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Hepatozoon Infecting Bats in the Southeastern Brazilian Rainforest

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ABSTRACT: Tick-borne protozoans of the genus *Hepatozoon* are obligate hemoparasites that can infect domestic and wild terrestrial vertebrates. Main hepatozoonosis affects canids and involves mainly *Hepatozoon canis* and *Hepatozoon americanum*. However, molecular studies revealed the capacity of *H. canis* to infect a wide range of wild mammals. In July 2018, we conducted an epidemiological survey for tick-borne pathogens in wild hosts, assaying *Hepatozoon* sp. occurrence of in 34 bats captured in different habitats within a conservation unit in the state of Espírito Santo, southeastern Brazil. Blood and spleen tissue DNA samples were submitted to PCR amplifications of *Babesia/Theileria* and *Hepatozoon* 18S rRNA gene and 21% (7/34) were positive for *Hepatozoon* sp. Phylogenetic inferences grouped the obtained sequences from Seba's short-tailed bat (*Carollia perspicillata*) with the *H. canis* cluster, and from the great fruit-eating bat (*Artibeus lituratus*) with rodent-associated *Hepatozoon* cluster. Further studies are needed to characterize the epidemiological role of Seba's short-tailed bat and the great fruit-eating bat in the wild transmission cycle of these hemoparasites in Brazil.

Key words: Bat, hemoparasite, protozoa, southeast Brazil, tick-borne pathogen.

The genus *Hepatozoon* comprises obligate hemoparasites that can infect domestic and wild terrestrial vertebrates. The *Hepatozoon* life cycle has a definitive hematophagous invertebrate host, and an intermediate vertebrate host (Serra-Freire 1979; Alencar et al. 1997; Dantas-Torres 2008), which becomes infected through a trophic cycle involving the ingestion of infected invertebrates or vertebrate prey, which can lead to hepatozoonosis (Smith 1996; Almeida et al. 2013; Maia et al, 2014).

Hepatozoonosis affects canids and typically involves *Hepatozoon canis* and *Hepatozoon americanum*, whose transmission occurs by ingestion of infected ticks (Rubini et al. 2005; Demoner et al. 2016). However, molecular studies revealed the potential of *H. canis* to infect a wide range of wild mammals such as the white-eared opossum (*Didelphis albiventris*), hoary fox (*Pseudalopex vetutus*), and bush dog (*Speothos venaticus*; Silva et al. 2017). The first evidence of *Hepatozoon* infection in Chiroptera was during a molecular survey of *Trypanosoma* sp. in mammals' tissues, which resulted in detection of *Hepatozoon* sp. in fawn leaf-nosed bat (*Hipposideros cervinus*) from Malaysia, without sequencing (Pinto et al. 2013). In Brazil, although molecular studies have shown the occurrence of *Hepatozoon* species in wild canines and felids, rodents, coatis, marsupials, crocodiles and amphibians (André et al. 2010; Azevedo et al. 2018; Perles et al. 2019), *Hepatozoon*-like organisms were detected in Chiroptera (by morphological analysis) in little yellow-shouldered bat (*Sturnira lilium*) from São Paulo state (Torres et al. 1983).

Between 28 July and 10 August 2018, an epidemiological survey was conducted for tick-borne pathogens inhabiting wild hosts and, in this study, we evaluate the occurrence of *Hepatozoon* in bats captured in different habitats types from a biological reserve of Espirito Santo state, southeastern Brazil. During an epidemiological survey of *Babesia* occurrence, field expeditions were conducted to collect ectoparasites, blood, and spleen tissues from bats, in the Biological Reserve of

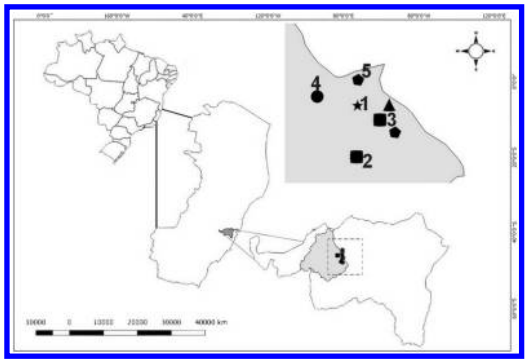

FIGURE 1. Map of Brazil (A), highlighting Espírito Santo State (B), at Brazil's Southeast Region, with the municipality of Cariacica underlined (C), emphasizing the investigated sites of bat sampling in Duas Bocas Biological Reserve (D), during the babesiosis epidemiologic research in July 2018. The numbers indicate the collection sites within the Reserve: accommodation for researchers (1 and 2), anthropized area trail (3), preserved forest trail (4), and cave with pipes (5). The bat species captured are indicated as: square, *Artibeus lituratus;* pentagon, *Carollia perspicillata;* triangle, *Desmodus rotundus;* star, *Molossus rufus;* and circle, *Myotis lucifugus*.

