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Original article

Diversity of piroplasmids among wild and domestic mammals and ectoparasites in Pantanal wetland, Brazil

Keyla Carstens Marques de Sousa[a], Marina Pugnaghi Fernandes[a], Heitor Miraglia Herrera[b], Carla Roberta Freschi[c], Rosangela Zacarias Machado[a], Marcos Rogério André[a,*]

[a] Universidade Estadual Paulista (Unesp), Faculdade de Ciências Agrárias e Veterinárias, Jaboticabal, SP, Brazil
[b] Universidade Católica Dom Bosco, Campo Grande, MS, Brazil
[c] Imunodot Diagnósticos Ltda, Jaboticabal, SP, Brazil

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ABSTRACT

Piroplasmoses are one of the most prevalent arthropod-borne diseases of animals. The present work aimed to investigate the occurrence of piroplasmid in wild mammals, domestic dogs and ectoparasites in southern Pantanal region, central-western Brazil. For that purpose, blood or tissue samples from 31 *Nasua nasua*, 78 *Cerdocyon thous*, 7 *Leopardus pardalis*, 42 dogs, 110 wild rodents, and 30 marsupials, and 1582 ticks were submitted to PCR assays for piroplasmid targeting 18SrRNA and hps70 genes. Seven dogs, one *C. thous,* five *L. pardalis*, three *N. nasua*, six wild rodents, eight *Amblyomma parvum*, two *Amblyomma sculptum* and one *Amblyomma ovale* were positive for piroplasmid-PCR assays. Genotypes closely related to *Babesia vogeli* were detected in six dogs and five wild rodents. While genotypes closely related to *Babesia caballi* were detected in one *C. thous*, one dog, one *A. ovale* and one *A. sculptum,* genotypes closely related to *Babesia bigemina* and *Babesia bovis* were detected in four *A. parvum* ticks. Four sequences obtained from *A. parvum*, three coatis and one wild rodent were closely related to *Theileria equi. Cytauxzoon* spp. was detected in four ocelots. The present study revealed that wild and domestic animals in Brazilian southern Pantanal are exposed to different piroplasmid species.

1. Introduction

Piroplasmid (Piroplasmida) are apicomplexan protozoa including the genera *Babesia*, *Theileria*, *Cytauxzoon* and *Rangelia* (Yabsley and Shock, 2013). These agents are tick-borne protozoans that parasitize blood cells of numerous wild and domestic vertebrates worldwide (Alvarado-Rybak et al., 2016). These parasites have a great economic and veterinary impact, being considered the second most commonly parasites found in the blood of mammals after trypanosomes (Schnittger et al., 2012). In the vertebrate hosts, the infection is usually characterized by fever, anemia and hemoglobinuria, and in severe cases, can lead to death (Kuttler, 1988). Although some of these parasites can cause diseases in animals and humans, the vectors are still unknown for many piroplasm species (Kjemtrup et al., 2000; Hersh et al., 2012).

Previously, the classification of piroplasmids relied only on host of origin, size and shape of trophozoites (small or large) and the number of merozoites within erythrocytes. However, the identification based on host origin has been invalidated, since many of these parasites are not host-specific (Penzhorn, 2006; Criado-Fornelio et al., 2003; Yabsley and Shock, 2013). Besides, the diagnosis based only on direct observations of blood smears does not always allow species identification and usually molecular assays are necessary in order to identify the etiological agent involved (Criado-Fornelio et al., 2003). In the last few years, the advent of molecular techniques has contributed to an expressive increase in the number of studies reporting infection with piroplasmids in wild animals worldwide (Alvarado-Rybak et al., 2016).

In Brazil, there are few reports concerning the seroprevalence and molecular detection of piroplasmid in wild carnivores. For instance, André et al. (2011) found a seroprevalence of 31.7% and 10.3% against *B. vogeli* antigen among wild felines and canids maintained in captivity, respectively. Additionally, André et al. (2011) detected a genotype closely related to *B. leo* in a neotropical wild cat (*Oncifelis colocolo*) and Cape genet (*Genetta tigrina*) also maintained in captivity in zoos in the state of São Paulo, Brazil. In addition to this, fatal cases of cytauxzoonosis were reported in two lions maintained in capitivity in a zoo in the

* Corresponding author at: Laboratório de Imunoparasitologia, Departamento de Patologia Veterinária, Faculdade de Ciências Agrárias e Veterinárias Júlio de Mesquita Filho (UNESP), Campus de Jaboticabal, Via de Acesso Prof. Paulo Donato Castellane, s/n, Zona Rural, CEP: 14884-900, Jaboticabal, São Paulo, Brazil.
E-mail address: marcos_andre@fcav.unesp.br (M.R. André).

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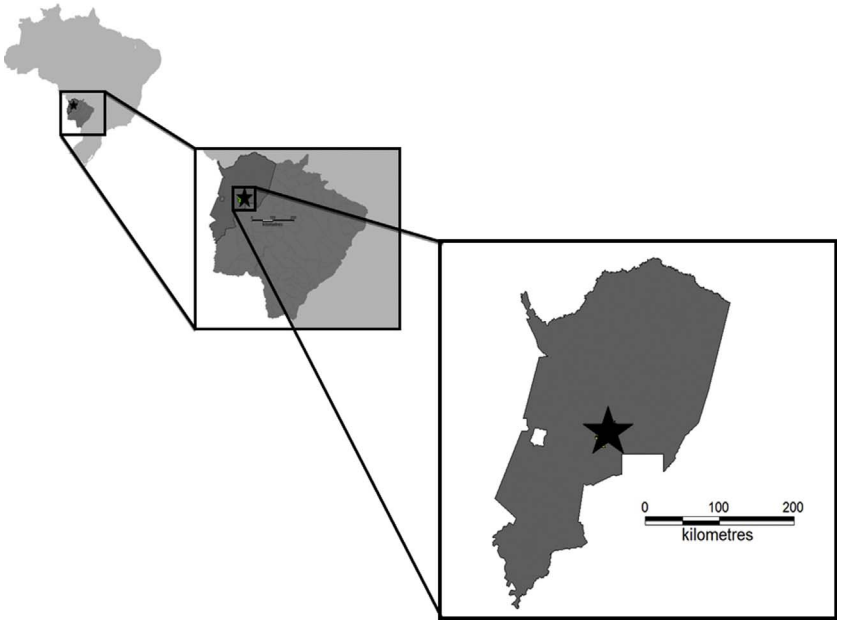

