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PROTOZOOLOGY - SHORT COMMUNICATION

Detection of *Rangelia vitalii* (Piroplasmida: Babesiidae) in asymptomatic free-ranging wild canids from the Pampa biome, Brazil

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Abstract

Canine rangeliosis is a tick-borne disease caused by the protozoan *Rangelia vitalii*, which has only been reported in South America. With this knowledge, we hypothesized that neotropical foxes could act as asymptomatic natural carriers of *R. vitalii*. To test this, we captured 44 free-ranging foxes and investigated the presence of *R. vitalii* DNA, and whether the infected animals presented any clinical findings or hematological changes. Eight foxes (18%), seven *Cerdocyon thous* (7/27–25%), and one *Lycalopex gymnocercus* (1/17–5%) were positive for *R. vitalii*. All foxes were clinically healthy and showed no hematological abnormalities. Thus, we propose that neotropical canids, particularly *C. thous*, could be the natural carriers of *R. vitalii*.

Keywords *Cerdocyon thous* · Rangeliosis · *Lycalopex gymnocercus* · Carrier · Hemoparasite

Introduction

The growth of human populations and the consequent increase in domestic animals around natural areas have enhanced the opportunities for pathogen transmission between wild and domestic animals (Daszak et al. 2000; Cleaveland et al. 2001; Bengis et al. 2002). Therefore, permanent surveys of pathogens among wild animals are essential to improve our understanding of the natural cycle of diseases, for early detection of risks to human/animal health and biodiversity, and to prevent and control zoonotic outbreaks through the One Health approach (Williams et al. 2002; Grogan et al. 2014).

In this context, wild carnivores play an important role in ecosystems and can be considered "bioaccumulators" of pathogens (Macdonald and Kays 2005; Jorge et al. 2010; Rocha et al. 2013). Wild canids could be seen as especially important due their ecological roles as keystone species, predators, or scavengers, in addition to their abundance and probable role as hosts for agents of emergent diseases (Aguirre 2009; Grogan et al. 2014).

The crab-eating fox (*Cerdocyon thous*) and pampas fox (*Lycalopex gymnocercus*) are the most common neotropical canids found in the Brazilian part of Pampa biome, which comprises the southernmost areas of Brazil (Rio Grande do Sul state), Uruguay, and part of Argentina. These species of foxes are considered generalists with opportunistic habits, meaning they can even adapt to anthropized environments (Faria-Corrêa et al. 2009).

One of the major concerns regarding the conservation of wild canids is the risk of infection with pathogens from domestic dogs. Conservation strategies must address both cosmopolitan pathogens, such as canine distemper virus and *Leishmania* spp., as well as locally important infectious agents. In the Pampa biome, one of the most common infectious diseases of dogs is canine rangeliosis. It is a neglected

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and re-emerging tick-borne disease caused by the protozoan *Rangelia vitalii* (Piroplasmida: Babesiidae) which, so far, has primarily been reported in the southern cone of South America (Loretti and Barros 2002; Eiras et al. 2014; Soares et al. 2015). Rangeliosis causes hemolytic and hemorrhagic disorders in dogs (Silva et al. 2013). The clinical findings may include anemia, jaundice, fever, splenomegaly, enlarged lymph nodes, gastrointestinal bleeding, persistent bleeding of the tips of ears, nose and oral cavity, and even death (Loretti and Barros 2002; Martins et al. 2016).

Previous studies with captive animals and carcasses of *C. thous* have revealed *R. vitalii* infection in this species (Soares et al. 2014). While some authors have proposed that wild canids could show clinical findings associated with rangeliosis, there is also a suspicion that they could be natural carriers. To date, there is no available data concerning potential hematological abnormalities associated with the infection in foxes. The suggestion of a neotropical carnivore as a natural host of *R. vitalii* is reinforced by the fact that canine rangeliosis has only been reported in South America to date.

Thus, the aim of this work was to investigate the occurrence of *R. vitalli* in free-ranging wild canids from the Pampa biome to support the hypothesis that *C. thous* and *L. gymnocercus* could act as asymptomatic natural carriers of *R. vitalii*. For this purpose, we investigated the presence of *R. vitalii* DNA in blood samples from free-ranging wild canids and its relationship with body conditions and clinical pathology parameters.