Duas Bocas, municipality of Cariacica, Espírito Santo State, Southeast Brazil (Fig. 1). The area is comprised of protected Atlantic Rainforest biome, with lightly anthropized fragments.

Bats were captured and euthanized for this study by the authorization of State Institute for the Environment and Water Resources of Espírito Santo (IEMA-ES) (Research License GRN Number 026ª-2017, Process Number 755612/1/16—Atualização) and by Chico Mendes Institute for Biodiversity Conservation of Brazilian Ministry of Environment (ICMBio/MMA) (Authorization for Scientific Purpose Activity Number 63023-1). Bats were captured for 7 consecutive d, between 0800 hours and 1600 hours with nets at sleeping sites, in artificial tunnels and natural caves, and at night, with mist nets. The nets were set up before dusk, around 1700 hours, and were checked every 20 min until 2300 hours, when they were closed. Captured bats were sedated with an intraperitoneal injection of 10% ketamine (200 mg/kg) and 2% xylazine (20 mg/kg) solution at a ratio of 2:1, and blood was collected by cardiac puncture and stored at –20 C until DNA purification. Taxonomic identifications were based on analyses of skull and dental characters and on morphometric measurements (Moratelli et al. 2011; Reis et al. 2017; Loureiro et al. 2018). Subsequently, specimens were euthanized with 2.5% sodium thiopental (120 mg/kg) and spleen samples were collected and fixed in 99% pure isopropyl alcohol and submitted to DNA purification.

We isolated DNA from chiropteran blood and spleen samples using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) and used as template for semi-nested PCR amplifications of *Babesia/Theileria* and *Hepatozoon* partial nuclear small-subunit ribosomal RNA (*18S rRNA*) gene (Criado-Fornelio et al. 2003b). The PCR products of the expected size (approximately 410 base pairs; Criado-Fornelio et al. 2003a) were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega, Madison, Wisconsin, USA) and sequenced with the BigDye™ Terminator–Cycle Sequencing Ready Reaction kit (Applied Biosystems, Carlsbad, California, USA) in an automatic sequencer (Applied Biosystems 3730xl DNA Analyzer). Sequences were edited using SeqMan software (DNASTAR Lasergene, Madison, Wisconsin, USA), and identity values were obtained using the Basic Local Alignment Search Tool (BLAST; National Center for Biotechnology Information 2018). Sequence alignment was performed using Clustal Omega (Madeira et al. 2019) and phylogenies were assessed applying the maximum-likelihood method, with T92+G correction model selected by MEGA version 7 software (Kumar et al. 2016), which was also used to produced phylogenetic trees from 1,000 bootstrap replicates for node support estimation.

We collected 34 Chiroptera specimens identified as the great fruit-eating bat (*Artibeus lituratus*), Seba's short-tailed bat (*Carollia perspicillata*), vampire bat (*Desmodus rotundus*), black mastiff bat (*Molossus rufus*), and black myotis (*Myotis nigricans*; Table 1). Of all examined bats, only one black mastiff bat was found parasitized by a female of Mesostigmata mite; however, the specimen

TABLE 1. Detection of partial nuclear small-subunit ribosomal RNA (18S rRNA) gene of Hepatozoon sp. in bats from Biological Reserve of Duas Bocas, Espírito Santo State, Southeast Brazil, between 28 July and 10 August 2018.

Species	Collected		PCR-positive			
	No.	%	Sample	No.	%	95% Confidence Interval
Artibeus literatus	4	12	Blood	0	00.00	0.00, 0.60
			Spleen	2	50.00	0.15, 0.85
			Total	2	50.00	0.15, 0.85
Carollia perspicillata	11	32	Blood	1	09.09	0.01, 0.43
			Spleen	4	36.36	0.12, 0.68
			Total	5	45.45	0.18, 0.75
Desmodus rotundus	2	6	Blood	0	00.00	0.00, 0.80
			Spleen	0	00.00	0.00, 0.80
			Total	0	00.00	0.00, 0.80
Molossus rufus	16	47	Blood	0	00.00	0.00, 0.24
			Spleen	0	00.00	0.00, 0.24
			Total	0	00.00	0.00, 0.24
Myotis nigricans	1	3	Blood	0	00.00	0.00, 0.94
			Spleen	0	00.00	0.00, 0.94
			Total	0	00.00	0.00, 0.94
Total	34	100	Blood	1	02.94	0.01, 0.58
			Spleen	6	17.65	0.42, 0.99
			Total	7	20.59	0.09, 0.38

was damaged and further identification could not be performed.

