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Original Article

Molecular survey of tick-borne pathogens in small mammals from Brazilian Amazonia

Levantamento molecular de patógenos transmitidos por carrapatos em pequenos mamíferos da Amazônia brasileira

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Abstract

Small non-volant mammals (marsupials and small rodents) were captured at three different timepoints from 23 forest fragments across three municipalities (Alta Floresta, Sinop and Cláudia) covering the Amazonian biome of the Mato Grosso State in Midwestern Brazil. The animal tissues (liver and spleen) and blood were screened using molecular tools for the detection of *Babesia*, *Coxiella*, *Cytauxzoon*, *Hepatozoon*, *Theileria*, and Anaplasmataceae agents. A total of 230 specimens (78 rodents and 152 marsupials) were trapped. *Hepatozoon* and Piroplasmorida agents were detected in the common opossums (*Didelphis marsupialis*). In turn, all samples (blood, liver, or spleen) collected from the small mammals were negative for the genus *Coxiella* and the family Anaplasmataceae, as detected by polymerase chain reaction (PCR). Phylogenetic analyses inferred from partial sequences of the 18S rRNA gene highlighted the occurrence of new *Hepatozoon* and Piroplasmorida haplotypes. Future studies determining the role of common opossum (*D. marsupialis*) in the epidemiological cycles of *Hepatozoon* and *Babesia* under natural conditions in the Amazonian biome are necessary.

Keywords: *Hepatozoon*, *Babesia*, Amazonian biome, Mato Grosso state.

Resumo

Pequenos mamíferos não voadores (marsupiais e pequenos roedores) foram capturados em três diferentes períodos, ao longo de 23 fragmentos florestais de três municípios (Alta Floresta, Sinop e Cláudia), localizados no bioma amazônico do Estado de Mato Grosso, no centro-oeste do Brasil. Os tecidos dos animais (fígado e baço) e sangue foram selecionados e submetidos a ensaios moleculares para a detecção do DNA de *Babesia*, *Coxiella*, *Cytauxzoon*, *Hepatozoon*, *Theileria* e agentes Anaplasmataceae. Um total de 230 espécimes (78 roedores e 152 marsupiais) foram capturados. *Hepatozoon* e agentes Piroplasmorida foram detectados em gambás (*Didelphis marsupialis*). Ao contrário, todas as amostras (sangue, fígado ou baço) coletadas dos pequenos mamíferos foram negativas para o gênero *Coxiella* e a família Anaplasmataceae, conforme detectado pela reação em cadeia da polimerase (PCR). Análises filogenéticas inferidas pelas sequências parciais do gene 18S rRNA evidenciaram a ocorrência de novos haplótipos de *Hepatozoon* e Piroplasmorida. Futuros estudos determinando a importância do gambá-comun (*D. marsupialis*) nos ciclos epidemiológicos de *Hepatozoon* e *Babesia* em condições naturais, no bioma amazônico, são necessários.

Palavras-chave: *Hepatozoon*, *Babesia*, bioma Amazônico, estado de Mato Grosso.

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Introduction

The interactions between animals, humans, and the environment in which both live are currently recognized as the drivers for a unified health concept, contemporary known as 'One Health' (ROBERTSON et al., 2014). Small mammals are widely distributed vertebrates and ticks constitute common parasites (DURDEN, 2006). A wide variety of vertebrate pathogens (especially certain viruses, bacteria and protozoa) evolved strategies to thrive in transmission cycles that include ticks and associated small mammals hosts (DURDEN & KEIRANS, 1996; SONENSHINE et al., 2002; DURDEN, 2006).

Vector-borne protozoa of the order Piroplasmorida (e.g. *Babesia*, *Theileria*, *Cytauxzoon*) and suborder Adelorina (e.g. *Hepatozoon*) represent a group of mammalian blood parasites with highly significant economic, veterinary, and medical impacts (MODRÝ et al., 2017; JALOVECKA et al., 2018). Obligate intracellular coccobacilli pathogenic for certain mammals, including humans, are present in the Anaplasmataceae family of alpha-proteobacteria (DUMLER et al., 2001). Apart from several *Candidatus* taxa, this family of bacteria currently comprises four recognized genera, namely *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia*, of which, the first two are recognized as tick-borne pathogens with relevance in veterinary and public health (DUMLER et al., 2001). The gamma-proteobacterium *Coxiella burnetii* is the causative agent of Q fever/coxiellosis and chronic endocarditis in humans globally (MAURIN & RAOULT 1999). Molecular evidence has revealed that *C. burnetti* infects humans in Brazil (LEMOS et al., 2011), and that wild mammals could act as natural reservoirs for this agent (ROZENTAL et al., 2017; FERREIRA et al., 2018; ZANATTO et al., 2019).

The state of Mato Grosso is located in Midwestern Brazil encompassing an area of approximately 1,000.000 km^2 and is a natural habitat for biotic elements of the Amazonia (Amazonian rainforest), Cerrado (Brazilian savannah), and Pantanal (wetlands). The southern Amazonian forests are spread as far as the northern portion of the state and cover nearly 53% of its territory (IBGE, 2004). It is noteworthy that the Amazonia biome is one of the largest reserves of biodiversity globally (JENKINS et al., 2013) and the largest ecosystem in Brazil (BRASIL, 2010).

