Ciências Agrárias
SEMINA

DOI: 10.5433/1679-0359.2022v43n6p2403

Hemoparasites in *Didelphis aurita* from Seropédica, Rio de Janeiro State, Brazil

Hemoparasitos em *Didelphis aurita* capturados em Seropédica, Estado do Rio de Janeiro, Brasil

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Highlights

Opossums captured in Seropédica, RJ were negative for tick-borne bacteria.
An opossum captured in Seropédica, RJ positive for Theileria sp.
Opossums in Seropédica, RJ were infested by ticks and fleas.

Abstract

The present study aimed to use polymerase chain reaction (PCR) to detect species of the order Piroplasmida, such as *Anaplasma* spp., *Borrelia* spp., and *Ehrlichia* spp., circulating in the blood of *Didelphis aurita* in a peridomiciliary environment. Blood samples collected from big-eared opossum (*Didelphis aurita*) were screened for hemoparasites using PCR. The extracted DNA was tested for tick-borne hemoparasites. We were unable to detect hemoparasites, such as *Ehrlichia* spp., *Babesia* spp., *Anaplasma* spp., and *Borrelia* spp. *Theileria* DNA was detected in only one sample screened using PCR for an approximately 650-base pair fragment of the 18S rRNA gene. Sequencing and BLAST analysis of a subset of the PCR amplicons revealed 97% (535/553 bp) identity with *Theileria bicornis.* The detection of *Theileria* sp. in *D. aurita* challenges us to pursue more in-depth studies of marsupial piroplasmosids and to evaluate the morphological aspects of the findings and their possible involvement in zoonoses.

Key words: Marsupial. *Theileria.* Ticks. Fleas. Vector-borne.

Resumo

O objetivo do presente estudo foi utilizar a reação em cadeia da polimerase (PCR) para detectar espécies da ordem Piroplasmida, como *Anaplasma* spp., *Borrelia* spp. e *Ehrlichia* spp., circulando no sangue de *Didelphis aurita* em ambiente peridomiciliar. Amostras de sangue coletadas de gambá da orelha preta (*Didelphis aurita*) foram triadas para hemoparasitas por meio da PCR. O DNA extraído foi testado para

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Received: Apr. 04, 2022 - Approved: Nov. 18, 2022

alguns hemoparasitas transmitidos por carrapatos. Não conseguimos detectar hemoparasitos, como *Ehrlichia* spp., *Babesia* spp., *Anaplasma* spp. e *Borrelia* spp. O DNA de *Theileria* foi detectado apenas em uma amostra. Um fragmento de ~650 pares de bases do gene 18S rRNA foi sequenciado. e a análise pelo BLAST (um subconjunto de amplicons de PCR) revelou uma identidade de 97% (535/553 pb) com *Theileria bicornis.* A detecção de *Theileria* sp. in *D. aurita* nos desafia a aprofundar estudos sobre piroplasmosídeos marsupiais e avaliar os aspectos morfológicos dos achados e seu possível envolvimento com zoonoses.
Palavras-chave: Marsupial. *Theileria.* Carrapatos. Pulgas. Vetores.

Introduction

Synanthropic wild mammals can generate potential economic and health problems relevant to humans, participate in the epidemiological chain of disease agents, and act as natural reservoirs of their etiological agents (McFarlane et al., 2012). Didelphinae is an outstanding natural host for fleas and ticks, harboring a significant number of species with a high degree of ectoparasite infestation (Milagres et al., 2010; Mendonça et al., 2020; Cáceres, 2012).

