International Journal for Parasitology: Parasites and Wildlife 6 (2017) 115–121

Contents lists available at ScienceDirect

International Journal for Parasitology: Parasites and Wildlife

journal homepage: www.elsevier.com/locate/ijppaw

Novel piroplasmid and *Hepatozoon* organisms infecting the wildlife of two regions of the Brazilian Amazon

Herbert S. Soares [a], Arlei Marcili [b], Amália R.M. Barbieri [a], Antonio H.H. Minervino [c], Thiago Rocha Moreira [c], Solange M. Gennari [a], Marcelo B. Labruna [a, *]

[a] Department of Preventive Veterinary Medicine and Animal Health, Faculty of Veterinary Medicine, University of São Paulo, Av. Prof. Orlando Marques de Paiva 87, Cidade Universitária, 05508-270 São Paulo, SP, Brazil
[b] Mestrado em Medicina e Bem estar animal, Universidade Santo Amaro, Av. Prof. Eneas de Siqueira Neto, 340, São Paulo, 04529-300, Brazil
[c] Institute of Biodiversity and Forest, Federal University of Western Pará, Avenida Vera Paz S/N, Salé, 68000-000 Santarém, PA, Brazil

ARTICLE INFO

Article history:
Received 8 March 2017
Received in revised form
22 May 2017
Accepted 23 May 2017

Keywords:
Babesia
Theileria
Cytauxzoon
Hepatozoon, wildlife
Amazon

ABSTRACT

During 2009–2012, wild animals were sampled in two areas within the Amazon biome of Brazil, in the states of Mato Grosso and Pará. Animal tissues and blood were molecularly tested for the presence of Piroplasmida (genera *Babesia, Theileria, Cytauxzoon*) or *Hepatozoon* DNA. Overall, 181 wild animals comprising 36 different species (2 reptiles, 5 birds, and 29 mammals) were sampled. The following Piroplasmida agents were detected: *Cytauxzoon felis* in one ocelot (*Leopardus pardalis*), *Theileria cervi* in two red brocket deer (*Mazama americana*), *Theileria* spp. in three nine-banded-armadillos (*Dasypus novemcinctus*), one agouti (*Dasyprocta* sp.), and four lowland pacas (*Cuniculus paca*), *Babesia* spp. in one common opossum (*Didelphis marsupialis*) and one white-lipped peccary (*Tayassu pecari*). The following *Hepatozoon* agents were detected: *Hepatozoon* sp. (possibly *Hepatozoon caimani*) in three spectacled caimans (*Caiman crocodilus*), *Hepatozoon felis* in an ocelot (*Leopardus pardalis*), and *Hepatozoon* spp. in one scorpion mud turtle (*Kinosternon scorpioides*) and one lowland paca (*Cuniculus paca*). Phylogenetic analyses inferred by the 18S rRNA gene partial sequences supported these results, highlighting at least five novel Piroplasmida agents, and two novel *Hepatozoon* agents. This study screened the presence of tick-borne protozoa in a number of wildlife species from the Amazon for the first time. Our results indicate that a variety of genetically distinct Piroplasmida and *Hepatozoon* organisms circulate under natural conditions in the Amazonian wildlife.

1. Introduction

The Amazon is the largest and most diverse tropical forest of the world, covering over 6.6 million km^2 in South America, with more than 50% of its original distribution occurring in Brazil (Mittermeier et al., 2003). Despite of a rich vertebrate fauna, although yet poorly explored and with a great potential for discovery of many new species, almost nothing is known about infection of the Amazonian wildlife by vector-borne protozoa of the order Piroplasmida (e.g., genera *Babesia, Theileria, Cytauxzoon*) or hemogregarines of the genus *Hepatozoon*. While there have been scarce reports of the felid *Panthera onca* (jaguar) infected by both *Hepatozoon felis* and *Cytauxzoon felis* (Furtado et al., 2017a, 2017b), and *Hepatozoon caimani* infecting the caiman *Caiman crocodilus* (Lainson et al., 2003) in the Amazon, to our knowledge, there have been no additional reports of Piroplasmida or *Hepatozoon* agents infecting free-ranging wildlife from the Amazon biome. This scenario motivated the present study, which performed a preliminary investigation of the infection of these protozoan agents in different free-ranging vertebrate wild species (reptiles, birds, and mammals) that were conveniently obtained in two large areas of the Brazilian Amazon.

