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Natural infection of the wild canid, *Cerdocyon thous*, with the piroplasmid *Rangelia vitalii* in Brazil

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ABSTRACT

Canine rangeliosis, caused by the piroplasmid protozoon *Rangelia vitalii*, is currently recognized as a reemerging disease that affects domestic dogs in Brazil. In the present study, piroplasmid infection was searched in wild canids (20 *Cerdocyon thous* and 4 *Lycalopex gymnocercus*) in Brazil. Molecular analysis, based on PCR and DNA sequencing of a portion of the 18S rRNA gene, revealed that 30% (6/20) *C. thous* were infected by *R. vitalii*. Blood and bone marrow samples from one of the *R. vitalii*-infected *C. thous* were inoculated into a domestic dog, which developed clinical rangeliosis that was confirmed by molecular tests. However, the *C. thous* donor showed no clinical, hematological or biochemical alterations, even though its *R. vitalii* infection status was confirmed for at least 80 days. These observations suggest that *R. vitalii* is not as highly pathogenic for *C. thous* as it is for domestic dogs. Phylogenetic analysis inferred by the 18S rRNA gene placed *R. vitalii* embedded in the clade '*Babesia* sensu stricto', consisting of a number of species that represent truly the genus *Babesia*. It is proposed that the species *R. vitalii* should be transferred to the genus *Babesia*. The present study expands our knowledge on the natural history of *R. vitalii*, suggesting that it might have a natural cycle involving the wild canid *C. thous*. Further studies are needed to confirm that *C. thous* is a natural reservoir of *R. vitalii* in Brazil.

1. Introduction

Piroplasmids are tick-borne protozoan parasites that infect blood cells of numerous wild and domestic vertebrates worldwide. The piroplasmid *Rangelia vitalii* is the etiologic agent of rangeliosis, a canine disease that was described in the beginning of the previous century in the state of São Paulo, southeastern Brazil (Pestana, 1910; Carini and Maciel, 1914). Because a number of subsequent authors (Wenyon, 1926; Doflein and Reichenow, 1929; Moreira, 1938, 1939; Levine, 1973; Peirce, 2000) considered *R. vitalii* a synonym of *Babesia vogeli* (reported as *Babesia canis*), rangeliosis was widely neglected during the

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second half of the 20th century. During the last decade, a number publications from southern Brazil highlighted *R. vitalii* as a reemerging agent of a severe canine piroplasmosis, especially among rural dogs (Krauspenhar et al., 2003; Loretti and Barros, 2005; Fighera, 2007; Fighera et al., 2008, 2010; França et al., 2010). In 2011, the validity of the taxon *R. vitalii* was proposed by molecular methods based on phylogenetic analyses inferred by partial sequences of the 18S rRNA and *hsp70* genes (Soares et al., 2011). The infection by *R. vitalii* has been confirmed solely in domestic dogs from southern and southeastern Brazil (Soares et al., 2011, 2013a,b; Lemos et al., 2012).

The Brazilian fauna of wild canids is composed by six native species (Cheida et al., 2011). Literature records on piroplasmids infecting these native canids are very scarce, and have been based solely on morphological identification of intraerythrocytic piroplasmid forms in blood smears from *Chrysocyon brachyurus* (Serra-Freire et al., 1995; Cansi et al., 2012), *Lycalopex vetulus* (Martins et al., 2006), *Lycalopex gymnocercus* (Ruas et al., 2003), and *Cerdocyon thous* (Paraense and Vianna, 1948; Massard et al., 1981). The crab-eating fox, *C. thous*, is widely distributed in South America, from Uruguay and northern Argentina to the lowlands of Bolivia and Venezuela, also occurring in Colombia, Guyana and Suriname. In Brazil, it is found in all major biomes except for the Amazon (Cheida et al., 2011). The Pampas fox, *L. gymnocercus*, has a more restricted distribution, occurring in the southern cone of South America, except for Chile (Cheida et al., 2011). In the present study, piroplasmid infection was searched for in *C. thous* and *L. gymnocercus* from Brazil. We report the first molecular detection of a piroplasmid agent in South American free-ranging wild canids, as well as the effects of the inoculation of this agent in a domestic dog.