Tick-borne diseases are of high importance in veterinary medicine, especially when associated with a pet's clinic. When evaluating wildlife, the concern is even more significant because these diseases involve animals as reservoirs and hemoparasitic amplifiers that can potentially infect domestic animals and humans (Dantas-Torres & Otranto, 2016). Tick-borne diseases include babesiosis, ehrlichiosis, anaplasmosis, and borreliosis, involving zoonotic and non-zoonotic agents (Dantas-Torres, 2008). Yoshinari et al. (2010) suggested the participation of marsupials as a potential reservoir animal for human borreliosis in Brazil; however, the molecular detection or isolation of bacteria of the genus *Borrelia* in marsupials has not yet been possible. Regarding the agents Anaplasmataceae, Guimarães et al. (2019) detected a putative novel genotype *Ehrlichia* sp. closely related to *E. canis* that circulates in opossums from Rio de Janeiro state, Brazil. However, many other studies have detected agents of the piroplasmid order in marsupials (Paparini et al., 2012; Wolf et al., 2016; Silva et al., 2017; Soares et al., 2017; Colle et al., 2019; Gonçalves et al., 2021).

The present study aimed to use polymerase chain reaction (PCR) to detect species of the order Piroplasmida, such as *Anaplasma* spp., *Borrelia* spp., and *Ehrlichia* spp., circulating in the blood of *Didelphis aurita* in a peridomiciliary environment.

Material and Methods

The study was conducted in the municipality of Seropédica (22º44'S, 43º42'W), Rio de Janeiro, Brazil (Figure 1). The marsupials were captured between February 2013 and October 2014. The capture, maintenance, and collection of samples were approved by the Research Ethics Committee of UFRRJ under protocol no. 255/2012 and authorized by the Brazilian Institute of Environment and Renewable Natural Resources under protocol no. 34701-2.

To capture marsupials, Tomahawk-type wire traps were used with dimensions

of 20 × 20 × 40 cm. The traps were installed in the afternoon and checked in the morning for two consecutive days. Locations near human dwellings were selected, including garages, food storage sheds, trees, animal houses, bamboo groves, rubbish dumps, and small forest fragments. Marsupials were attracted to bananas with peanut butter. After capture, the animals were identified (N. R. Reis et al., 2010) and weighed following chemical containment with ketamine hydrochloride and xylazine at a dosage of 30 mg/kg and 4 mg/kg, respectively. Each animal was examined thoroughly to collect ectoparasites, and the collected specimens were stored in isopropyl alcohol and identified in the laboratory using a dichotomous key for ticks (Martins et al., 2010) and fleas (Linardi & Guimarães, 2000).