Several studies on the Amazonian wildlife have assessed the occurrence of vector-borne protozoans and bacteria. Molecular studies performed on free-ranging vertebrates (HARRIS et al., 2015; FURTADO et al., 2017a, b; SOARES et al., 2017a, b; GOMES et al., 2018) and their associated ticks (SOARES et al., 2015) have already detected the presence of tick-borne agents belonging to the genera *Babesia*, *Coxiella*, *Cytauxzoon, Hepatozoon* and *Theileria*, and the Anaplasmataceae family of bacteria. While these studies shed light on the occurrence of putative new species of microorganisms and on the implied roles of vertebrate reservoirs, the analyzed samples belonged mainly to reptiles, birds, and large mammals (HARRIS et al., 2015; FURTADO et al., 2017a, b; SOARES et al., 2017a, b; GOMES et al., 2018). In order to assess their roles as carriers for different tick-borne pathogens, in this study, we conducted molecular analyses for the detection of *Hepatozoon*, *Babesia*, *Theileria*, *Cytauxzoon*, *Coxiella* and Anaplasmataceae agents in the small, non-volant, understudied mammals (marsupials and small rodents) inhabiting the Amazonia region of the Mato Grosso State.

Materials and Methods

Study area and sampling procedures

Samples were collected at three different timepoints from 23 forest fragments across three municipalities covering the Amazonian biome of the Mato Grosso State in Midwestern Brazil (Figure 1). One field expedition was conducted in the Alta Floresta municipality from 29 April to 15 May 2014. The municipalities of Sinop and Cláudia were included in the surveys and were visited simultaneously from 3 November to 1 December 2016, and from 3 July to 1 August 2017. Small non-volant mammals were captured using wire cage traps (16.5 cm × 16.5 cm × 35 cm) and Sherman-like traps (9.5 cm × 8 cm × 25 cm), which were distributed as mentioned below.

At each prospected point, we set a line of trap stations comprising alternatively of one Sherman-like and one wire cage trap displayed on the ground or onto vines up to 1.5 m high. At each of the six points in the Alta Floresta and 17 forest fragments surveyed at Sinop and Cláudia, 13 trap stations (with a total of 26 traps) and 30 trap stations (with a total of 60 traps), respectively were set. Each station was separated from the other by a distance of 10 – 15 m. The traps were baited with a piece of banana and a mixture of peanut butter, corn flour, sardine oil, and vanilla flavor. All traps were kept active for 16 consecutive days in the Alta Floresta, and 8 consecutive days in the Sinop and Cláudia.

Apart from the Sherman-like and wire cage traps, pitfall traps (60-liter buckets) were installed at each station in the Alta Floresta. For this purpose, we set a line of 10 traps, each separated by a distance of 8 m from the next and linked by a 60-cm-high drift fence to direct small vertebrates toward the pits. The pitfall traps were kept active for 16 consecutive days.

The traps were checked daily and rebaited whenever necessary. The captured animals were anesthetized with an intramuscular injection of ketamine hydrochloride/xylazine solution. After the onset of anesthesia, blood was collected from the animals via cardiac puncture and preserved with ethanol (1:1 ratio) in sterile polypropylene tubes. All specimens were subsequently euthanized by increasing the anesthetic doses. Thereafter, fragments of their spleens and livers were also collected through laparotomy and placed in separate sterile polypropylene tubes. Blood and tissue samples were stored by freezing at –20 °C prior to their transfer to the laboratory for DNA extraction and molecular analyses by polymerase chain reaction (PCR) assays, as detailed below.

Animal handling procedures were conducted as per the guidelines of the American Society of Mammologists for the use of wild mammals in research (SIKES et al., 2016). The marsupials were identified based on the criteria provided by Gardner (2007), except those belonging to the *Gracilinanus, Marmosa, Marmosops*, and *Monodelphis* genera, which were identified as per the criteria defined by Semedo et al. (2015), Díaz-Nieto & Voss (2016), Pavan et al. (2017) and Pavan (2019), respectively, and subgenus *Micoureus* that was identified based on the criteria provided by Silva et al. (2019). The rodents were identified according to the criteria provided by Patton et al. (2015), except *Neacomys*, *Oecomys*, and *Oligoryzomys*, which were identified based on those provided