Fig. 1. Capture sites. Map of Mato Grosso do Sul State, central-western Brazil, showing the Pantanal region, where mammals' blood and spleen samples and ticks were collected in the present study.

state of Rio de Janeiro (Peixoto et al., 2007). Besides, André et al. (2009) detected *Cytauxzoon* DNA closely related to *Cytauxzoon felis* in asymptomatic neotropical felines also maintained in captivity in zoos in the cities of São Paulo and Brasília. Furthermore, *Cytauxzoon* sp. has also been molecularly detected in domestic cats from the states of Rio de Janeiro (Maia et al., 2013) and Mato Grosso do Sul (André et al., 2015).

Due to the lack of information about the epidemiology and transmission routes of piroplasms among wild animals in Brazil, the present study aimed to investigate the occurrence of piroplasmids in wild mammals and domestic dogs and their respective ectoparasites in the region of Pantanal, state of Mato Grosso do Sul, central-western Brazil.

2. Materials and methods

2.1. Study area

The fieldwork was conducted at the Nhumirim ranch (56°39′ W, 18°59′ S), located in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul, central-western Brazil (Fig. 1). This region is characterized by a mosaic of semi-deciduous forest, arboreal savannas, seasonally flooded fields covered by grasslands with dispersed shrubs and several temporary and permanent ponds. The Pantanal is the largest Neotropical floodplain, being well known for its rich biodiversity. Two well-defined seasons are recognized in that region: a rainy summer (October to March) and a dry winter (April to September) (Sousa et al., 2017a, 2017b).

2.2. Biological sampling

Between August 2013 and March 2015, a total of 256 animals were captured in the central region of the Pantanal, municipality of Corumbá, state of Mato Grosso do Sul: 158 carnivores, among them 78 crab-eating foxes (*C. thous*), 31 coatis (*N. nasua*) and seven ocelots (*L. pardalis*); 140 small mammals, among them 110 wild rodents (77 *Thrichomys fosteri*, 25 *Oecomys mamorae* and 8 *Clyomys laticeps*) and 30 wild marsupials (14 *Thylamys macrurus,* 11 *Gracilinanus agilis*, 4 *Monodelphis domestica* and 1 *Didelphis albiventris*). Additionally, 42 blood samples from domestic dogs cohabiting the same studied area were collected. Blood samples were collected from carnivores and domestic dogs by puncture of the cephalic vein and stored in Vacutainer® tubes with EDTA and without EDTA, in order to obtain total blood and serum samples for molecular and serological assays, respectively. Spleen samples were collected from small mammals and stored in absolute ethanol (Merck®, Kenilworth, Nova Jersey, USA) for molecular assays. All blood and serum samples were stored at −20 °C. The DNA extraction and serological assays were performed one week after the captures. All animal captures were in accordance with the licenses obtained from the Brazilian Government Institute for Wildlife and Natural Resources Care (IBAMA) (license numbers 38145, 38787-2) and endorsed by the Ethics Committee of FCAV/UNESP University (Faculdade de Ciências Agrárias e Veterinárias, Universidade Estadual Paulista "Júlio de Mesquita Filho", Câmpus Jaboticabal) n° 006772/13 (Sousa et al., 2017a, 2017b).

One thousand five hundred and eighty-two ticks parasitizing the sampled mammals were collected, of which 1033 (65.2% [115 adults and 918 nymphs]) belonging to *A. sculptum* Berlese, 241 (15.2% [78 adults and 163 nymphs]) belonging to *A. parvum* Aragão, 32 (2%) *A. ovale* Koch adults, one (0.06%) *Amblyomma tigrinum* Koch adult, one (0.06%) *Rhipicephalus (Boophilus) microplus* (Canestrini) adult, one (0.06%) *Rhipicephalus sanguineus* s.l. (Latreille) adult, four (0.2%) *Amblyomma auricularium* (Conil) nymphs, and 269 (17%) *Amblyomma* larvae. Besides, a total of 80 *Polygenis* (*Polygenis*) *bohlsi bohlsi* (Wagner) fleas were collected (Sousa et al., 2017a, 2017b).

2.3. Giemsa-stained blood smears

Blood smears were performed using peripheral blood collected from wild carnivores and domestic dogs, fixed with methanol and stained with Giemsa (Giemsa stain, modified, Sigma-Aldrich®, St. Louis, MO, USA).

2.4. Enzyme-linked immunosorbent assay (ELISA)

In order to detect IgG antibodies to *B. vogeli*, canids (*C. thous* and dogs) serum samples were individually tested by an ELISA assay using a commercial kit (IMUNODOT, Diagnósticos Ltda®, Jaboticabal, SP, Brazil), according to the manufacturer's instructions.

2.5. DNA extraction

DNA was extracted from 200 µL of each whole blood (158 wild

Table 1
Oligonucleotides sequences, target genes, cycling conditions and PCR products sizes used in conventional PCR assays targeting mammals and ticks endogenous genes, and 18S rRNA and heat shock protein 70 (*hsp70*) piroplasmid genes in biological samples from wild mammals domestic dogs and their respective ectoparasites, sampled in Pantanal wetland, Brazil.