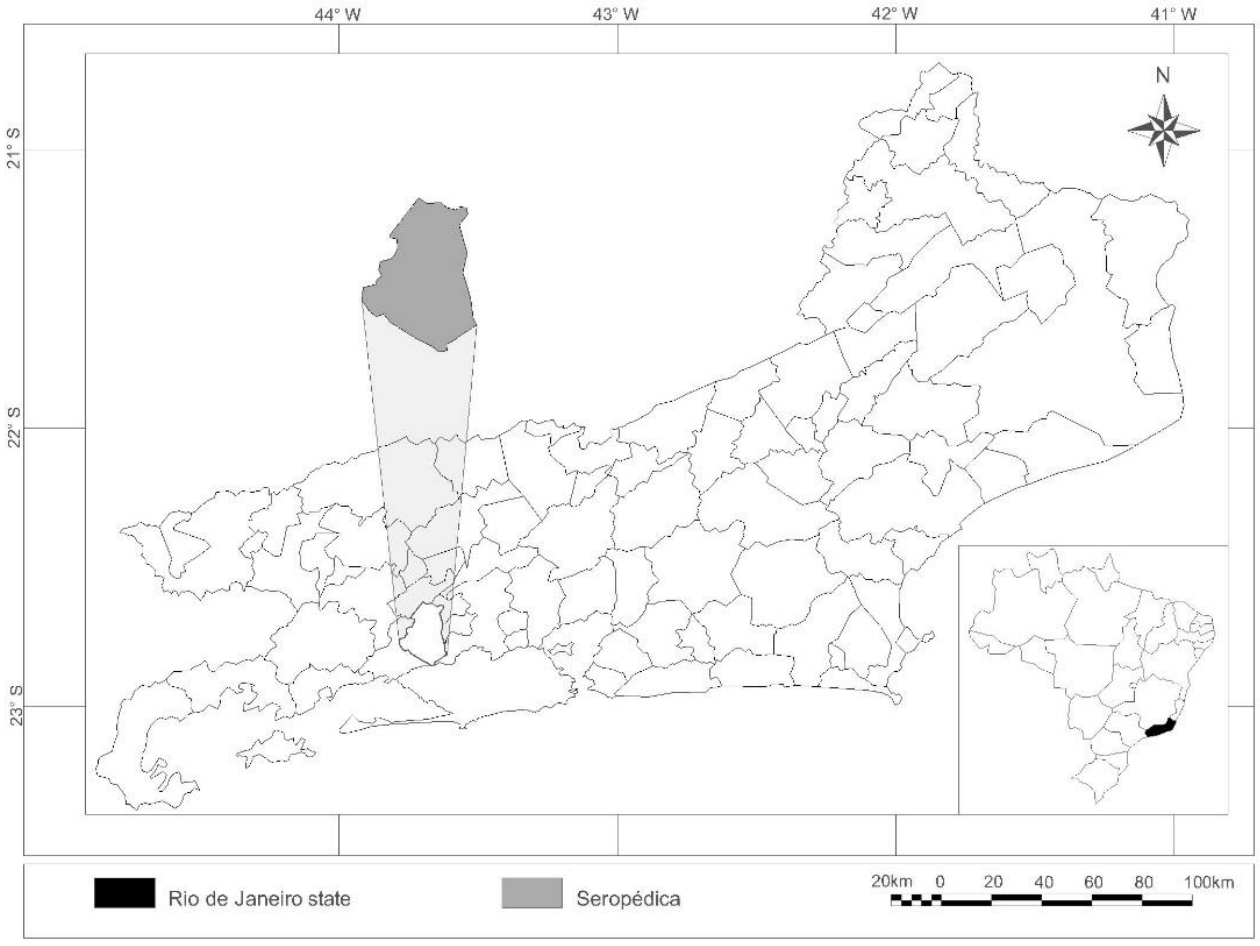

Figure 1. Map of the Federative Republic of Brazil illustrating the location of the city from which *Didelphis aurita* were collected in the Rio de Janeiro state.

Blood was drawn by puncture from the intracaudal vein and stored in tubes containing 7.2 mg of K2 EDTA as an anticoagulant. The marsupials were marked with an ear cut (left) (2 mm) so that they could be identified if they were captured again. After checking for the complete return of the animal from the anesthesia state, the animals were released in the same location from where they were trapped.

DNA was extracted from the blood using a Qiagen® DNeasy blood and tissue kit, following the manufacturer's recommendations. To ensure the reliability of the negative results, all samples were tested for the presence of amplifiable DNA using the primer set GAPDH-F/GAPDH-R, which amplifies a fragment of the gene encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH), present in all mammals (Birkenheuer et al., 2003).

DNA was extracted from blood samples and screened for the following infectious agents: *Borrelia* spp., *Anaplasma bovis, Anaplasma platys, Anaplasma phagocytophilum, Ehrlichia* spp., and Piroplasmida order. PCR was performed using the primers listed in Table 1. For each reaction, the DNA of *Borrelia anserina* strain AL, *Babesia bigemina, Anaplasma platys,* and *Ehrlichia canis* from the culture, cattle, and dog positives, respectively, and two negative controls (water) were used. All the reactions were performed according to the protocol described in the original article.

Amplicons of the expected size were used for sequencing. The products were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega®, Madison, WI, USA) and sequenced using the Sanger method (Sanger et al., 1977), using an ABI Prism 310 Genetic Analyzer (Applied Biosystems®, Perkin Elmer, CA, USA). The Basic Local Alignment Search Tool (BLAST) was used to evaluate the similarity of the genes with the nucleotide sequences available in GenBank. Fragments of the nucleotide sequences were obtained from GenBank for phylogenetic trees.