2. Materials and methods

Wild animals were sampled in two areas within the Amazon biome of Brazil. In one area in Mato Grosso state (central-western Brazil), samples comprised animals that were hunted by Indians during September 2010 to June 2012 in the Tapirapé Indian Reserve, within Confresa Municipality (10°36′ to 10°52′S; 51°10′ to

* Corresponding author.
E-mail address: labruna@usp.br (M.B. Labruna).

http://dx.doi.org/10.1016/j.ijppaw.2017.05.002

51°21′W). In another area in Pará state (northern Brazil), samples comprised road-killed animals during February 2009 to November 2011, alongside the BR163 highway, between Km 50 and 217 within Santarém (02°24′S; 54°42′W) and Rurópolis (04°05′S; 54°54′W) municipalities. Further details on these animal samplings have been reported elsewhere (Soares et al., 2015).

From each animal, fragments of internal organs or blood samples were collected and kept frozen at −20 °C until being processed in the laboratory. DNA extraction of blood or fragments of lung or liver was conducted with the DNeasy Tissue and Blood Kit (Qiagen, Chatsworth, CA, USA) according to manufacturer's instructions. Blank tubes containing water were always included as a contamination control during DNA extraction. The concentration of extracted DNA was measured in a spectrophotometer UV (Bio Photometer plus, Eppendorf, Hamburg, Germany). Only samples with at least 20 ng/μl of DNA were subjected to PCR assays.

Tissue or blood DNA samples were tested by two polymerase chain reaction (PCR) protocols. One protocol employed primers BAB2 143–167 (5′-CCG TGC TAA TTG TAG GGC TAA TAC A-3′) and BAB2 694-667 (5′-GCT TGA AAC ACT CTA RTT TTC TCA AAG-3′), targeting a ≈551-bp of the 18S rRNA gene of tick-borne Piroplasmida (genera *Babesia, Theileria, Cytauxzoon*). The second protocol employed primers HEP2 144–169 (5′- GGT AAT TCT AGA GCT AAT ACA TGA GC-3′) and HEP2 743-718 (5′-ACA ATA AAG TAA AAA ACA YTT CAA AG-3′), targeting a 574-bp of the 18S rRNA gene of *Hepatozoon* spp. Reactions were performed as previously reported (Almeida et al., 2012).

For each reaction, both positive (DNA of *Babesia vogeli* or *Hepatozoon canis*) and negative (water) controls were included. Amplified products were analyzed after electrophoresis in 1.5% agarose gels stained by SYBR Safe DNA Gel Stain (Life Technologies, Grand Island, NY, USA) and visualized under U.V. transilluminator. All PCR products of the expected size were purified with ExoSap (USB, Cleveland, OH, USA) and DNA-sequenced (bi-directional Sanger sequencing) in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyser, Foster City, CA, USA) with the same primers used for PCR. Generated sequences were compared to each other and submitted to BLAST analysis (www.ncbi.nlm.nih.gov/blast) to infer closest similarities available in GenBank.

Partial sequences of the 18S rRNA gene generated in this study by the Piroplasmida-targeted PCR were aligned with corresponding sequences of related genotypes and Piroplasmida species available in GenBank. Similarly, partial sequences of the 18S rRNA gene generated in this study by the *Hepatozoon*-targeted PCR were aligned with corresponding sequences of related genotypes and *Hepatozoon* species available in GenBank. Sequences were aligned using ClustalX (Thompson et al., 1997) and secondary structure comparative analysis, and were adjusted manually using GeneDoc (Nicholas and Nicholas, 1997). The 18S rRNA sequences were used to construct a phylogenetic tree using maximum parsimony, as implemented in PAUP version 4.0b10 (Swofford, 2002) with heuristic search in 1000 replicates and 500 bootstrap replicates, random stepwise addition starting trees (with random addition sequences) and TBR branch swapping.

This work was authorized by the Brazilian Institute of Environment and Natural Resources (IBAMA authorization no. 23225-1 and 21526-1) and the Indian National Foundation (FUNAI authorization no. 45/AAEP/10 – Process no. 2433/07), and was approved by the Ethical Committee of Animal Use of the Faculty of Veterinary Medicine of the University of São Paulo (protocol no. 1747/2009).