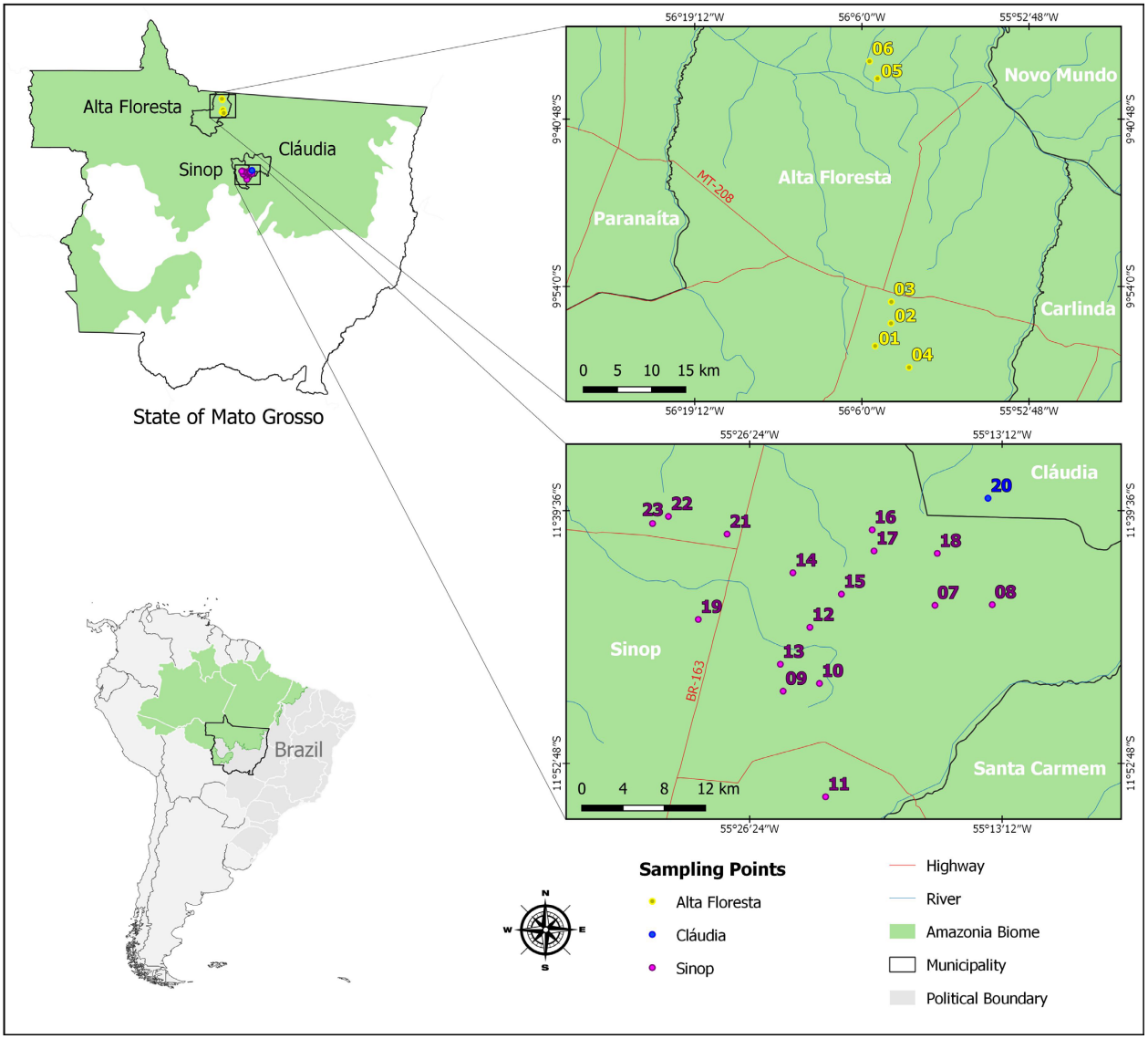

Figure 1. Map showing the sites where captures were performed in Alta Floresta (09°58'41.9"S, 56°04'57.2"W), Cláudia (11º 30' 55" S, 54º 53' 29" W), and Sinop (11°49'1.71"S, 55°24'39.05"W) municipalities within Brazilian Amazonia of the Mato Grosso State, Brazil.

by Hurtado & Pacheco (2017), Suárez-Villota et al. (2018), and Weksler et al. (2017), respectively. Voucher specimens were deposited in the "Coleção Zoológica da Universidade Federal de Mato Grosso (UFMT)", Cuiabá, Mato Grosso, Brazil.

Procedures in this study were previously approved by the Ethics Committee on Animal Research of the Federal University of Mato Grosso (CEUA protocol no. 23108.076870/2015-41) and "Instituto Chico Mendes de Conservação da Biodiversidade" (ICMBio permit no. 8863-1).

Molecular analyses

DNA extraction from the blood, spleen, and liver tissues was carried out using the DNA extraction PureLink™ Genomic DNA Mini Kit (Thermo Fisher Scientific, Waltham, Massachusetts, USA), according to the manufacturer's instructions. Mock extractions containing nuclease-free water were included as contamination controls. In order to verify the success of extraction, an initial PCR targeting a fragment of the mammalian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene was performed as described previously (BIRKENHEUER et al., 2003). The extracted samples were then subjected to a battery of PCR assays for amplifying fragments of the following three loci: 18S rRNA of *Hepatozoon* and Piroplasmorida members (*Babesia*, *Theileria,* and *Cytauxzoon*), *Coxiella* CTP synthase (*pyrG*), and 16S rRNA from members of the Anaplasmataceae family. Negative controls (nuclease-free water) and appropriate positive controls were included in each reaction as follows: *Babesia vogeli*, *Hepatozoon canis*, both obtained from blood of naturally infected dogs (MAIA et al., 2019), *Coxiella burnetii* (PACHECO et al., 2013) or *Ehrlichia canis* (AGUIAR et al., 2008). All primer sets used in the present study are listed in Table 1. The PCR assays were performed in a total volume of 50 uL, which contained at least 150 ng uL of target DNA, 20 pmol of each forward and reverse primer (Thermo Fisher Scientific™, Waltham, Massachusetts,

Table 1. Primers used in the present study.