Multiple sequence alignments were performed with the sequences obtained from this study and sequences from GenBank using MAFFT v.7 software program (Katoh & Standley, 2013). The best-fit evolutionary model was determined using MEGA version 11 using the Bayesian information criterion (Tamura et al., 2021). Phylogenetic relationships were estimated by maximum likelihood (ML) phylogenetic inference using PhyML, which was implemented in SeaView v.5 (Gouy et al., 2021). Statistical support for the clades was measured using a heuristic search with 1000 bootstrap replicates.

Table 1
Sequences of oligonucleotide primers used for the detection of microorganisms of the genera *Borrelia*, *Ehrlichia,* and *Anaplasma* and the phylum Apicomplexa (genera *Babesia* and *Theileria*) in the DNA of ticks from the municipality of Seropédica, Rio de Janeiro, Brazil

Primers	Gene	Organism	Nucleotides sequences (5'-3')	Expected Amplicon length	References
BorFlaF1	*fla*B	*Borrelia* spp.	TACATCAGCTATTAATGCTTCAAGAA	740 pb	Blanco et al. (2017)
BorFlaR1			GCAATCATWGCCATTGCRGATTG		
BorFlaF2			CTGATGATGCTGCTGGWATGG		
BorFlaR2			TCATCTGTCATTRTWGCATCTT		
BT-F3	18S rRNA	Ordem Piroplasmida	TGGGGGGAGTATGGTCGCAAG	650 pb	Seo et al. (2013)
BT-R3			CTCCTTCCTTTAAGTGATAAG		
DSB-330	*Dsb*	*Ehrlichia* spp.	GATGATGCTTGAAGATATSAAACAAAT	349 bp	Almeida et al. (2013)
DSB-380			ATTTTTAGRGATTTTCCAATACTTGG		
DSB-720			CTATTTTACTTCTTAAAGTTGATAWATC		
ge3A	16S rRNA	*Anaplasma bovis, Anaplasma platys and Anaplasma phagocytophilum*	CACATGCAAGTCGAACGGAT TATTC	546 bp	Massung et al. (1998)
ge10R			TTCCGTTAAGAAGGATCTAATCTCC		
ge9f			AACGGATTATTCTTTATAGCTTGCT		
ge2			GGCAGTATTAAAAGCAGCTCCAGG		

Results and Discussion

Twenty-six marsupials of the species *D. aurita* were captured, 10 males and 16 females, weighing an average of 845.45 ± 386.35 g. Among them, 10 were adults and the remaining 16 were young. Regarding the presence of ectoparasites, 50% (13/26) of animals were infested with fleas. Only 7.69% (2/26) of the marsupials were tick-infested, and these were co-infested with fleas. A total of 36 adult fleas, 100% of which were identified as belonging to the species *Ctenocephalides felis felis* and 26 *Amblyomma sculptum* tick nymphs were collected.

In Brazil, several tick species, such as *Rhipicephalus sanguineus* (Reck et al., 2018), *Amblyomma incisum* (Reck et al., 2018), *Amblyomma* sp. (F. S. Reis et al., 2008; Maia et al., 2018; Mendonça et al., 2020), *Amblyomma fuscum* (Dantas-Torres et al., 2012), *Amblyomma aureolatum* (Müller et al., 2005; Reck et al., 2018), *A. sculptum* (Wolf et al., 2016; Ueno et al., 2020), *Amblyomma scalpturatum* (Mendonça et al., 2020), *Amblyomma cajennense* strictu sensu (Mendonça et al., 2020), *Amblyomma humerale* (Mendonça et al., 2020), *Amblyomma coelebs* (Mendonça et al., 2020), *Amblyomma ovale* (Reck et al., 2018), *Ixodes amarali* (Guitton et al., 1986), *Ixodes loricatus* (Guitton et al., 1986; Dantas-Torres et al., 2012), *Amblyomma dubitatum* (Ueno et al., 2020; Gonçalves et al., 2021), *Amblyomma auricularium* (Maia et al., 2018), *Amblyommma parvum* (Maia et al., 2018),

Ornithodoros guaporensis (Wolf et al., 2016), and *Haemaphysalis juxtakochi* (Reck et al., 2018), have already been described in marsupials, in addition to the fleas of the species *Ctenocephalides canis, C. felis felis* (Milagres et al., 2010), *Rhopalopsyllus* spp. (Mendonça et al., 2020), and *Xenopsylla cheopis* (Milagres et al., 2010).