Oligonucleotides sequences (5′-3′)	Target gene	Cycling conditions	PCR Product Size	References
GAPDH-F (CCTTCATTGACCTCAACTACAT) GAPDH-R (CCAAAGTTGTCATGGATGACC)	GAPDH/Mammals	95 °C for 5 min; 35 cycles of 95 °C for 15 s, 50 °C for 30 s and 72 °C for 30 s; and final extension of 72 °C for 5 min.	400 pb	Birkenheuer et al. (2003)
16S + 1 (CTGCTCAATGATTTTTTAAATTGCTGTGG) 16S − 1 (CCGGTCTGAACTCAGATCAAGT)	16SrRNA/Ticks	10 cycles of 92 °C for 1 min, 48 °C for 1 min and 72 °C for 1 min, followed by 32 cycles of 92 °C for 1 min, 54 °C for 35 s and 72 °C for 1,35 min, and final extension of 72 °C for 7 min.	460 pb	Black and Piesman (1994)
HC02198 (TAAACTTCAGGGTGACCAAAAAATCA) LCO1490 (GGTCAACAAATCATAAAGATATTGG)	COX1/Fleas	95 °C for 1 min, 35 cycles of 95 °C for 15 s, 55 °C for 15 s and 72 °C for 10 s, and final extension of 72 °C for 5 min.	710 pb	Folmer et al. (1994)
HepF300 (GTTTCTGACCTATCAGCTTTCGACG) HepR900 (CAAATCTAAGAATTTCACCTCTGAC)	18SrRNA/*Hepatozoon*	94 °C for 3 min, 35 cycles of 94 °C for 30 s, 60 °C for 30 s and 72 °C for 1 min, and final extension of 72 °C for 7 min.	600 pb	Ujvari et al. (2004)
BTF1 (GGCTCATTACAACAGTTATAG) BTR1 (CCCAAAGACTTTGATTTCTCTC) BTF2 (CCGTGCTAATTGTAGGGCTAATAC) BTR2 (GGACTACGACGGTATCTGATCG)	18SrRNA/*Babesia* sp., *Theileria* sp., *Rangelia vitalii* and *Cytauxzoon* sp.	1°Round: 94 °C for 3 min, 1 min for 58 °C and 2 min for 72 °C; 45 cycles of 94 °C for 30 s, 58 °C for 20 s and 72 °C for 30 s; and final extension of 72 °C for 5 min. 2° Round: 94 °C for 3 min, 1 min for 62 °C and 2 min for 72 °C; 45 cycles of 94 °C for 30 s, 64 °C for 20 s and 72 °C for 30 s; and final extension of 72 °C for 5 min.	800 pb	Jefferies et al. (2007)
Cy-F (GCGAATCGCATTGCTTTATGCT) Cy-R (CCAAATGATACTCCGGAAAGAG)	18SrRNA/*Cytauxzoon* sp.	94 °C for 5 min; 40 cycles of 95 °C for 45 s, 59 °C for 45 s and 72 °C for 1 min; and final extension of 72 °C for 5 min.	300 pb	Birkenheuer et al. (2006)
hsp70F1 (CATGAAGCACTGGCCHTTCAA) hsp70 R1 (GCNCKGCTGATGGTGGTGTTGTA)	*hsp70/Babesia* spp., *Rangelia vitalii* and *Theileria* sp.	95 °C for 5 min; 35 cycles of 95 °C for 15 s, 60 °C for 30 s and 72 °C for 30 s; and final extension of 72 °C for 5 min.	740 pb	Soares et al. (2011)

carnivores and 42 domestic dogs) and 10 mg of spleen (140 small mammals) samples using the QIAamp DNA Blood Mini kit (QIAGEN®, Valencia, CA, USA), according to the manufacturer's instructions. The total number of individual tick samples or pools was 523, of which 228 (43.5%) were from adults, 256 (48.9%) pooled nymphs, and 39 (7.4%) from pooled larvae. DNA extraction from ticks was processed in pools for nymphs (up to 5 individuals) and larvae (up to 10 individuals), while the adults were processed individually. A total of 39 pooled fleas samples were submitted to DNA extraction. DNA extraction from fleas was also performed in pools consisting of up to five individuals. Ticks and fleas were macerated and submitted to DNA extraction, using the same kit before mentioned (Sousa et al., 2017a, 2017b).

2.6. PCR for endogenous genes

In order to verify the presence of amplifiable DNA in the samples, internal control PCR assays targeting fragments of mammalian glyceraldehyde-3-phosphatedehydrogenase (GAPDH), ticks mitochondrial 16S rRNA and fleas cytochrome-c oxidase subunit I (COX1) genes were performed (Table 1). All 298 DNA animal samples amplified the predicted product for GAPDH gene. Out of 523 sampled ticks, 31 (5.9% [23 *A. parvum* adults, 4 *A. sculptum* adults, 1 *A. ovale* adult, 1 *A. parvum* nymph and 2 pooled *Amblyomma* larvae]) showed negative results for the tick mitochondrial 16S rRNA gene and were excluded from subsequent analyses. Only one flea DNA sample did not amplify the predicted product for cox-1 and was also excluded from subsequent analyses (Sousa et al., 2017a, 2017b).

2.7. PCR assays

Previously described PCR protocols based on 18SrRNA gene and heat shock protein 70 gene (*hsp70*) were performed in order to amplify *Babesia* spp., *Cytauxzoon* sp. and *Theileria* sp. DNA (Ujvari et al., 2004; Birkenheuer et al., 2006; Jefferies et al., 2007; Soares et al., 2011) (Table 1). Each sample of extracted DNA was used as a template in 25 µL PCR reactions. The mixture containing 10X PCR buffer (Life Technologies®, Carlsbad, CA, USA), 1.0 mM $MgCl_2$ (Life Technologies®, Carlsbad, CA, USA), 0.2 mM deoxynucleotide triphosphate (dNTPs) mixture (Life Technologies®, Carlsbad, CA, USA), 1.5 U Taq DNA Polymerase (Life Technologies®, Carlsbad, CA, USA), and 0.5 µM of each primer (Integrated DNA Technologies®, Coralville, IA, USA). *Babesia vogeli, Cytauxzoon* sp. and *Theileria* sp. DNA positive controls were obtained from naturally infected cats (André et al., 2015). Ultra-pure sterile water (Life Technologies®, Carlsbad, CA, USA) was used as negative control. PCR products were separated by electrophoresis on 1% agarose gel stained with ethidium bromide (Life Technologies®, Carlsbad, CA, USA). In order to prevent PCR contamination, DNA extraction, reaction setup, PCR amplification and electrophoresis were performed in separated rooms. The gels were imaged under ultraviolet light using the Image Lab Software version 4.1 (Bio-Rad®). The reaction products were purified using the Silica Bead DNA gel extraction kit (Thermo Fisher Scientific®, Waltham, MA, USA). The sequencing was carried out 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).