Material and methods

This study comprised six different municipalities of the Brazilian Pampa, located in Southern Brazil in the state of Rio Grande do Sul (RS). Two of them are protected areas: APA (Environmental Protection Area) do Ibirapuitã (municipality of Santana do Livramento; 30°25′53.1″S 55°28′09.9″W), and Refúgio de Vida Silvestre Banhado dos Pachecos (RVSBP, municipality of Viamão; 30°05′32.0″S 50°51′00.0″W). The others comprise unprotected regions of natural remaining vegetation, located within rural areas of the municipalities of Triunfo (29°51′58.1″S 51°21′54.5″W), Candiota (31°28′59.8″S 53°48′44.1″W), Arroio Grande (32°19′55.2″S 52°54′05.8″W), and Alegrete (30°04′00.0″S 55°31′00.0″W).

The foxes were captured between April 2014 and August 2016 with Tomahawk live traps baited with meat, bacon, and sardines. After capture, the animals were anesthetized using a combination of ketamine (10 mg/kg) and xylazine (1 mg/kg), and underwent a physical examination by a veterinarian. Blood samples were collected by a single jugular vein puncture. After full recovery from anesthesia, the animals were released at the same site of capture. All procedures were authorized by the Brazilian authorities (SISBIO-ICMBio license numbers 47,357-1 and 38,803-2, CEUA-IPVDF license number 14/13).

Blood samples were submitted to hematological analysis using an automated veterinary hematology analyzer (Bio-1800 Vet; Bioeasy Diagnostica S/A, Belo Horizonte, Brazil). The following parameters were analyzed: red blood cell (RBC) count, hemoglobin (Hb), packed cell volume (PCV), and white blood cell (WBC) count. DNA from whole blood samples were extracted using the Invitrogen PureLink Genomic DNA kit according to the manufacturer's protocol. Polymerase chain reaction (PCR) for *R. vitalii* detection was performed using the primers BAB143-167 (5′-CCGT GCTAATTGTAGGGCTAATACA-3′) and BAB649-667 (5′-GCTTGAAACACTCTARTTTTCTCAAAG-3′), targeting a 500-bp fragment of the 18S rRNA gene specific to the Babesiidae family, according to Soares et al. (2011). Briefly, for the amplification, initial denaturation was performed at 95 °C for 3 min, followed by 34 cycles of denaturation at 95 °C for 30 s. Annealing occurred at 63 °C for 1 min, and extension at 72 °C for 1 min. The final extension was performed at 72 °C for 7 min. The PCR products were submitted to electrophoresis and purified for sequencing. The DNA of *Babesia canis*, kindly provided by Dr. Marcelo Labruna (USP), was used as a positive control in all PCR reactions. Ultrapure water was used as a negative control. PCR targeting the mammalian glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene (Maciel et al. 2018) was performed to confirm that all potential negative results were not due to the presence of DNA inhibitors or a lack of DNA in the sample. All positive samples of the expected sizes were purified using a commercial kit (Purelink Quick Gel Extraction and Purification Combo Kit; Thermo Fisher Scientific, Vilnius, Lithuania) and sequenced using an automatic sequencer (Applied Biosystems/Perkin Elmer, CA, USA). The sequences obtained were submitted to BLAST analysis to confirm their identity with other *R. vitalii* sequences deposited in GenBank. Statistical analysis (*P* value calculation for comparison tests, values of mean, standard deviation, median, frequency, and its 95% confidence interval, 95%CI) were performed at GraphPad Prism.

Results and discussion

A total of 44 wild canids were captured, including 27 *C. thous* and 17 *L. gymnocercus*. Eight animals (18%) were positive for *R. vitalii* DNA. A higher frequency of infection was observed in *C. thous*, with 25% (95%CI, 13–44%) of the specimens being positive (7/27) compared with only 5% (95%CI, 1–26%) of *L. gymnocercus* (1/17). Positive *C. thous* individuals were sampled from APA do Ibirapuitã ($n = 5$), RVSBP ($n = 1$), and Triunfo ($n = 1$), while the positive *L. gymnocercus* was from Candiota (Fig. 1). All sequences of positive samples

were identical to each other and showed 100% identity with *R. vitalii* isolate 108 (GenBank accession number KT323931), previously identified in a dog with rangeliosis in the municipality of Passo Fundo in RS state.