Among the samples tested, none of the *D. aurita* samples yielded detectable DNA of *Borrelia* spp., *Ehrlichia* spp., or *Anaplasma* spp. However, an adult male animal, weighing 1 kg, trapped on the campus of Federal Rural University, was positive according to PCR for the target gene 18S rRNA of Piroplasmida. After analysis, the obtained sequence was deposited in GenBank under the code MN715140 and showed 97% (535/553 bp) identity with *Theileria bicornis* (MF536661), 96% (538/558 bp) with *Babesia ardae* (KY436057), and 96% (538/558 bp) with a species of *Cytauxzoon* (KM025200) when compared to sequences deposited in GenBank. Phylogenetic analysis (Figure 1) revealed that this sequence joined a clade belonging to the genus *Theileria.* In this study, it was referred to as *Theileria* sp. strain GR8.

The positive adult male *Theileria* sp. strain GR8 was not parasitized by ectoparasites at the time of capture. In general, piroplasmosids are transmitted by ixodid ticks. In total, 26 nymphs of *A. sculptum* ticks were found, although they were typically found on capybaras and tapirs. According to Labruna et al. (2001), *A. sculptum* has a low host specificity, particularly during the larval and nymph stages. Moreover, *A. sculptum* is considered the main tick species that attacks humans in Brazil and is the primary Brazilian spotted fever vector in humans (Krawczak et al., 2014). *Ctenocephalides felis felis* fleas were found in 50% of the animals. There is no record in the literature of fleas transmitting from piroplasmids to mammals.

In Central-Western Brazil, Gonçalves et al. (2021) detected 22 out of 67 (16.4%) *Didelphis albiventris* positive for piroplasmid. Additionally, intra-erythrocytic oval ring-shaped organisms, similar to piroplasmid merozoites, were detected in blood smears from 10 *D. albiventris.* These amplified sequences showed 97.2-99.4% identity with *Babesia* sp. previously detected in a capybara from south Brazil (Gonçalves et al., 2021). *Babesia* sp. number MW290046 (Reported in GenBank as *Theileria* sp. LPerles 2020-a isolate GP1) detected in *Didelphis albiventris* by Gonçalves et al. (2021) had 98.92% (552/558) identity with *Theileria* sp. isolate GR8, and phylogenetic analysis (Figure 2) showed a high evolutionary relationship between the two species.

Other authors have also found piroplasmids in marsupials in Brazil, which include *Hepatozoon* spp. (Silva et al., 2017; Colle et al., 2019) and e *Babesia* spp. (Wolf et al., 2016; Soares et al., 2017; Colle et al., 2019). In Australia, one animal was identified as positive for *Theileria* in one of three boodies (*Bettongia lesueur*) screened from a wildlife rehabilitation center, and more than seven *Babesia* positives were found in the woylies (*Bettongia penicillata ogilbyi*) among the wild animals captured (Paparini et al., 2012).

Based on the morphological characteristics, Regendanz and Kikuth (1928) identified a protozoan in the erythrocytes of a small marsupial of the species *Philander opossum* quica and named it *Nuttallia brasiliensis.* Later, Reichenow (1953) classified this same agent as *Theileria*

brasiliensis without even visualizing the evolutionary forms in lymphocytes. Piroplasmida in marsupials was last recorded by Sampaio and Massard (2003), suggesting that the previously reported evolutionary forms in erythrocytes were organisms of the genus *Babesia* because no evolutionary forms were found in the leukocytes. However, none of these studies had molecular data that could help to elucidate this question.