Targeted DNA	Gene (PCR nucleotides size)	Primer sequence (5'-3')	Cycling conditions of PCR assays	Reference
Mammal	GAPDH (590 bp)	Forward (CCTTCATTGACCTCAACTACAT) Reverse (CCAAAGTTGTCATGGATGACC)	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.	Birkenheuer et al. (2003)
Hepatozoon spp.	18S rRNA (574 bp)	Forward (GGTAATTCTAGAGCTAATACATGAGC) Reverse (ACAATAAAGTAAAAAACAYTTCAAAG)	95 °C for 5 min; 35 cycles of 95 °C for 30 s, 57 °C for 30 s and 72 °C for 60 s; and final extension of 72 °C for 5 min	Almeida et al. (2012)
Piroplasmida	18S rRNA (551 bp)	Forward (CCGTGCTAATTGTAGGGCTAATACA) Reverse (GCTTGAAACACTCTARTTTTCTCAAAG)	95 °C for 5 min; 35 cycles of 95 °C for 30 s, 61 °C for 30 s and 72 °C for 30 s; and final extension of 72 °C for 7 min	Almeida et al. (2012)
Coxiella spp.	CTP synthase (504 bp)	Forward (TTATTTACCAACGTTCCTGAGCCG) Reverse (TTTATCCCGAGCAAATTCAATTATGG	95 °C for 5 min; 40 cycles of 95 °C for 60 s, 60 °C for 60 s and 72 °C for 60 s; and final extension of 72 °C for 10 min.	Reeves et al. (2008)
Anaplasmataceae	16S rRNA (344 bp)	Forward (GGTACCYACAGAAGAAGTCC) Reverse (TAGCACTCATCGTTTACAG)	95 °C for 5 min; 34 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 90 s; and final extension of 72 °C for 5 min.	Inokuma et al. (2000)

USA), 2U of Taq DNA polymerase (Sigma-Aldrich™, Darmstadt, Germany), 1 × reaction buffer (Sigma-Aldrich™, Darmstadt, Germany) containing 1.5 mM $MgCl_2$ and 0.5 mM of deoxynucleotide triphosphate (dNTPs) mixture (Thermo Fisher Scientific™, Waltham, Massachusetts, USA). The PCR products were resolved in 1.5% agarose gels stained with the GelRed™ Nucleic Acid Gel Stain (Biotium, Fremont, California) and visualized in a ChemiDoc XRS system (Bio-Rad, Hercules, California). Amplicons of the expected sizes were purified using the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare, Chicago, Illinois) and prepared for sequencing as per the instructions provided with the BigDye™ kit (Applied Biosystems, Foster, California). An ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster, California) was employed for conducting the sequencing procedures with the same primers used for the PCR. In order to evaluate the quality of the sequences, electropherograms were verified with CLC Genomics Workbench software (Qiagen®). Obtained sequences were then subjected to a BLAST analyses (ALTSCHUL et al., 1990) in order to infer closest identities with organisms available in GenBank.

Phylogenetic analyses

Sequences of 18S rDNA generated in this study and homologue sequences retrieved from the GenBank were used to construct two alignments, one for the *Hepatozoon* spp. and another for the Piroplasmorida representatives. The selected sequences were aligned using Clustal X (THOMPSON et al., 1997), and adjusted manually with GeneDoc (NICHOLAS et al., 1997). Two phylogenetic analyses for each alignment were performed. Inferences by maximum parsimony were constructed as per their implementation in PAUP version 4.0b10 (SWOFFORD, 2002), using a heuristic search in 1000 replicates, 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR (tree bisection and reconnection) branch swapping. MrBayes v3.1.2 was used to perform Bayesian analyses (HUELSENBECK & RONQUIST, 2001) with four independent Markov chain runs for 1,000,000 metropolis-coupled MCMC generations, sampling a tree every 100th generation. The first 25% of trees represented burn-in, and the remaining trees were used to calculate Bayesian posterior probability. The GTR+I+G was the standard model used in MrBayes software. Sequences of *Neospora caninum* (L24380; U03069) and *Toxoplasma gondii* (U03070; L37415) were used as an out-group for the *Hepatozoon* tree, and *Babesia* sp. (AF188001) rooted the Piroplasmorida tree. GenBank accession numbers for all sequences used for the phylogenetic analyses were embedded in each tree.

Results

Capture of mammals, PCR and sequence analyses

Table 2 provides a list of all captured animals and results of their molecular analyses grouped according to the locality, species and forest fragment. A total of 82 specimens of mammals (32 rodents and 50 marsupials) belonging to at least 11 different species of the order Rodentia and 10 species of the order Didelphimorphia were trapped in the Alta Floresta. At Sinop, 145 specimens (44 rodents and 101 marsupials) belonging to six species of the order Rodentia and seven species of the order Didelphimorphia were captured. Finally, three specimens (2 rodents and one marsupial) belonging to two species of the order Rodentia and one species of the order Didelphimorphia were captured in the areas of the Cláudia municipality. Some of the captured animals perished inside the traps before performing sampling procedures. In these cases, we were unable to take all the samples per specimen (blood, liver, and spleen). Total accounts of samples obtained for each of the captured species are specified in Table 2.