3. Results

In Mato Grosso state, 49 wild animals comprising 29 different species (2 reptiles, 5 birds, and at least 22 mammals) were sampled (Table 1). In Pará state, 132 wild animals comprising 18 mammal species were sampled (Table 2). The Piroplasmida 18S rRNA gene-targeted PCR showed that among 49 evaluated animals from Mato Grosso, and among 132 evaluated animals from Pará, 5 (10.2%) and 11 (8.3%), respectively, contained Piroplasmida DNA (Tables 1 and 2). All PCR-positive animals were mammals. Among Mato Grosso samples, only the lung yielded Piroplasmida DNA; for Pará samples, both lung and liver yielded Piroplasmida DNA. In only two animal species, the detected Piroplasmida agent was a known agent since the resulting 18S rRNA gene sequences of the PCR amplicons were 100% identical to GenBank available sequences; these include *Cytauxzoon felis* detected in one ocelot (*Leopardus pardalis*), and *Theileria cervi* detected in two red brocket deer (*Mazama americana*) (Table 2). All remaining piroplasmid sequences are new haplotypes, with closest matches in GenBank varying from 95 to 98%, as shown in Tables 1 and 2. These novel agents were detected in three nine-banded-armadillos (*Dasypus novemcinctus*), one agouti (*Dasyprocta* sp.), four lowland pacas (*Cuniculus paca*), one common opossum (*Didelphis marsupialis*), and one white-lipped peccary (*Tayassu pecari*). No piroplasmida haplotype was shared by different vertebrate species. On the other hand, armadillos (*D. novemcinctus*) from Mato Grosso and Pará states had identical Piroplasmida haplotypes. The phylogenetic tree inferred by Piroplasmida 18S rRNA gene partial sequences showed that these two armadillo (*D. novemcinctus*) haplotypes grouped with the agouti (*Dasyprocta* sp.) haplotype under 100% branch support and 2.40% sequence divergence; the clade composed by *Theileria* parasites included the deer haplotype (identical to *T. cervi*; 70% bootstrap and 1.02% sequence divergence) detected in this study; the paca (*C. paca*) haplotype grouped with a piroplasmid sequence from capybara (a rodent species closely related to paca) from southern Brazil under 97% bootstrap support and 1.28% sequence divergence (Fig. 1). The opossum (*D. marsupialis*) haplotype formed a clade with a *Babesia* sp. recently reported in another opossum species (*Monodelphis domestica*) from the Brazilian Pantanal (76% bootstrap, 0.96% sequence divergence). Finally, the peccary (*T. pecari*) haplotypes grouped within a clade (97% bootstrap support) composed by several piroplasmid agents phylogenetically distinct from the classic *Babesia* or *Thelieria* organisms (70% bootstrap and 0.83% sequence divergence).

The *Hepatozoon* 18S rRNA gene-targeted PCR showed that among 49 evaluated animals from Mato Grosso, and among 132 evaluated animals from Pará, 5 (10.2%) and 2 (1.5%), respectively, contained *Hepatozoon* DNA (Tables 1 and 2). PCR-positive animals included 2 reptile species and 2 mammal species. From the lung of three spectacled caimans (*Caiman crocodilus*), the *Hepatozoon* 18S rRNA partial DNA sequence was 100% identical to GenBank *Hepatozoon* sp. MRA-2014b clone C23 (KJ413113), previously detected in *Caiman yacare* from the Brazilian Pantanal. From the liver of an ocelot (*L. pardalis*), the *Hepatozoon* 18S rRNA partial DNA sequence was 100% identical to GenBank *Hepatozoon felis* sequence (Table 2). The remaining *Hepatozoon* sequences generated in this study are new haplotypes, with closest matches in GenBank varying from 95 to 96% (Tables 1 and 2). These novel agents were detected in the blood of one scorpion mud turtle (*Kinosternon scorpioides*), and in the lung of one lowland paca (*C. paca*). The phylogenetic tree inferred by *Hepatozoon* 18S rRNA gene partial sequences showed that the mud turtle haplotype grouped with *Hepatozoon catesbianae* (AF130361) in a large clade composed by either rodent- or reptile-associated *Hepatozoon* organisms, including the caiman *Hepatozoon* haplotype generated in this study (Fig. 2). The paca (*C. paca*) haplotype branched separately, basal to a large clade of *Hepatozoon* organisms associated with reptiles and marsupials.

The GenBank nucleotide sequence accession numbers for the