2.8. Bioinformatics/Phylogenetic analysis

The sequences obtained from positive samples were first submitted to a screening test using Phred-Phrap software version 23 (Ewing and Green, 1998; Ewing et al., 1998) in order to evaluate the electropherogram quality and obtain consensus sequences from the alignment of sense and antisense sequences. The BLAST program (Altschul et al., 1990) was used to analyze the sequences of nucleotides (BLASTn), aiming to browse and compare with sequences from international database (GenBank) (Benson et al., 2002). All sequences that showed appropriate quality standards and identity with piroplasmid species were deposited in GenBank. Samples showing positive results for both PCR protocols (18SrRNA and *hsp70*) had their sequences concatenated, using the Fragment Merger software version 1 (Bell and Kramvis, 2013). The sequences were aligned with sequences published in GenBank using MAFFT software, version 7 (Katoh and Standley, 2013).

Phylogenetic inference was based on Bayesian Inference (BI). The BI

analysis was performed with MrBayes 3.1.2 (Ronquist and 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 jModelTest 2 (version 2.1.6) on XSEDE (Darriba et al., 2012), under the Akaike Information Criterion (AIC) (Posada and Buckley, 2004). All phylogenetic analyses were performed using CIPRES Science Gateway (Miller et al., 2010). The trees were examined in Treegraph 2.0.56-381 beta (Stover and Muller, 2010).

3. Results

The average absorbance of negative sera provided in ELISA kit was 0.157 (0.198 ± 0.129), resulting in a cut-off value of 0.393. Thirty-nine (92.8%) dogs and 42 (53.8%) *C. thous* were seroreactive to *B. vogeli* antigen. The mean antibodies absorbance values of seropositive dogs and crab-eating foxes were 0.734 (1.431 ± 0.450) and 0.691 (1.211 ± 0.411), respectively.

Seven (16.6%) dogs, one (1.2%) crab-eating fox, three (42.8%) ocelots, six (7.8%) *T. fosteri* wild rodents, eight (6%) *A. parvum* (5 adults and 3 nymphal pools), two (0.6%) *A. sculptum* (1 adults and 1 nymphal pool) and one (3.1%) *A. ovale* adult (1.2%) were positive for 18SrRNA-PCR based on Jefferies et al. (2007) protocol. Three (9.6%) coatis and one (14.2%) ocelot were positive for 18SrRNA-PCR based on Ujvari et al. (2004) and Birkenheuer et al. (2006) protocols, respectively. Three out of seven positive dogs in 18SrRNA-PCR and two out of eight positive *A. parvum* in 18SrRNA-PCR were also positive in *hsp70*-PCR based on Soares et al. (2011) protocol. All the sampled fleas were negative in PCRs assays for piroplasmids. All sequences obtained from the positive samples were deposited in GenBank international database under the accession numbers KY450716-KY450752.

Regarding the presence of inclusions suggestive of piroplasmid in Giemsa-stained blood smears, piroplasms and Maltese cross forms were observed within one ocelot́s erythrocytes. Additionally, piroplasms were found in one coatís erythrocytes (Fig. 2). Both animals were positive in 18SrRNA PCR assays for piroplasmid.

Six out of seven 18S rRNA-piroplasmid sequences obtained from domestic dogs showed 100% of identity with *B. vogeli* previously deposited in GenBank (KT323934) by BLAST analysis. One 18S rRNA-piroplasmid sequence obtained from a domestic dog showed 100% of identity with *B. caballi* (EU642512). While five out of six 18S rRNA-piroplasmid sequences obtained from *T. fosteri* showed 99% of identity with *B. vogeli* (KT323934), one of them showed 99% of identity with *T. equi* (KU672386) by BLAST analysis. One 18S rRNA-piroplasmid sequence obtained from a crab-eating fox, one sequence obtained from an *A. ovale* adult and one sequence obtained from an *A. sculptum* adult showed 100% of identity with *B. caballi* previously deposited in GenBank (EU642512). While four 18S rRNA-piroplasmid sequences obtained from *A. parvum* adults showed 99% of identity with *T. equi* (KU672386), four others 18S rRNA-piroplasmid sequences obtained from *A. parvum* (1 adult and three nymphal pools) showed 97% of identity with a sequence of *Babesia* sp. obtained from *Odocoileus virginianus*, from USA (HQ264119). An 18S rRNA-piroplasmid sequence obtained from an *A. sculptum* nymphal pool showed 99% of identity with a sequence of *Theileria* sp. obtained from *Rangifer tarandus tarandus*, also from USA (JN086224).

Surprisingly, three coatis' blood samples amplified piroplasmid DNA sequences using a PCR protocol for *Hepatozoon* based on 18SrRNA gene (Ujvari et al., 2004). The amplified sequences showed 100% of identity with a *Theileria* sp. sequence obtained from a domestic cat sampled in Brazil (KF970930). These results were obtained in a previous study that aimed to perform the molecular detection of *Hepatozoon* spp. in the same animal samples (Sousa et al., 2017a). These results showed that the primers previously designed by Ujvari et al. (2004) may also anneal to piroplasmid DNA.

All 18S rRNA-piroplasmid sequences obtained from ocelots (n = 4) showed 99% of identity with *C. felis* previously deposited in GenBank (GU903911) by BLAST analysis (Tables 2 and 3). Three *hsp70*-piroplasmid sequences obtained from domestic dogs showed 100% of identity with *B. vogeli* (AB248733). Finally, two *hsp70*-piroplasmid sequences obtained from *A. parvum* nymphal pools showed 90% of identity with *B. canis* (AB248735), by BLAST analysis.