DNA of all 585 samples subjected to *gapdh* internal control amplified the predicted product. In total, 5.8% (34/585) of the mammals' samples analyzed were infected by *Hepatozoon*, while co-infection with Piroplasmorida agents was detected in 0.34% (2/585) of small non-volant mammals trapped. DNA of *Hepatozoon* was not detected in the samples of mammals captured from the Alta Floresta and Cláudia municipalities; however, at least one tested sample from approximately half (45.16%; 14/31) of the common opossums (*D. marsupialis*) captured at Sinop was positive for this genus of protozoa (Table 2). Among the animals that exhibited positive results for this protozoa, three specimens (field numbers RVR 134, 168 and 255) were positive in all tested samples (blood, spleen and liver); in four specimens (RVR 118, 135, 228 and 256), *Hepatozoon* DNA was present in both the blood and liver samples; two specimens (field numbers RVR 128 and 130) were positive for *Hepatozoon* in the blood and spleen; and finally, in five specimens (RVR 153, 154, 156, 159 and 210) only blood samples were positive, as determined by the PCR assay. Because of the quality and the insufficient DNA of amplified samples, it was not possible to sequence 18S rRNA amplicons for all *Hepatozoon*-positive samples. However, only one haplotype was obtained after sequencing products from 14 blood, seven liver, and six spleen samples. This trend was confirmed after aligning all sequences in Clustal X, from which a consensus of 544 base pairs (bp) was retrieved. After BLAST comparisons, this consensus sequence was 96% identical (522/544 bp) to the following four sequences: 1) *Hepatozoon* sp. isolate HepIxo-281 (MH174345) detected in *Ixodes* sp. (Acari: Ixodidae) from Chile; 2) *Hepatozoon* sp. (KY684007) detected in *Caiman crocodilus* (Crocodylia: Alligatoridae) from Brazilian Amazonia; 3) *Hepatozoon* sp. DMA-2015 strain Rodent MT (KP757838) detected in *Calomys callosus* (Rodentia: Cricetidae) from Brazilian Pantanal; and 4) *Hepatozoon* sp. AS7 (FJ719819) detected in *Abrothrix sanborni* (Rodentia: Cricetidae) from Chile. The *Hepatozoon* haplotype generated in the present study was designated as *Hepatozoon* sp. isolate MTopossum and has been deposited in the GenBank under the accession number MK257775.

Amplicons for Piroplasmorida were not found in any of the samples obtained from mammals captured in the Alta Floresta and Cláudia regions (Table 2). By contrast, Piroplasmorida DNA was detected in two out of 31 blood samples (6.45%) of *D. marsupialis* (RVR 130 and 168) trapped at Sinop, both previously positive for *Hepatozoon* (Table 2). Partial sequences of the 18S rRNA gene obtained from these two specimens were identical to each other and presented different identities (94-100%) against other piroplasmid sequences from the *Babesia* and *Theileria* genera. Highest identities

Table 2. Wild mammals captured and samples (blood, liver, spleen) tested by PCR assays for detection of *Hepatozoon*, Piroplasmorida, Anaplasmataceae, and *Coxiella* within Alta Floresta, Sinop, and Cláudia municipalities, in Brazilian Amazon of Mato Grosso State, Brazil.

Municipality	Mammal species (n)	Field numbers (RVR)	Forest fragment occurrence[a]	Number of infected sample/ Number of tested sample (%)		
				Blood	Liver	Spleen
Alta Floresta						
	ORDER RODEN/TIA					
	Euryoryzomys nitidus (1)	84	2	0/1	0/1	n/t[b]
	Hylaeamys megacephalus (4)	77, 83, 88, 100	1, 4, 62	0/4	0/4	0/3
	Makalata sp. (1)	85	2	0/1	0/1	n/t
	Neacomys amoenus (7)	6, 7, 46, 47, 74, 87, 109	3, 4	0/3	0/6	0/6
	Necromys lasiurus (2)	48, 76	3, 4	0/1	0/2	0/2
	Oecomys cleberi (3)	49, 50, 75	1, 2	0/1	0/2	0/2
	Oecomys paricola (2)	38, 94	6	0/1	0/2	0/2
	Oecomys aff. *catherinae* (1)	89	4	0/1	0/1	0/1
	Oligoryzomys cf. *mattogrossae* (5)	42, 54, 90, 98, 99	2, 4, 6,	0/3	0/5	0/2
	Oxymycterus amazonicus (1)	39	6	n/t	0/1	0/1
	Proechimys sp. (5)	1, 18, 35, 63, 92	1, 5	0/2	0/5	0/5
	ORDER DIDELPHIMORPHIA					
	Caluromys philander (1)	25	2	n/t	0/1	0/1
	Cryptonanus sp. (10)	2, 16, 17, 23, 27, 37, 55, 57, 64, 104	1-5	0/2	0/10	0/10
	Didelphis marsupialis (2)	78,79	4	0/1	0/1	0/1
	Glironia venusta (1)	107	2	0/1	0/1	0/1
	Marmosa constantiae (11)	3, 10-12, 14, 15, 26, 29, 34, 36, 105	1-4	0/1	0/10	0/10
	Marmosa murina (2)	31, 80	1, 3	0/1	0/1	0/1
	Marmosops bishopi (13)	5, 9, 13, 28, 53, 56, 60, 67, 93, 101-103, 110	1-4	0/6	0/10	0/9
	Marmosops aff. *pinheiroi* (4)	24, 58, 62, 95	1, 5, 6	0/2	0/3	0/3
	Monodelphis glirina (4)	4, 33, 65, 82	2, 4	0/1	0/4	0/3
	Monodelphis saci (2)	8, 59	1, 3	n/t	0/2	0/2
Sinop						
	ORDER RODENTIA					
	Mesomys hispidus (3)	165, 230, 245	8, 10, 21	0/3	0/3	0/3
	Mus musculus (1)	178	23	n/t	0/1	0/1
	Oecomys bicolor (18)	133, 142, 145, 146, 148, 169, 186, 192, 205, 214, 233, 241, 244, 248, 249, 253, 260, 264	14-15, 18, 23	0/14	0/17	0/18
	Oecomys paricola (1)	167	22	0/1	0/1	0/1
	Oecomys roberti (7)	122, 123, 143, 147, 149, 211, 215, 263	8, 9, 13, 15, 16, 18	0/7	0/6	0/5
	Proechimys roberti (14)	120, 129, 160, 177, 179, 180, 185, 191, 199, 200, 206, 212, 220, 223	10, 12, 22, 23,	0/12	0/11	0/11