Under physical evaluation, all foxes were considered clinically healthy, with no clinical findings compatible with rangeliosis or any other disease. A summary of information about the captured animals is presented in Table 1. Unfortunately, due to clot formation, hematological analysis of the only positive *L. gymnocercus* was not possible. Thus, a comparison of hematological parameters between infected and uninfected foxes was only performed for *C. thous*. The four hematological parameters were evaluated independently using Mann–Whitney test for unpaired samples, and no significant differences were observed between infected and uninfected foxes for the tested parameters. The two groups showed very similar means for all parameters, with all values within the reference interval reported for this species in Mattoso et al. (2012) (Table 2).

Other studies have previously reported the detection of *R. vitalii* in neotropical wild canids (Soares et al. 2014; de Quadros et al. 2015; Fredo et al. 2015; Silveira et al. 2016). However, to date, no data from free-ranging animals were available, with the available reports limited to captive animals, rescued animals or those found dead.

Herein, we observed the presence of this hemoparasite in approximately 20% of foxes sampled in different areas of the Pampa. It supports the hypothesis of a wild enzootic cycle of *R. vitalii* in neotropical wild canids. A drawback of our study was the low number of *L. gymnocercus* foxes sampled in the studied area. Although *C. thous* and *L. gymnocercus* are sympatric in all regions of the state of Rio Grande do Sul, their abundance significantly changes according to the microenvironment, as crab-eating foxes are more frequently recorded in forest habitats, whereas pampas foxes prefer open grasslands. As these species overlap in their distribution and even parasites, we cannot rule out that both of these fox species could act as natural carriers for *R. vitalii*. Indeed, further studies should address the *R. vitalii* prevalence in larger populations of *L. gymnocercus*.

Moreover, considering that foxes found to be positive for *R. vitalii* did not present clinical signs of the disease, it is reasonable to suggest that this protozoan organism has an asymptomatic or low virulence natural cycle involving the wild canids, probably as a result of co-adaptations between the hosts and pathogens. These results support previous suggestions that the pathogenicity of *R. vitalii* is markedly different in neotropical canids compared with the domestic dog (Soares et al. 2014).

Based only on the postmortem histopathological findings of a *C. thous* fox that tested positive for *R. vitalii*, Fredo et al. (2015) suggested that this microorganism was pathogenic for neotropical canids. In their case, a rescued fox admitted to a veterinary hospital died with clinical signs compatible with an acute neurological disease (including myoclonus characteristic of distemper). In the case reported by Fredo et al. (2015), we believe that the clinical manifestations cannot be directly