The phylogenetic analysis based on 18S rRNA gene fragments clustered six piroplasmid sequences obtained from dogs' blood samples (KY450732, KY450733, KY450734, KY450735, KY450736, KY450737) and five sequences obtained from *T. fosteri* spleen samples (KY450738,

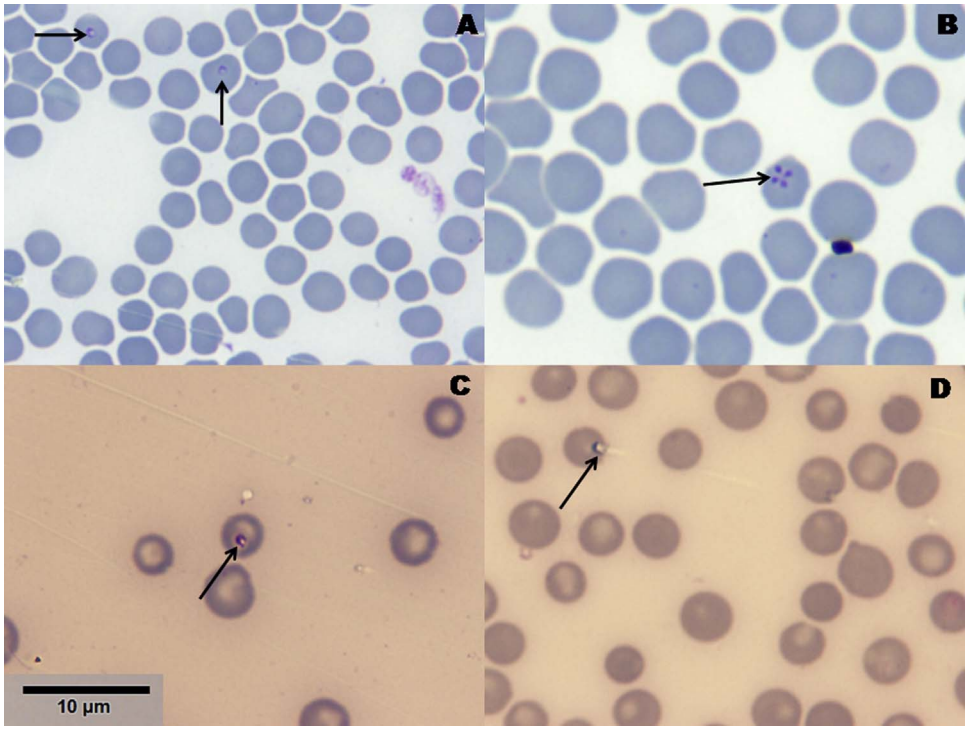

Fig. 2. Forms morphologically similar to piroplasmids found in Giemsa-stained blood smears from sampled mammals in Brazilian southern Pantanal. (A) Individual forms found within erythrocytes from an ocelot. (B) Maltese-cross inclusion found within erythrocytes from an ocelot. (C; D) Individual forms found within erythrocytes from coatis sampled in southern Pantanal.

Table 2
Maximum identity of 18S rRNA and *hsp70* piroplasmid sequences detected in wild and domestic animals in Brazilian Pantanal by BLAST analysis.

Host	Number of sequences analyzed	Target gene	% identity by BLAST®- analysis
Cerdocyon thous	1	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Thrichomys fosteri	5	18SrRNA	99% *Babesia vogeli* (KT323934)
Thrichomys fosteri	1	18SrRNA	99%*Theileria equi* (KU672386)
Canis familiaris	5	18SrRNA	100% *Babesia vogeli* (KT323934)
Canis familiaris	1	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Nasua nasua	3	18SrRNA	100% *Theileria* sp. (KP410272)
Leopardus pardalis	4	18SrRNA	99% *Cytauxzoon felis* (GU903911)
Canis familiaris	3	*hsp70*	100% *Babesia vogeli* (AB248733)

KY450739, KY450740, KY450741, KY450742) in a branch comprising *B. vogeli* sequences (AY371196, HM590440) previously deposited in GenBank, with clade support value of 100, based on BI analysis. 18SrRNA-piroplasmid sequences obtained from one *C. thous* blood sample (KY450725), one domestic dog blood sample (KY450731), one *A. ovale* adult (KY450726), and one *A. sculptum* adult (KY450730) were closely related to *B. caballi* (EU888901, EU642512), with clade support value of 100, based on BI. Four 18SrRNA-piroplasmid sequences obtained from *A. parvum* (1 adult [KY450743], three sequences obtained from nymphal pools [KY450727, KY450728, KY450729]), and a *Babesia* sp. sequence, previously deposited in GenBank and obtained from *Odocoileus virginianus* sampled in the USA (HQ264119), were grouped in the same clade of *B. bigemina* (JQ723014) and *B bovis* (L19077) based on BI analysis. 18SrRNA-piroplasmid sequences obtained from four *A. parvum* adults (KY450716, KY450717, KY450718, KY450716), three coatis blood samples (KY450722, KY450723, KY450724) and one *T. fosteri* spleen sample (KY450720) were grouped into the same large branch of *T. equi* sequences (KU672386, AY150064, AB515315), with clade support value of 99 in BI analysis. One 18SrRNA-piroplasmid sequence obtained from an *A. sculptum* nymphal pool (KY450721) was placed in the same clade of *Theileria* sp. sequence obtained from *Rangifer tarandus tarandus* (JN086224) sampled in the USA, with clade support value of 100, based on BI analysis. Four 18SrRNA-piroplasmid sequences obtained from ocelots blood samples (KY450744, KY450745, KY450746, KY450747) was pooled in the same clade of *C. felis* sequences previously deposited in GenBank (DQ382277, L19080), with clade support value of 100, based on BI analysis (Fig. 3). 18SrRNA-phylogenetic inference based on BI method was performed using the evolutionary model GTR + G + I and *Coccidia* sp. (HM117907), *Isospora suis* (U97523), *Sarcocystis* sp. (U97524), and *Adelina bambarooniae* (AF494059) as outgroups.

Regarding the concatenated phylogenetic analysis of piroplasmid based on 18SrRNA and *hsp70* genes fragments, two piroplasms sequences obtained from *A. parvum* nymphal pools were pooled in the same branch of *R. vitalii* sequences (JF279603; KF218606), with clade support value of 94, based on BI analysis. The remaining three piroplasms *hsp70* + 18SrRNA concatenated sequences obtained from dogs blood samples were grouped in the same clade of *B. vogeli* sequences (AB248733; AY371196), with clade support value of 100 in BI analysis (Fig. 4). Concatenated 18SrRNA + *hsp70*-phylogenetic inference based on BI method were performed using the GTR + G + I evolution model and *Plasmodium falciparum* (M19753; JQ627152) as outgroup.