[a]Forest fragment numbers represented in Figure 1; [b]Sample non-tested; [c]Results refer to samples testing positive for *Hepatozoon* PCR assay; [d]Results refers to samples testing positive for Piroplasmorida PCR assay.

Table 2. Continued...

Municipality	Mammal species (n)	Field numbers (RVR)	Forest fragment occurrence[a]	Number of infected sample/ Number of tested sample (%)		
				Blood	Liver	Spleen
	ORDER DIDELPHIMORPHIA					
	Caluromys philander (5)	183, 190, 216, 218, 226	12, 19	0/5	0/5	0/5
	Didelphis marsupialis (31)	118, 119, 128, 130, 132, 134, 135, 140, 141, 153-159, 163, 166, 168, 175, 210, 213, 228, 229, 238, 242, 246, 251, 255-257	7-10, 12-14, 16-22	14/31 (45.16)[c] 2/31 (6.45%)[d]	13/29 (44.82)[c] 0/29	7/29 (24.13)[c] 0/29
	Gracilinanus peruanus (5)	207, 231, 247, 250, 265	10, 14, 16	0/3	0/5	0/5
	Marmosa constantiae (53)	121, 124-127, 131, 136, 137, 144, 150-152, 161, 162, 164, 170-174, 182, 184, 187-189, 193-198, 201-204, 208, 209, 217, 219, 221, 222, 224, 225, 227, 232, 236, 237, 239, 240, 243, 252, 258, 261	7-10, 12-19, 21-23	0/52	0/53	0/49
	Marmosa murina (1)	139	14	0/1	0/1	0/1
	Marmosops aff. *pinheiroi* (1)	81	14	0/1	0/1	n/t
	Metachirus nudicaudatus (5)	138, 234, 235, 262, 266	8, 16-18	0/5	0/5	0/4
Cláudia						
	ORDER RODENTIA					
	Hylaeamys megacephalus (1)	254	20	0/1	0/1	0/1
	Oecomys paricola (1)	259	20	0/1	0/1	0/1
	ORDER DIDELPHIMORPHIA					
	Didelphis marsupialis (1)	176	20	0/1	0/1	0/1

[a]Forest fragment numbers represented in Figure 1; [b]Sample non-tested; [c]Results refer to samples testing positive for *Hepatozoon* PCR assay; [d]Results refers to samples testing positive for Piroplasmorida PCR assay.

matched (1) a *Babesia* sp. (KY684002, KP757839) detected in *D. marsupialis* from the Brazilian Amazonia, and *Monodelphis domestica* from Brazilian Pantanal; (2) *Theileria ornithorhynchi* (KT937391) detected in *Ornithorhynchus anatinus* from Australia; and (3) *Theileria bicornis* (AF499604) detected in the Black rhinoceros from South Africa.

The GenBank accession number for the partial sequence of 18S rRNA gene generated for the *Babesia* sp. isolate MTopossum in the present study is MK257776.

All samples (blood, liver, or spleen) collected from the small mammals of the Alta Floresta, Sinop and Cláudia municipalities were negative for the genus *Coxiella* and the family Anaplasmataceae when evaluated using PCR assays.

Phylogenetic analyses

The phylogenetic analyses inferred from a partial 18S rDNA obtained from *D. marsupialis* indicate that *Hepatozoon* sp. Mato Grosso forms a clade with a *Hepatozoon*. sp. recently detected in *Ornithodoros atacamensis*, a lizard-associated soft tick that occurs in the Atacama Desert, Chile (MUÑOZ-LEAL et al., 2019) (Figure 2). The phylogeny for the Piroplasmorida 18S rRNA gene showed that *Babesia* sp. Mato Grosso clusters with two congeneric haplotypes, a *Babesia* sp. also detected in *D. marsupialis* (KY684002) from the Brazilian Amazonia (SOARES et al., 2017b) and *Babesia* sp. Monodelphis, characterized from *M. domestica* (KP757839) in the Pantanal biome (WOLF et al., 2016) (Figure 3).

