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Original article

Molecular evidence of *Babesia* species in *Procyon cancrivorus* (Carnivora, Procyonidae) in Uruguay

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ABSTRACT

The crab-eating raccoon (*Procyon cancrivorus*) is a carnivore widely distributed from southern Central America to all South American countries except Chile. In the Southern cone of America, *P. cancrivorus* has been found parasitized by several *Amblyomma* spp. Particularly, in Uruguay, *A. aureolatum* is the only tick found in this wild carnivore. Piroplasmid hemoparasites were found in *Procyon lotor* from North America and Japan. In this work, molecular evidence *Babesia* sp. DNA was found in blood and tissues from road-killed *P. cancrivorus* from different locations in Uruguay. PCRs targeting 18S rRNA gene were carried out. Subsequently, the obtained amplicons were sequenced and full-length sequences was assembled. A phylogenetic tree was constructed and revealed that the *Babesia* sp. found in this work clustered with other 18sRNA sequences of *Babesia* spp. obtained from *P. lotor* from Japan and USA, along with *Babesia* spp. of maned wolf and *I. ovatus*. This is the first report of molecular evidence of *Babesia* sp. parasitizing *P. cancrivorus*.

1. Introduction

The crab-eating raccoon, *Procyon cancrivorus* (Cuvier, 1798) (Carnivora, Procyonidae), is a carnivore widely distributed from southern Central America to all South American countries except Chile (Canevari and Vaccaro, 2007). It is an omnivorous mammal that inhabits forests and open areas near to waterways. In Uruguay, it is widely distributed throughout the country (González and Martínez-Lanfranco, 2010).

In the Southern cone of America *P. cancrivorus* has been found parasitized by the following tick species: *Amblyomma aureolatum* (Pallas, 1772), *A. calcaratum* Neumann, 1899, *A. ovale* Koch, 1844, *A. parvum* Aragão, 1908, and *A. sculptum* Berlese, 1888 (Labruna et al., 2005; Nava et al., 2017). However, in Uruguay the only tick found in *P. cancrivorus* was *A. aureolatum* (Venzal et al., 2003).

There are many reports of piroplasmids found in raccoons *Procyon lotor* (Linnaeus, 1758) from North America (Wenyon and Scott, 1926; Anderson et al., 1981; Telford Jr. and Forrester, 1991; Birkenheuer et al., 2006) and Japan (Jinnai et al., 2009). In some cases, these hemoparasites were described as *Babesia* spp., and were reported as highly prevalent in raccoon populations.

Nevertheless, the nomenclature and the scientific name given to these *Babesia*-like parasites are still confusing (Jinnai et al., 2009). In the present work, molecular evidence for the presence of *Babesia* sp. in blood and tissues from road-killed *P. cancrivorus* from different locations in Uruguay was described. Also, comparison among different *Babesia* species was performed using the 18S rRNA sequence.

2. Materials and methods

Between 2015 and 2017 samples from blood, tissues and ticks of thirteen road-killed *P. cancrivorus* were obtained from different locations in Uruguay (Table 1). Due the specimens were found dead along the roads the integrity of the carcasses varied, thus, the tissue sample collection could differ. Tissues collected for each specimen were listed on Table 2. The animals were also inspected for the presence of ticks. The ticks were manually retrieved from the animals and preserved in ethanol 95°. For tick identification, taxonomic keys described by Nava et al. (2017) we used.

DNA from blood, tissues and ticks were extracted using Pure Link™ Genomic DNA Kit (Invitrogen™, USA) according to the instructions specified by the manufacturer. Ticks collected from the same *P. cancrivorus* were pooled.

Molecular screening of *Babesia* spp. was done by PCR targeting the

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Table 1
Data from the road-killed *P. cancrivorus* used in the study.

Sample	Date	Site - Department	Latitude, Longitude
W1	October 10, 2015	Route 3, km 552 - Salto	30°52′47″S, 57°41′25″W
W2	November 16, 2015	Route 3, km 454, Chapicuy - Paysandú	31°39′02″S, 57°53′24″W
W3	November 19, 2015	Route 9, km 125 - Maldonado	34°43′58″S, 55°02′50″W
W5	February 22, 2016	Route 2, km 199, Santa Catalina - Soriano	33°46′58″S, 57°29′46″W
W10	April 24, 2016	Route 3, km 470 - Paysandú	31°30′56″S, 57°54′34″W
W14	May 20, 2016	Route 31, km 16, San Antonio - Salto	31°22′53″S, 57°46′15″W
W15	July 19, 2016	Route 3, km 424 - Paysandú	31°54′40″S, 57°51′29″W
W16	August 4, 2016	Route 3, km 419 - Paysandú	31°57′05″S, 57°51′16″W
W17	August 8, 2016	Route 3, km 168 - Flores	33°41′29″S, 56°48′43″W
W19	August 23, 2016	Route 3, km 474 - Paysandú	31°28′29″S, 57°54′01″W
W28	January 15, 2017	Route 3, km 386, Constancia - Paysandú	32°11′32″S, 57°59′02″W
W30	April 30, 2017	Route 26, km 218 - Tacuarembó	31°46′08″S, 56°05′39″W
W32	February 11, 2017	Route 3, km 454, Chapicuy - Paysandú	31°39′02″S, 57°53′24″W

Table 2
Samples of blood, tissues and ticks obtained from *P. cancrivorus*. PCR results with the primers BAB143-167 and BAB694-667 are shown as positive (+) and negative (−).

