIA et al., 2014).

In conclusion, the present work emphasizes the need for molecular confirmation of co-infection by multiple vector-borne pathogens in cats presenting non-specific clinical signs and inclusions resembling hemoparasites in blood smears.

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