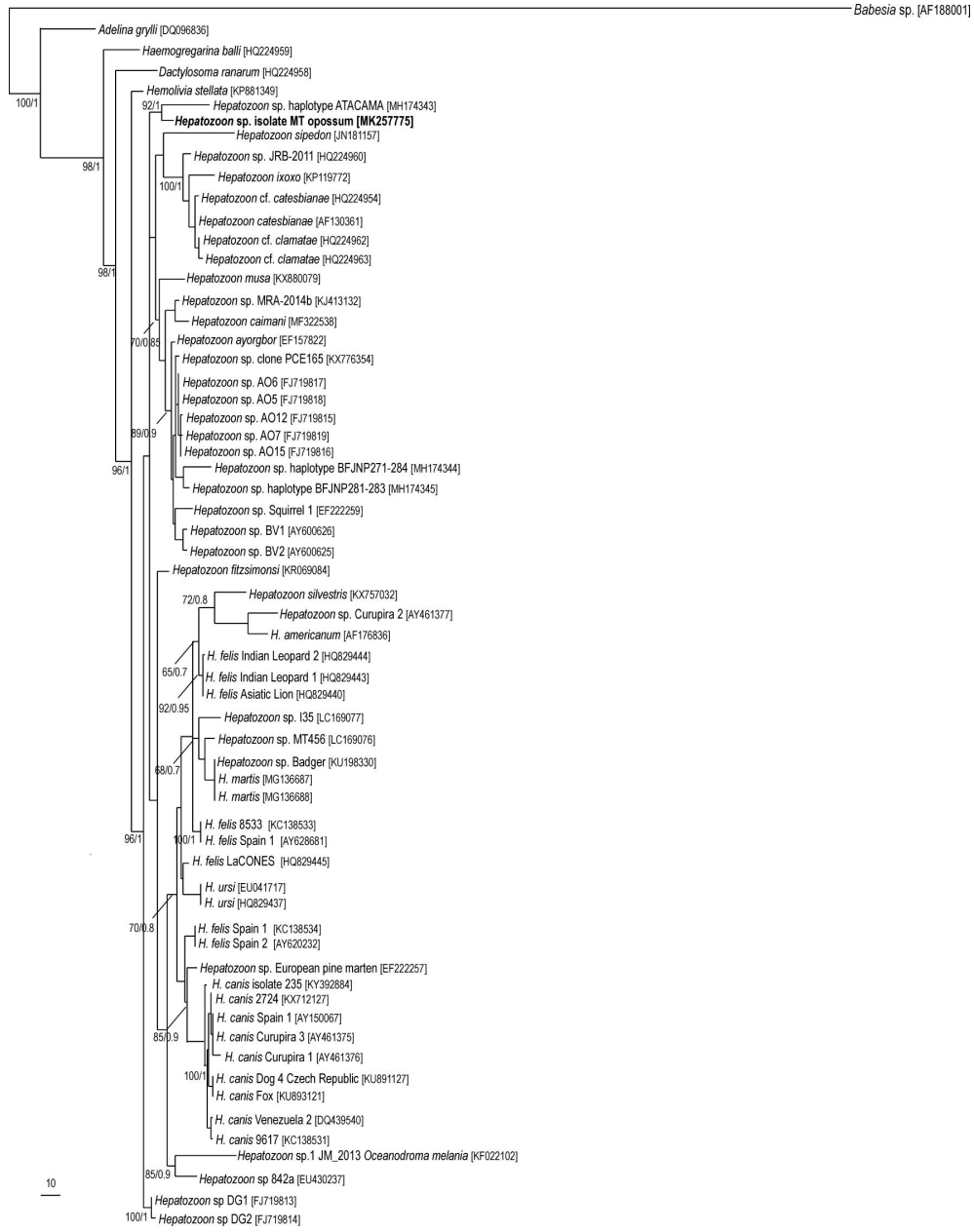

Figure 2. Maximum parsimony and Bayesian tree constructed for an alignment of sequences of *Hepatozoon* spp. using 18S rRNA gene sequences (467 bp). Numbers at nodes are the support values for the major branches (bootstrap over 500 replicates). The sequence obtained in this study is highlighted in bold. Numbers in brackets correspond to GenBank accession numbers.

Figure 3. Maximum parsimony and Bayesian tree constructed for an alignment of sequences of Piroplasmorida (*Babesia* spp., *Theileria* spp., and *Cytauxzoon* spp.) species using 18S rRNA gene sequences (374 bp). Numbers at nodes are the support values for the major branches (bootstrap over 500 replicates). The sequence obtained in this study is highlighted in bold. Numbers in brackets correspond to GenBank accession numbers.

Discussion

With the exception of *Hepatozoon* and *Babesia*, DNA from tick-borne pathogens (*Coxiella*, *Anaplasma,* and *Ehrlichia*) was not detected in the blood or tissues sampled from the rodents and marsupials. Contrarily, previous studies performed on reptiles, birds, and mammals exposed a variety of genetically distinct organisms of the Anaplasmataceae family circulating in the Amazonian wildlife under natural conditions, which included those of the *Cavia* sp. (Rodentia: Caviidae) and *Marmosa demerarae* (Didelphimorphia: Didelphidae) (SOARES et al., 2017a). Therefore, *M. demerarae* encompassed the host species sampled in the present study.

Our negative results may be related to the low specificity and sensitivity of the conventional PCR (cPCR) assays used for screening. On the other hand, the role of small mammals (rodents and marsupials) as natural reservoirs of these pathogens in nature has not yet proven in Brazil. Indeed, Benevenute et al.