4. Discussion

The present study showed the presence of piroplasmid DNA in blood or spleen samples from wild carnivores, domestic dogs, rodents and ticks sampled in the southern region of Pantanal, state of Mato Grosso do Sul, central-western Brazil. Molecular analyses based on 18S rRNA and *hsp70* genes revealed an occurrence of different piroplasmid species among sampled mammals and arthropods.

Babesia vogeli is a widespread tick-borne protozoan in dogs in Brazil (Passos et al., 2005; O'Dwyer et al., 2009; Sousa et al., 2013; da Costa et al., 2015; Moraes et al., 2015), in accordance with the wide distribution of its vector, *R. sanguineus* s.l., mainly in urban and peripheral urban areas (Labruna and Pereira, 2001). In the present study, the molecular occurrence of *B. vogeli* (16.6%) among sampled dogs was higher than that found (3.3%) in a previous study involving dogs in an urban area from the same state of Mato Grosso do Sul (Sousa et al., 2013) and dogs (3.13%) sampled in northern Pantanal, state of Mato Grosso (Melo et al., 2016). Herein, the high seroprevalence (92.8%) to *B. vogeli* found among dogs was similar to that found (81.6%) among dogs sampled in an urban area from the same state of Mato Grosso do Sul (Sousa et al., 2013). Infection by *B. vogeli* in dogs seems to be common among dogs from the state of Mato Grosso do Sul.

On the other hand, reports of seropositivity to *B. vogeli* among wild canids are scarce in Brazil (André et al., 2011). A higher seroprevalence (53.8%) was found among free-living crab-eating foxes sampled in the present study when compared to that found among with wild canids (10%) maintained in captivity in Brazilian zoos (André et al., 2011). Although this work showed the first serological evidence of exposure to

Table 3
Maximum identity of 18S rRNA and *hsp70* piroplasmid sequences detected in ticks collected from wild animals in Brazilian Pantanal by BLAST analysis.

Tick specimen	Host	Target gene	% identity by BLAST® analysis
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	99%*Theileria equi* (KU672386)
Amblyoma parvum adult	*Cerdocyon thous*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyoma parvum nymph	*Thrichomys fosteri*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyoma parvum nymph	*Thrichomys fosteri*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyoma parvum nymph	*Clyomys laticeps*	18SrRNA	97% *Babesia* sp. from *Odocoileus virginianus* (HQ264119)
Amblyomma sculptum adult	*Cerdocyon thous*	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Amblyomma sculptum nymph	*Cerdocyon thous*	18SrRNA	99% *Theileria* sp. from *Rangifer tarandus tarandus* (JN086224)
Amblyomma ovale adult	*Cerdocyon thous*	18SrRNA	100% *Babesia caballi* genotype A_CABEQ30 (EU642512)
Amblyoma parvum nymph	*Thrichomys fosteri*	*hsp70*	90% *Babesia canis* (AB248735)
Amblyoma parvum nymph	*Clyomys laticeps*	*hsp70*	90% *Babesia canis* (AB248735)

