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Original article

Novel *Babesia* and *Hepatozoon* agents infecting non-volant small mammals in the Brazilian Pantanal, with the first record of the tick *Ornithodoros guaporensis* in Brazil

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ABSTRACT

Taking into account the diversity of small terrestrial mammals of the Pantanal, the present study aimed to verify the occurrence of infection by *Ehrlichia* spp., *Anaplasma* spp., *Rickettsia* spp., *Hepatozoon* spp., *Babesia* spp. and parasitism by ticks in non-volant small mammals collected in the Brazilian Pantanal. Samples of blood, liver and spleen were collected from 64 captured animals, 22 marsupials and 42 rodents. Pathogen detection was performed by the use of genus-specific Polymerase Chain Reaction (PCR) assays. Ticks collected from the animals consisted of *Amblyomma sculptum* and *Amblyomma triste* nymphs, and *Ornithodoros guaporensis* larvae. None of the vertebrate samples (blood, liver, or spleen) yielded detectable DNA of *Rickettsia* spp. or *Ehrlichia* spp. The blood of the rodent *Hylaeamys megacephalus* yielded an *Anaplasma* sp. genotype (partial 16S rRNA gene) 99% similar to multiple *Anaplasma* spp. genotypes around the world. The blood of three rodents of the species *Calomys callosus* were positive for a novel *Hepatozoon* sp. agent, phylogenetically related (18S rDNA gene) to distinct *Hepatozoon* genotypes that have been detected in rodents from different parts of the world. One marsupial (*Monodelphis domestica*) and three rodents (*Thrichomys pachyurus*) were positive to novel piroplasmid genotypes, phylogenetically (18S rDNA gene) related to *Theileria bicornis*, *Cytauxzoon manul*, and *Cytauxzoon felis*. The present study provides the first molecular detection of *Hepatozoon* sp. and piroplasmids in small mammals in Brazil. Additionally, we expanded the distribution of *O. guaporensis* to Brazil, since this tick species was previously known to occur only in Bolivia.

1. Introduction

Pantanal is a one of the largest wetland in the world located in the center of South America between Brazil, Bolivia and Paraguay. The region is seasonally flooded and has a complex mosaic of habitats such as flooded and non-flooded forest patches, seasonally flooded grasslands, and permanent or temporary lagoons (Nunes da Cunha and Junk, 2009). Pantanal is considered one of the most important sources of life of the Platine basin and is world widely recognized by its vast biodiversity (Ussami et al., 1999). Approximately 26% of the total mammal diversity of Pantanal (~152 mammal species) belongs to the non-volant small mammal group (NVSM) (Tomas et al., 2010), namely Didelphimorphia, Rodentia, and Lagomorpha (Chupel and Aragona, 2010). NVSM are important hosts for immature stages of Ixodidae and Argasidae ticks (Nieri-Bastos et al., 2004; Barros-Battesti et al., 2006; Linardi, 2006; Reis et al., 2008). While a number of acarologic surveys on NVSM have been conducted in different Brazilian biomes – Atlantic rainforest (Dantas-Torres et al., 2012; Szabó et al., 2013), Caatinga (Horta et al., 2011), and Cerrado (Saraiva et al., 2012; Sponchiado

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et al., 2015) – at least one study covered parts of the Pantanal biome (Sponchiado et al., 2015).

Ticks are important vectors of numerous bacteria and protozoa for animals and humans. Different species of *Rickettsia* (Rickettsiales: Rickettsiaceae) belonging to the spotted fever group have been detected by PCR or cellular isolation in ticks of the *Amblyomma* genus in the Pantanal (Alves et al., 2014; Nieri-Bastos et al., 2014; Melo et al., 2015a,b). Moreover, to date at least three *Ehrlichia* (Rickettsiales: Anaplasmataceae) species are known to occur in the Pantanal: *Ehrlichia canis* and a closely related agent (*Ehrlichia* sp. strain UFMT-BV) were detected in dogs and cattle, respectively (Santos et al., 2013; Aguiar et al., 2014), while an *Ehrlichia ruminantium*-closely related species (*Ehrlichia* sp. strain jaguar) was detected in free-ranging jaguars (*Panthera onca*) (Widmer et al., 2011).

The tick-borne protozoans of the genus *Babesia* (Sarcodina: Babesiidae) and *Hepatozoon* (Eucoccidiorida: Hepatozoidae) are associated with different infections and diseases of domestic and wild mammals (Serra-Freire, 1979; Alencar et al., 1997; Souza et al., 2000; Dantas-Torres, 2008). While most of the *Hepatozoon* reports in Brazil refer to *Hepatozoon canis* infecting domestic dogs (reviewed by O'Dwyer, 2011), there have been reports of other species or genotypes infecting domestic and wild Felidae (Rubini et al., 2006; Andre et al., 2010) and wild Canidae (Soares et al., 2007; Criado-Fornelio et al., 2006; Andre et al., 2010; Almeida et al., 2013). Similarly, the vast majority of *Babesia* reports from Brazil have been on domestic dogs, cattle and horses (Passos et al., 2005; Souza et al., 2000; Barros et al., 2005). Reports of these two protozoa genera in the Pantanal have been restricted to *Babesia canis vogeli* and *H. canis*, both infecting domestic dogs (Melo et al., 2015b).