(2017) surveyed the presence of Anaplasmataceae agents in wild and synanthropic rodents from five different biomes in the country, and observed a low occurrence of *Ehrlichia* (0.44%) and *Anaplasma* (2.4%) among sampled rodents, while low occurrence of *Coxiella* (4.6%) have also been described by Rozental et al. (2017) infecting wild rodents in eight municipalities of Rio de Janeiro state. This trend has also evidenced in other studies conducted in the Brazilian Pantanal biome (WOLF et al., 2016; SOUSA et al., 2017). Furthermore, Benevenute et al. (2017) observed differences between cPCR assay targeting 16S rRNA gene and a real-time multiplex PCR (qPCR) focusing amplifications of a fragment of *groEL* gene, probably given the lower sensitivity of cPCR assays when compared to qPCR assays.

Unlike Perles et al. (2019) that have reported the occurrence of six haplotypes after analyses performed with available 18S rRNA *Hepatozoon* sequences detected in rodents from five Brazilian biomes, in the present study, despite the high prevalence of *Hepatozoon* (45.16%) among *D. marsupialis* from Sinop, a single haplotype of *Hepatozoon* was retrieved from all positive common opossums. Based on phylogenetic analyses, this haplotype could possibly correspond to a new species. In Brazil, *Hepatozoon* DNA has already been detected in rodents (i. e. *Calomys callosus*, *Oecomys mamorae*) and marsupials (*Thylamys macrurus*) from the Pantanal biome of the Mato Grosso and Mato Grosso do Sul states (WOLF et al., 2016; SOUSA et al., 2017). Likewise, in the southeastern region of the country (São Paulo state), two distinct *Hepatozoon* genotypes (*Hepatozoon* sp. genotype Rodent SP-1 and *Hepatozoon* sp. genotype Rodent SP-2) have been recorded in *Hepatozoon*-positive rodents (i.e. *Oligoryzomys nigripes*, *Oligoryzomys flavescens*, *Akodon* sp., *Necromys lasiurus*, and *Sooretamys angouya*) by Demoner et al. (2016), while *Hepatozoon canis* and a new species, named *Hepatozoon milleri* sp. nov. have been detected in *Didelphis albiventris* and *Akodon montensis*, respectively (SILVA et al., 2017; DEMONER et al., 2019). However, to the best of our knowledge, here we provide the first report on the molecular detection of *Hepatozoon* in *D. marsupialis*. A common species of *Hepatozoon*, namely *Hepatozoon didelphydis*, that infects neotropical marsupials (such as *D. albiventris*, *D. aurita*, *D. marsupialis*, *Philander opossum,* and *Metachirus nudicaudatus*) has already been described in French Guiana (THOISY et al., 2000), Colombia (AYALA et al., 1973), and Brazil (SILVA & ARANTES, 1916). However, these reports were based only on morphological analyses through microscopical observations of blood smears, and could therefore represent different *Hepatozoon* species (MERINO et al., 2008). A study of blood smears from the opossum *Thylamys elegans* captured in Central Chile, using light microscopy, conducted by the later authors, identified *H. didelphydis* as well. Based on molecular analyses, this organism was subsequently assigned to the family Sarcocystidae (MERINO et al., 2008). Therefore, molecular methods should be used to identify *Hepatozoon* agents discovered by optical observation and to further assess the geographical distribution of new haplotypes among marsupials.

The piroplasmid haplotype detected in the common opossum (*D. marsupialis*), herein designated *Babesia* sp. isolate MTopossum, was identical to a *Babesia* haplotype reported recently in a common opossum from the state of Pará (SOARES et al., 2017b). This fact suggests that the currently detected agent could have a broad distribution within the Amazonia. Additionally, infection by the piroplasmid *Babesia brasiliensis*, determined through morphological characterization, was described in *D. marsupialis* from the Caribbean coast until the Amazon forest (DEANE & DEANE, 1961; HERRERA & URDANETA-MORALES, 1991). Soares et al. (2017b) had suggested that the *Babesia* haplotypes detected in opossums belonging to the Amazon and Pantanal biomes (WOLF et al., 2016) could correspond to *B. brasiliensis*. However, until a genetic characterization of *B. brasiliensis* from its type host and locality is available, any comparison based on morphology should be considered as speculative.

Despite recent reports of infection by piroplasmid and *Hepatozoon* agents among Brazilian wildlife (HARRIS et al., 2015; FURTADO et al., 2017a,b; SOARES et al., 2017a,b; GOMES et al., 2018), our knowledge on these agents is still insufficient. Notwithstanding, the involvement of common opossums (*D. marsupialis*) in the epidemiology of *Hepatozoon* spp. and *Babesia* spp. in the Amazonian biome is a remarkable fact that needs further studies.

Since negative results were observed in at least 224 small mammals tested for Anaplasmataceae and *Coxiella* agents, this finding is contrary to the results obtained among other vertebrate groups belonging to the same biome (SOARES et al., 2017a). This fact suggests that the role of species screened in this study as reservoirs of these agents is limited, particularly in the areas studied by us.

Conclusion

The present study provided the first report on the molecular detection of *Hepatozoon* in common opossum (*D. marsupialis*) and showed a high occurrence of *Hepatozoon* spp. among *D. marsupialis* from Brazilian Amazonia. Furthermore, phylogenetic analyses inferred from partial sequences of the 18S rRNA gene highlighted the occurrence of new *Hepatozoon* and Piroplasmorida haplotypes infecting common opossum. Future studies determining the importance of *D. marsupialis* in the epidemiological cycles of *Hepatozoon* and *Babesia* under natural conditions, in the Amazonian biome, are necessary.

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