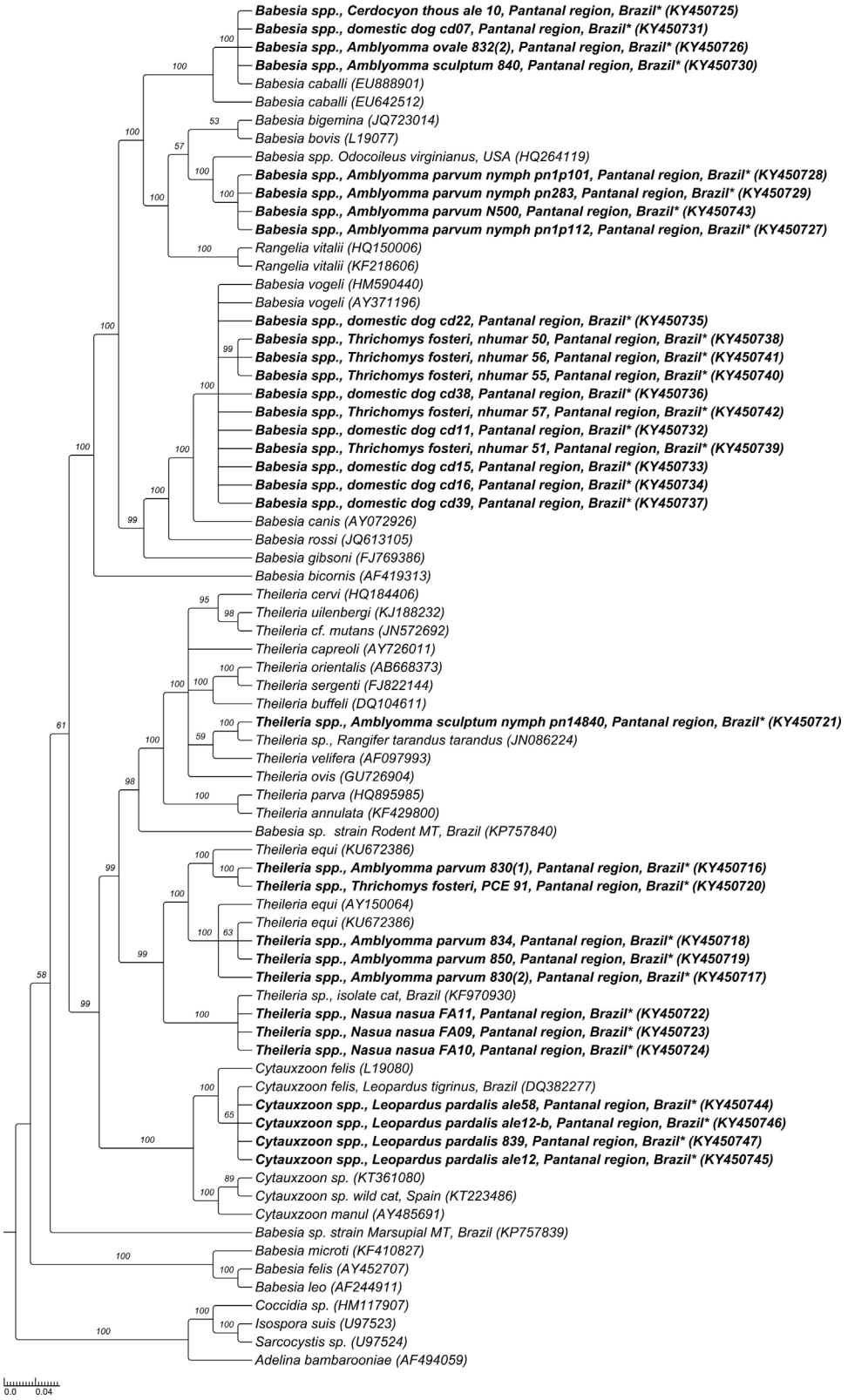

Fig. 3. Phylogenetic tree constructed with 800 pb of piroplasmid-18SrRNA sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50, using *Coccidia* sp. (HM117907), *Isospora suis* (U97523), *Sarcocystis* sp. (U97524), and *Adelina bambarooniae* (AF494059) as outgroups.

B. vogeli among free-living wild canids in Brazil, cross-reactions between *Babesia* and *Rangelia* could not be discarded. Furthermore, considering the high occurrence of *B. vogeli* found in domestic dogs sampled in the studied area, the role of these animals as a source of *B. vogeli* infection to wild carnivores cohabiting the same area should be better investigated.

In the present study, unusual findings were observed: genotypes closely related to *B. caballi* were detected in one dog and one crab-eating fox, and genotypes closely related to *T. equi* were detected in three coatis and in one wild *T. fosteri* rodent. In addition to this, *Theileria*-suggestive piroplasmid inclusions were found in a coati blood smear. This sort of unexpected DNA piroplasmid detection in non-usual

Fig. 4. Phylogenetic tree constructed with 1560 pb of piroplasmid 18SrRNA + *hsp70* sequences, using Bayesian method and GTR + G + I evolutionary model. Numbers at nodes correspond to Bayesian posterior probabilities over 50. *Plasmodium falciparum* (M19753; JQ627152) was used as outgroup.

hosts has already been reported. For instance, while *Theileria annulata*, *T. equi* and *B. caballi* DNA were detected in blood samples from dogs in Croatia (Beck et al., 2009), *B. canis* and *B. rossi* DNA were detected in blood samples from horses in France (Fritz, 2010). Besides, genotypes closely related to *T. equi* have also been detected in cats in Brazil (André et al., 2014; 2015). These unusual findings suggest that some piroplasm species may show a lack of host specificity. Alternatively, new piroplasm species closely related to those already described in domestic animals may circulate in different hosts. Furthermore, the small gene fragments used in the present study may not have allowed a robust phylogenetic positioning of the amplified sequences.

Although *Anocentor nitens* (Roby and Anthony, 1963) and *R.* (*B.*) *microplus* (Battsetseg et al., 2002) ticks are considered, respectively, the main vectors for *B. caballi* and *T. equi* in Brazil, some evidence has suggested the participation of *Amblyomma cajennense* sensu lato ticks in *T. equi* (Kerber et al., 2009; Ribeiro et al., 2011; Scoles and Ueti, 2013) and *B. caballi* (Vieira et al., 2013) transmission cycles. Herein, while *B. caballi* DNA was detected in *A. ovale* and *A. sculptum* ticks, *T. equi* DNA was detected in *A. parvum* ticks. These findings highlight the importance of future studies regarding the role of *Amblyomma* species in the epidemiological cycles of *B. caballi* and *T. equi.*

In South America, *Rangelia vitalii,* a piroplasm species associated with hemolytic disorders (Soares et al., 2011) in canids, has been reported in domestic and wild canids from southeastern and southern regions of Brazil (Soares et al., 2011, 2014; Gottlieb et al., 2016; Quadros et al., 2015; Fredo et al., 2015; Silveira et al., 2016), and in domestic dogs from Argentina (Eiras et al., 2014) and Uruguay (Soares et al., 2015). Herein, the concatenated phylogenetic analysis revealed the presence of a genotype closely related to *R. vitalii* in *A. parvum* ticks collected from *T. fosteri* and *C. laticeps* wild rodents. Previous studies suggested that *C. thous* is the natural reservoir for *R. vitalii* in Brazil (Soares et al., 2014; Fredo et al., 2015). Considering the fact that *C. thous* is frequent found in Pantanal (Mamede and Alho, 2006), future studies should be performed in order to assess the real significance of the circulation of this genotype in this Brazilian wetland.

A genotype closely related to *Theileria* sp. previously detected in a wild deer (*R. t. tarandus*) sampled in the USA was detected in *A. sculptum* nymphs collected from *C. thous* in the present study. Previously, a genotype showing 90–100% identicalness to *Theileria cervi* was detected in 52% of sampled pampas deer (*Ozotoceros bezoarticus*) in the same region of Pantanal (Silveira et al., 2013). Genotypes closely related to *Theileria* sp. associated to wild ruminants have also been detected in cats from Campo Grande, an urban area in the state of Mato Grosso do Sul (André et al., 2015). Future studies aiming at investigate the host and vectors range of wild ruminants-associated *Theileria* genotypes should be investigated in Brazilian Pantanal.

While the epidemiology of *Cytauxzoon* sp. has been extensively studied in the USA (Blouin et al., 1984; Meinkoth and Kocan, 2005; Yabsley et al., 2006; Haber et al., 2007), the occurrence of these parasites in other regions of the world has been scarcely reported. In fact, while clinical and fatal cytauxzoonosis has been well reported in the USA (Garner et al., 1996; Nietfield and Pollock, 2002), Germany (Jakob and Wesemeier, 1996) and Italy (Carli et al., 2012), the parasite has been rarely described and not associated with clinical disease in domestic cats in Brazil so far (Maia et al., 2013; André et al., 2015). Although *Cytauxzoon* sp. have already been reported in neotropical and exotic wild felids maintained in captivity in zoos in Brazil (Peixoto et al., 2007; André et al., 2009), the present work showed the first molecular detection of *Cytauxzoon* sp. among free-living wild felids in South America. Herein, a genotype closely related to *C. felis* was detected in four apparently asymptomatic ocelots. *Cytauxzoon*-suggestive maltese cross inclusions were found in one ocelot blood smear. The pathogenic potential of *Cytauxzoon* isolates in Brazil is still unknown. In fact, while fatal cytauxzoonosis has been reported in lions maintained in captivity in a zoo in the state of Rio de Janeiro (Peixoto et al., 2007), the parasite has been detected in apparently asymptomatic wild felids maintained in captivity in the state of São Paulo and Brasília (André et al., 2009). The role of neotropical wild felids as reservoirs for *Cytauxzoon* in Brazil should be further investigated. Although *A. parvum* ticks were found parasitizing the sampled ocelots, *Cytauxzoon* DNA was not detected in any collected tick specimen. While *Dermacentor variabilis* (Blouin et al., 1984) and *A. americanum* (Reichard et al., 2010) are responsible for transmitting *C. felis* among felids in the USA, the vectors involved in the *Cytauxzoon* epidemiological cycles in Brazil remains unknown.