Hepatozoon DNA was detected in one Seba's short-tailed bat blood sample and four spleen tissue samples; it was also detected in two great fruit-eating bat spleen tissue samples, totaling seven *Hepatozoon*-positive samples (Table 1). The BLAST analysis of Seba's short-tailed bat sequences that we obtained (MN369545, MN369546, and MN369547) showed 99% identity (410/416, 402/408, and 403/409 base pairs, respectively) with *H. canis* (KU893123). In addition, the great fruit-eating bat-derived sequences (MN369548 and MN369549) showed 99% identity (407/411 and 406/411, respectively) with *Hepatozoon* sp. strain BV2 (AY600625) and BV1 (AY600626), originally isolated from a cricetid rodent in Spain. Indeed, our phylogenetic reconstructions supported grouping our *Hepatozoon* 18S rRNA sequences from Seba's short-tailed bat with the *H. canis* cluster from canids, and our *Hepatozoon* sequences from great fruit-eating bat with the *Hepatozoon* sp. BV2/BV1 cluster, from rodents (Fig. 2).

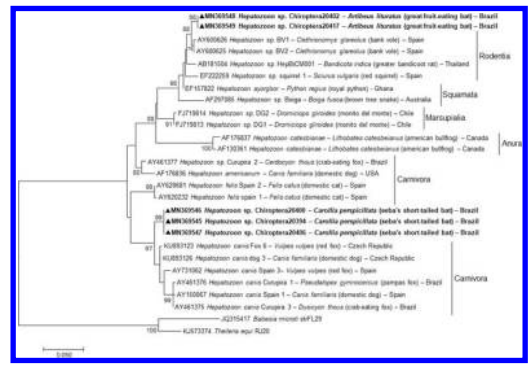

FIGURE 2. Phylogenetic inferences by maximum-likelihood method from 1,000 replicated trees based on nucleotide sequences of *Hepatozoon* 18s rRNA gene isolated of bats from Biological Reserve of Duas Bocas, Espírito Santo State, Southeast Brazil, between 28 July and 10 August 2018. Evolutionary distances were estimated by T92+G model. Bootstrap values greater than 70% are shown. Sequences obtained are highlighted with black triangle and GenBank accession numbers precede the sequence names. Scale bar indicates nucleotide substitutions per site.

Immunologically, spleen is an important organ for vector-borne pathogen control, because it is the organ where several stages of *H. canis* are found and, together with the bone marrow, it is one of the most frequently parasitized organs, harboring chronic infections (Levine 1973; Levi et al. 2018). Here, we recorded a higher number of positive samples (six of seven positive samples) of *Hepatozoon* strains from spleen (Table 1) than blood, which was a better tissue for detection than blood in this study, as has been observed for other mammalian species (Hodžić et al. 2018; Levi et al. 2018). It was not possible to include the *Hepatozoon* sequences of Chiroptera from Malaysia (Pinto et al. 2013) in our alignments (Fig. 2) because these sequences represent a different portion of 18S rRNA gene fragment than the sequences obtained in this study, impeding identification and comparison of the Chiroptera isolates.

In our study, nearly half of the great fruit-eating bat and Seba's short-tailed bat captured were infected individuals (Table 1). This intrinsic relationship of *Hepatozoon* strains detected in the two infected Chiroptera species might be related to the enzootic cycle of each group, because spleen can harbor chronic infections (O'Dwyer 2011; Movilla et al. 2017; Levi et al. 2018), and due to the feeding habits and behavior of bats in the investigated area. The great fruit-eating bat is distributed throughout the Neotropical region, from Mexico to northern Argentina, including all regions of Brazil. The species is abundant in conserved areas as well as in altered and urban environments and, although primarily frugivores, can also feed on insects. Similarly, Seba's short-tailed bat is known to occur widely in the Neotropics, including all regions of Brazil, predominantly in altered and urban environments, feeding mainly on pepper plants (Piperaceae), but also able to feed on nectar and insects (Reis et al. 2007).

Although few studies investigating the tick-borne protozoans infection have reported the presence of *Hepatozoon* in bats (Torres et al. 1983; Pinto et al. 2013), our results indicated that great fruit-eating bat and Seba's short-tailed bat are susceptible to infection by organisms genetically related to *Hepatozoon* sp. strain BV2 and *H. canis*, respectively, and probably, the hygienic practices of these mammals could increase *Hepatozoon* infection mechanisms via ingestion of infected hematophagus ectoparasites (Pinto et al. 2013).

It is not possible to establish a relation with vectors, paratenic, and reservoir hosts for *Hepatozoon* in this region, because these remain unknown. Further studies are needed to clarify whether Seba's short-tailed bat and great fruit-eating bat have any eco-epidemiologic importance in the cycle of these hemoparasites in Brazil.

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