2. Materials and Methods

2.1. Wild canids

In May 2012, a free-ranging, young, adult female (approximately 5 kg) of *C. thous* (animal #1) was rescued in Carazinho Municipality, state of Rio Grande do Sul, southern Brazil, and taken to the nearby Veterinary Teaching Hospital of the University of Passo Fundo due to a traumatic amputating injury in the hind limb. Clinical evaluation was performed, and blood samples collected 0, 69, and 80 days after admission were used for molecular detection for piroplasmids (described below), whereas blood samples collected in both dry and EDTA tubes at the 21st day were used for biochemical and hematological evaluations, respectively. At the 80th day, the animal died of unknown etiology; bone marrow aspirates and spleen samples were collected.

From July 2012 to August 2013, tissue samples (blood or internal organs) were collected from 23 wild canids from different areas of the states of Rio Grande do Sul and São Paulo (southeastern Brazil) (Table 1). Animal #2 was rescued due to trauma caused by fighting with domestic dogs; animals #3–13 and 21–23 were road-killed, whereas animals #14–20, and 24, were sampled in captivity at zoos. From road-killed animals, the collected material varied accordingly to the availability of tissues found in the carcasses. From living animals sampled at the zoos, only blood in EDTA was collected.

2.2. Inoculation of domestic dog

Three ml of bone marrow aspiration and 10 ml of blood was collected from *C. thous* #1 on the 80th day after its admission to the hospital, and sent to the University of São Paulo under refrigeration. Twenty hours later, the samples were intravenously inoculated into an 8-month domestic dog, derived from our Beagle experimental kennel, where dogs have never experienced tick infestations, are regularly vaccinated and dewormed, and are regularly tested to certify that they are free of tick-borne diseases. The domestic dog was clinically evaluated and had its rectal temperature measured daily, as well as blood samples taken in EDTA-tubes 3 times per week for blood cell and platelet counts, and PCR targeting piroplasmids during a period of 32 days after inoculation. An additional blood collection at 69 days post-inoculation (dpi) was used for PCR analysis. During this period, the dog was kept in a room with no environmental contamination with ticks.

2.3. Molecular and phylogenetic analyses

Animal blood or tissue samples were subjected to DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen®, Hilden, Germany), according to the manufacturer's instructions. All DNA samples from wild canids were initially tested by one of the following two PCR assays, one targeting a ≈700-bp fragment of the Carnivore mitochondrial DNA (mtDNA) control region containing the first hypervariable segment (HVS-I), using primers MTLPRO2 and CCR-DR1, as previously described (Tchaicka et al., 2007); or a PCR assay targeting a 359-bp fragment of the vertebrate mitochondrion cytochrome b gene (*cyt b*), as previously described (Steuber et al., 2005). For detection of piroplasmids, all samples were tested by a PCR protocol using primers BAB143-167 and BAB694-667, targeting a ≈500-bp fragment of the piroplasmid 18S rRNA gene, as previously described (Soares et al., 2011). PCR products were electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and examined by UV transillumination. Amplicons of the expected size were purified with ExoSap (USB, Cleveland, OH) and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 310 Genetic, Foster City, CA) according to the manufacturer's protocol. Generated sequences were submitted to BLAST analysis (Altschul et al., 1990) to determine the closest similarities in GenBank.

Partial sequences (549-nt) of the 18S rRNA gene of piroplasmids derived from the wild canids were aligned with corresponding 18S rRNA sequences from 53 genotypes of the genera *Babesia*, *Theileria*, *Cytauxzoon* and *Hepatozoon* retrieved from Genbank, using Clustal/W v.1.8.1 (Thompson et al., 1994). A maximum likelihood phylogenetic tree using GTR+G+I substitution model was generated using Mega 5.2.2 software (Tamura et al., 2011) with 100 bootstrap replicates. The substitution model was select using Mega 5.2.2 software (Tamura et al., 2011) according