Considering the high diversity of NVSM in the Pantanal (Tomas et al., 2010) and their relationship with the biological life cycle of ticks, as food supply, shelter and dispersal, and also considering that NVSM act as carriers for different tick-borne pathogens, the present study evaluated tick infestations and infection by tick-borne agents in NVSM captured in different habitat types in the northern region of the Brazilian Pantanal.

2. Materials and methods

2.1. Study area and sampling procedures

The study was performed in the Pantanal, sub region of Poconé, between the municipalities of Poconé and Nossa Senhora do Livramento. NVSM were sampled between October 2013 and February 2014 in eight different habitat types according to the definitions of Nunes da Cunha and Junk (2009) and Lopes et al. (2011): (1) *brejo*, marshy grassland; (2) *cambarazal*, monodominant (*Vochysia divergens* Pohl.) semideciduous flooded forest; (3) *campo de murundu*, flooded termite savanna; (4) *cordilheira*, deciduous dry forest; (5) *landizal*, evergreen flooded forest; (6) *pastagem*, flooded exotic pasture *Brachiaria* sp.; (7) *pombeiro*, monodominant (*Combretum* sp.) woodland flooded forest; and (8) *taquaral*, semideciduos dry savanna forest dominated by *Guadua* sp. woody bamboos (Fig. 1). Each area was sampled with a set of 50 hook cage traps (16.5 cm × 16.5 cm × 35 cm) and 50 Sherman-like traps (9.5 cm × 8 cm × 25 cm), which were arranged in a sampling grid (10 m × 10 m). Traps were baited with a fruit (pineapple for hook cages and banana for shermans) and a standart bait (peanut butter, corn flour, and sardine) (Aragona and Marinho-Filho, 2009). Traps were rebaited daily and remained open for five consecutive days totaling an effort of 500 trap-night in each of the eight areas. Captured animals were anesthetized by intramuscular injection of ketamine hydrochloride and euthanized with potassium chloride. Blood collection was performed by cardiac puncture, while liver and spleen were collected through laparotomy. Trapped animals were inspected for the presence of ticks, which were collected in polypropylene microtubes containing absolute isopropyl alcohol, and transported to the laboratory.

Mammals were trapped and collected under authorization by IBAMA (License number 33068-3) and according to the Ethical Guidelines for Animal Research established by the Brazilian Society of Laboratory Animal Science (SBCAL) and approved by the university's Animal Research Ethics Committee (Protocol number UFMT 23108. 043095/13-6).

2.2. Taxonomic identifications

Taxonomic identification of mammals followed Bonvicino et al. (2008) and Reis et al. (2011), while morphological identification of ticks followed Barros-Battesti et al. (2006) for genera, and Martins et al. (2010) and Nava et al. (2013) for ixodid species. Ticks identified as *Amblyomma cajennense* sensu lato (s.l.) or *Ornithodoros* sp. was submitted to a PCR assay targeting a portion of tick mitochondrial 16S rRNA gene, as previously described (Mangold et al., 1998) (Table 1). In addition, *Ornithodoros* sp. larvae were mounted in Hoyer's medium to make semi permanent slides and examined and photographed by light microscopy for morphological analyses by light microscopy (Olympus, Tokyo, Japan). Morphological identification of *Ornithodoros* larvae to species level was based on taxonomic keys (Kohls et al., 1969; Jones and Clifford, 1972), and original species descriptions of Neotropical Ornithodorinae (Endris et al., 1989; Venzal et al., 2008; Nava et al., 2013; Venzal et al., 2012, 2013). In addition, type specimens of *Ornithodoros guaporensis*, deposited at the tick collection "Coleção Nacional de Carrapatos" (CNC) under the accession number CNC-2307, were used for morphological comparisons with the *Ornithodoros* ticks collected in the present study.

2.3. Molecular analyses for tick-borne agents

NVSM blood, liver and spleen samples, and ticks were processed for DNA extraction using the phenol-chloroform method according to Sambrook and Russel (2001). DNA samples from blood, liver, spleen, and ticks were tested by a battery of PCR assays targeting the bacterial genera *Rickettsia* and *Ehrlichia* with primers described in Table 1. Blood DNA samples were additionally tested by PCR for the bacterial genus *Anaplasma*, and the protozoan genera *Babesia* and *Hepatozoon* (Table 1). In each PCR assay, an appropriate positive control DNA was employed, namely *Rickettsia* sp. strain NOD, *E. canis*, *Anaplasma platys*, *B. canis vogeli*, and *H. canis*. Amplicons were purified using illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare) according to manufacturer's instructions and sequenced in an automatic sequencer (ABI DNA Model 3500 Series Genetic Analyzer) according to the manufacturer's protocol. The sequences obtained were submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) to infer closest similarities available in GenBank.

2.4. Phylogenetic analysis

Partial sequences of *Hepatozoon* (558-bp) and *Babesia* (532-bp from a rodent and 556-bp from a marsupial) 18S rRNA genes generated by the *Hepatozoon*- and *Babesia*-PCR, respectively, were aligned by the ClustalX (Thompson et al., 1997) and manually refined by Genedoc (Nicholas et al., 1997) with corresponding sequences available in GenBank. The alignment of *Hepatozoon* 18S rRNA included 21 different sequences (593 characters), while the *Babesia* 18S rRNA alignment included 67 sequences (567characters). Two phylogenetic trees, one for the *Hepatozoon* 18S rRNA gene and another for the *Babesia* 18S rRNA gene, were inferred by the maximum-parsimony methods and were performed with