A genotype closely related to *B. vogeli* was also detected in five *T. fosteri* wild rodents. It seems that the host range for *B. vogeli* or a genotype closed related to is wider than previously supposed, since the protozoa has also been detected in cats (et al., 2014, 2015; et al., 2014, 2015), besides dogs and rodents, in Brazil. Changes in land use may facilitate the circulation of piroplasmid-infected ticks between wild rodents and domestic and wild animals, since those animals can share the same ecotypes. Recently, a genotype closely related to *Theileria bicornis* was detected, for the first time in the American continent, in *T. fosteri* rodents sampled in northern Pantanal (Wolf et al., 2016). Infections by *T. bicornis* have been reported only in black (*Diceros bicornis michaeli*) and white (*Ceratotherium simum simum*) rhinoceros from Africa (Otiende et al., 2015). The molecular detection of a genotype closely related to *T. bicornis* in America (Wolf et al., 2016) also represents an unusual finding. Indeed, the molecular analysis of a small subunit of 18SrRNA gene (349 pb) might not have been sufficiently robust to allow an accurate phylogenetic positioning of the piroplasmid species detected in wild rodents from northern Pantanal. Future molecular studies targeting different genes are much needed in order to access the real identity of the piroplasm species circulating among Brazilian wild rodents.

Molecular studies aiming at identifying the phylogenetic positioning of piroplasmids have been mainly based on the small subunit ribosomal

18S gene, due to its conserved nature, although others genes, such as cytochrome b and the heat-shock proteins encoding genes, have also been used (Irwin, 2009). However, a common problem observed when the molecular analyses are only based on 18S rRNA sequences is that the molecular findings usually do not reflect the piroplasm morphology (Allsopp et al., 1994; Schnittger et al., 2012). This discrepancy between molecular data and parasite morphology may be due to the complexity of 18S secondary structure that could lead to inconsistencies in gene alignment (Morrison and Ellis, 1997). Mitochondrial genome sequences and structures have been showed as an alternative to analyze the relationships and for delineating specimens to the species level (Schreeg et al., 2016).

Recently, phylogenetic analyses based on mitochondrial genome sequences concatenated along with 18S rRNA sequences identified five distinct Piroplasmida lineages with different biological features, namely: i. *Babesia* sensu stricto (*B. caballi*, *B. bigemina*, *B. canis*, *B. rossi*, *B. vogeli*, *B. gibsoni* and *B. bovis*); ii. *Theileria* sp. and *Cytauxzoon* sp.; iii. *T. equi*; iv. Western *Babesia* group (*B. conradae*); v. *B. microti* group (Schreeg et al., 2016). Although the mitochondrial genome analysis was not performed in the present study, sequences apparently belonging to three out of five Piroplasmida lineages were identified. Genotypes closely related to *B. caballi* and *B. vogeli,* obtained from *C. thous, T. fosteri, A. ovale* and *A. sculptum* from the present study seems to belong to *Babesia* sensu stricto lineage, which is characterized by infecting erythrocytes from different vertebrate species and transovarial and transstadial transmission in ticks hosts (Schreeg et al., 2016). On the other hand, the sequences closely related to *Theileria* sp. and *C. felis,* obtained from *A. sculptum* nymphs and ocelots seems to belong to *Theileria* sp. and *Cytauxzoon* sp. lineage. Protozoans belonging to this lineage have developed strategies to enhance their propagation, which has been evidenced by enlarged schizont-infected leukocytes, suggesting a blocking of host cell apoptosis (Hagiwara et al., 1997; Susta et al., 2009). Piroplasmid sequences obtained from three coatis, one *T. fosteri* four *A. parvum* ticks seems to belong to *T. equi* lineage, in which host leukocyte manipulation has not been observed, differently from *Theileria* sp. and *Cytauxzoon* sp. lineage (Schreeg et al., 2016). In fact, the mitochondrial genome structure of *T. equi* has showed to be radically divergent from any other Piroplasmida species, suggesting that *T. equi* was recovered as a unique distinct lineage (Schreeg et al., 2016). Finally, although a genotype closely related to *R. vitalii* was detected in *A. parvum* ticks in the present study, the mitochondrial genome structure of *R. vitalii* has not been analysed yet, precluding its positioning in piroplasmid phylogroups. Schreeg et al. (2016) suggested that new lineages would be reported if a higher number of newly described piroplasmid were included in phylogenetic analyses. From a preliminary point of view, based on 18S rRNA and *hsp70*-concatenated phylogenetic analyses, *R. vitalii* and associated genotype detected in the present study grouped with protozoans allocated in *Babesia* s.s. group.

In conclusion, the present study revealed that wild animals and ticks in southern Pantanal region, Brazil, are exposed to a high diversity of piroplasmid species. The role of domestic dogs as a source of *B. vogeli* infection to wild animals should be better investigated. Wild and domestic animals and *Amblyomma* ticks seemed to be eventually infected by genotypes closely related to equine piroplasmid. Genotypes closely related to *R. vitalii* and ruminants-associated *Theileria* species circulate in Pantanal region. Finally, *Cytauxzoon* sp. was detected among free-living wild felids for the first time in South America. Therefore, future studies are much needed in order to estimate the impact of piroplasmid infections among the Brazilian wildlife in Pantanal biome.

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