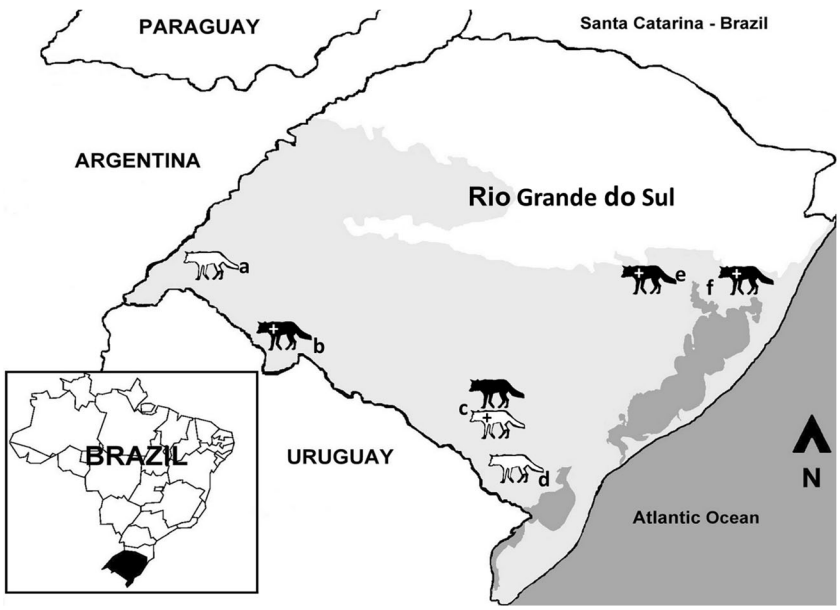

Fig. 1 Map of the studied area, Rio Grande do Sul state, Southern Brazil, and neighboring countries. The Pampa biome region is shown in light gray. Foxes were sampled from the following municipalities: (a) Alegrete, (b) Santana do Livramento (APA do Ibirapuitã), (c) Candiota, (d) Arroio Grande, (e) Triunfo, and (f) Viamão (Refúgio de Vida Silvestre Banhado dos Pachecos, RVSBP). Black foxes in the map indicate places where *Cerdocyon thous* were captured; white foxes, in turn, show places where *Lycalopex gymnocercus* were sampled. Animals positive for *Rangelia vitalii*-DNA were represented by a positive (+) symbol. In the insert, map of Brazil showing Rio Grande do Sul state (in black)

Table 1 Information on the wild canids sampled and the results of molecular detection of *R. vitalii*

Identification	Municipality	Species	Sex	Age	Weight (kg)	*R. vitalii* detection
C3	Santana do Livramento	*C. thous*	Male	Adult	6.0	−
C5	Santana do Livramento	*C. thous*	Male	Juvenile	5.0	+
C6	Santana do Livramento	*C. thous*	Male	Juvenile	6.0	−
C7	Santana do Livramento	*C. thous*	Female	Juvenile	5.0	+
C8	Santana do Livramento	*C. thous*	Female	Adult	6.0	−
C9	Santana do Livramento	*C. thous*	Male	Adult	6.75	+
C10	Santana do Livramento	*C. thous*	Female	Adult/senile	6.25	−
C11	Santana do Livramento	*C. thous*	Male	Adult	5.25	−
C12	Santana do Livramento	*C. thous*	Male	Adult	5.0	+
C13	Santana do Livramento	*C. thous*	Male	Adult	5.0	+
C16	Santana do Livramento	*C. thous*	Male	Adult	7.29	−
C14	Triunfo	*C. thous*	Male	Adult	6.75	−
C15	Triunfo	*C. thous*	Male	Adult	6.9	+
C17	Viamão	*C. thous*	Male	Adult	6.73	−
C18	Viamão	*C. thous*	Male	Adult/senile	8.37	−
C19	Viamão	*C. thous*	Female	Adult/senile	4.5	−
C20	Viamão	*C. thous*	Male	Adult	6.94	−
C21	Viamão	*C. thous*	Male	Adult	5.97	−
C22	Viamão	*C. thous*	Male	Adult	7.2	−
C23	Viamão	*C. thous*	Female	Adult	6.77	−
C24	Viamão	*C. thous*	Male	Adult	6.40	+
C26	Viamão	*C. thous*	Female	Adult	5.73	−
C27	Viamão	*C. thous*	Male	Adult/senile	5.55	−
C28	Viamão	*C. thous*	Female	Juvenile	5.27	−
Can29	Candiota	*C. thous*	Female	Juvenile	5.8	−
Can31	Candiota	*C. thous*	Male	Adult	4.8	−
Can37	Candiota	*C. thous*	Male	Adult	6.85	−
Can30	Candiota	*L. gymnocercus*	Female	Adult	5.30	−
Can32	Candiota	*L. gymnocercus*	Male	Adult	6.65	−
Can34	Candiota	*L. gymnocercus*	Female	Adult	5.40	−
Can38	Candiota	*L. gymnocercus*	Female	Adult	4.85	−
Can39	Candiota	*L. gymnocercus*	Male	Adult	4.80	−
Can42	Candiota	*L. gymnocercus*	Male	Adult	5.22	+
Can44	Candiota	*L. gymnocercus*	Female	Adult	4.00	−
Can45	Candiota	*L. gymnocercus*	Male	Adult	4.88	−
Can46	Candiota	*L. gymnocercus*	Male	Juvenile	1.35	−
Can47	Candiota	*L. gymnocercus*	Female	Juvenile	3.54	−
#1	Arroio Grande	*L. gymnocercus*	Male	Adult	5.45	−
FP1130	Arroio Grande	*L. gymnocercus*	Female	Adult	5.40	−
FP1132	Arroio Grande	*L. gymnocercus*	Male	Adult	5.55	−
FP1135	Arroio Grande	*L. gymnocercus*	Male	Adult	5.50	−
BPGY88	Alegrete	*L. gymnocercus*	Female	Adult	5.00	−
BPGY89	Alegrete	*L. gymnocercus*	Male	Adult	5.95	−
BPGY88	Alegrete	*L. gymnocercus*	Feamale	Adult	5.57	−