Sample	Blood	Spleen	Liver	Skeletal muscle	Ticks species
W1	(+)[b]	(+)			*A. aureolatum*[a] 1F, 2M
W2				(−)	*A. aureolatum* 1M
W3	(−)	(−)			No
W5	(−)	(+)[b]			No
W10	(+)[b]	(+)[b]			No
W14		(+)	(+)[b]		No
W15	(−)				No
W16	(−)	(+)	(+)[b]	(+)	No
W17	(−)	(−)			No
W19	(−)	(−)			*A. aureolatum* 1M
W28	(−)				*A. aureolatum* 1F, 2M
W30	(−)				*A. aureolatum* 1F, 2M
W32	(−)				*A. aureolatum* 2F

[a] *Amblyomma aureolatum*: F = female, M = male.
[b] Sequenced.

Table 3
Primers used to amplify the nearly full-length 18S ribosomal DNA by polymerase chain reaction.

Primer	Sequence	Fragment size (bp)	Source
BAB143-167	CCGTGCTAATTGTAGGGCTAATACA	551	Soares et al., 2011
BAB694-667	GCTTGAAACACTCTARTTTTCTCAAAG		
Bovisfor	AACCTGGTTGATCCTGCCAGTA	781	This work
RLB-R2	CTAAGAATTTCACCTCTGACAGT		Gubbels et al., 1999
Bab550for	GCTCCAATAGCGTATATTAAAC	650	This work
Bab1200R	CAACTAAGAACGGCCATGCACCA		
Babesia782for	GGTAATGGTTAATAGGAACGGTTG	935	This work
Bovisrev	GATCCTTCTGCAGGTTCACCTAC		

18S rRNA gene using primers BAB143-167 and BAB694-667 (Table 3), which amplify an approximately 551-bp fragment (Soares et al., 2011). Furthermore, the positive samples were used to amplify the full-length 18S ribosomal DNA. Three set of primers were designed for this purpose (Table 3). The amplification process was started with 5 min at 94 °C, followed by 35 cycles at 94 °C for 10 s, 57 °C for 20 s, and 72 °C for 30 s, and a final extension of 5 min at 72 °C. *Babesia bovis* DNA and distillate water were used as positive and negative controls, respectively. The amplicons were electrophoresed in a 1% agarose gel, stained with ethidium bromide and visualized under UV light. Finally, the DNA fragments were purified using a commercial kit (Promega®) and sequenced using dye terminator sequencing (INTA Castelar). Comparisons with GenBank available sequences were made by the basic local alignment search tool (BLAST) (Altschul et al., 1990). Multiple alignment was performed using Bioedit software (Hall, 1999) and phylogenetic analysis was performed with MEGA7.0 (Kumar et al., 2016) using the Neighbor-Joining approach (Tamura-Nei model). Bootstrap support was calculated with 1000 replicates. Sequences of *Theileria* spp. were employed as outgroup.

3. Results

Six of the thirteen *P. cancrivorus* obtained were parasitized by adults of *A. aureolatum* (Table 2). The PCR using primers BAB143-167/BAB694-667 were negative for all tick samples. However, blood and different tissues of five *P. cancrivorus* resulted positive using the same primers (Table 2). With the primers BAB143-167/BAB694-667 and Babesia782for/Bovisrev partial fragments were obtained with samples W5 (495-bp) and W1 (773-bp), W14 (894-bp), respectively. For sample W10, full-length sequence (1689-bp) was generated by assembling the amplicons obtained with primers Bovisfor/RLB-R2, Babesia782for/Bovisrev, and Bab550for/Bab1200R (GenBank accession number MG682489).

Partial sequences obtained from W1, W5, and W14 (GenBank accession numbers MG682492, MG682490, MG682491, respectively) were identical to W10, meanwhile, W16 sequence had too much noise thus was uninterpretable. The assemble sequence of W10 was used for the phylogenetic tree construction (Fig. 1). This sample clustered as a monophyletic group with other *Babesia*-like 18sRNA sequences obtained from raccoons from Japan and USA, *Babesia* sp. obtained of captive maned wolf (*Chrysocyon brachyurus*) from USA, and *Babesia* sp. from *Ixodes ovatus*, along with *B. odocoilei* and *B. divergens* (bootstrap support = 99%).

4. Discussion

Road-killed animals are an accessible and alternative source to live animals in research, providing feasible material to detect new pathogens by molecular techniques (Richini-Pereira et al., 2016). It is worth mentioning that the autolysis sometimes compromises the integrity of the erythrocytes, thus, blood smears for hemoparasites morphology studies cannot be achieved as it was the case in this work.

There are several reports in Brazil of Apicomplexa infecting blood and tissue samples of *P. cancrivorus*, such as *Toxoplasma gondii* and *Hepatozoon procyonis* (Richini-Pereira et al., 2016; Rodrigues et al., 2007). However, there are no reports with a molecular diagnosis of *Babesia* spp. in this species of wild carnivore. The results obtained herein, represent the first molecular-based evidence of a *Babesia* sp. hemoparasite in *P. cancrivorus*. Phylogenetically, the full-length sequence of 18 s rDNA of this *Babesia* sp. clustered with other 18sRNA