associated with rangeliosis. It should be emphasized that the presence or absence of clinical findings associated with the infection, particularly in wild animals, may depend on the interaction of a number of factors, such as habitat, captivity stress, physical and body condition, age, immunity, and the occurrence of simultaneous infections (Jorge et al. 2010).

Table 2 Hematological parameters of sampled *Cerdocyon thous* positive and negative for *Rangelia vitalii*

Parameter	Median [mean ± standard deviation]		*P* value[a]	Reference interval[b]	
	R. vitalii positive ($n = 7$)	Non-infected ($n = 20$)		Minimum	Maximum
Red blood cells (RBC) count (10^6/ μL)	4.06 [4.13 ± 0.23]	4.43 [4.31 ± 0.09]	0.21	3.05	6.08
Hemoglobin (g/dL)	12.9 [12.7 ± 0.52]	13.6 [13.01 ± 0.35]	0.08	10	18.1
Packed cell volume (%)	41.6 [40.17 ± 3.8]	40.1 [39.67 ± 5.39]	0.53	28	53
White blood cells (WBC) count (10^3/μL)	11.90 [15.06 ± 4.8]	16.70 [15.53 ± 5.0]	0.81	3.4	23.2

[a] Mann–Whitney test for unpaired samples

[b] Mattoso et al. 2012

Even in the case of clinical manifestations compatible with rangeliosis, other co-infections must be considered.

Given the intensity of the clinical findings observed in domestic dogs and the restricted distribution of rangeliosis cases, it is possible that dogs are, in fact, accidental hosts of *R. vitalii*. This is corroborated by the fact that the strains circulating among domestic dogs and neotropical canids present identical DNA sequences. The high frequency of infected *C. thous* foxes, concomitant with the high prevalence of the competent tick vector (*Amblyomma aureolatum*) in this canid species (Dall'Agnol et al. 2018), suggest that the *C. thous–A. aureolatum* is the most likely complex responsible for the maintenance of *R. vitalii* in the environment. Indeed, in a previous work of our group, Dall'Agnol et al. (2018) showed that animals studied here had a tick prevalence parasitism above 90%. Also, almost two-thirds of ticks recovered from foxes were *A. aureolatum*. The only proven vector of rangeliosis, *A. aureolatum* tick, (Soares et al. 2018), is one of the most common ticks in Rio Grande do Sul, Southern Brazil, known to be found in both the Atlantic Rainforest and grasslands areas (Pampa biome) (Reck et al. 2018). Its geographical distribution mainly includes the eastern area of South America, including Uruguay, eastern Argentina, and eastern Paraguay, as well as the southern and southeastern parts of Brazil (Guglielmone et al. 2003).

In accordance with these findings, the only *L. gymnocercus* specimen positive for *R. vitalii* DNA was in good physical condition with no clinical signs compatible with rangeliosis or any other disease. It is possible that *R. vitalii* may also have a natural and subclinical cycle in *L. gymnocercus*, as well as in other wild canid species. Considering the results presented here, we propose the hypothesis that *C. thous* is a natural host of *R. vitalii*, which, in the absence of stressful conditions, show no signs of clinical disease. This has an impact on our understanding of the ecology of rangeliosis, which was previously assumed to be an infection predominantly associated with domestic dogs. In addition to this, our findings will also affect the management of rescued wild canids, in which the detection of certain microorganisms (such as hemoparasites) may determine whether the animal should be treated/rehabilitated, returned to the wilderness, or kept in captivity.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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