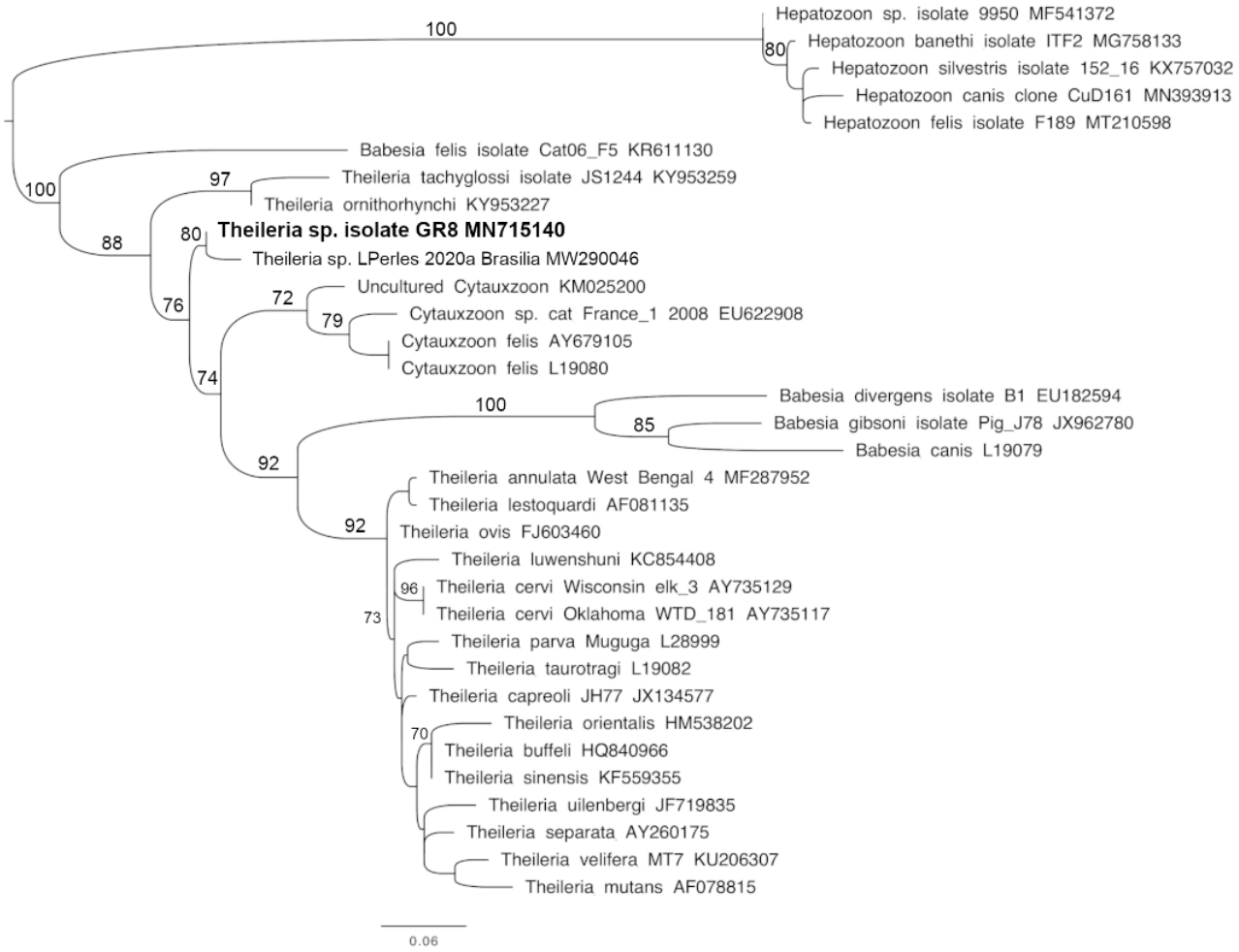

Figure 2. Phylogenetic analysis based on the 18S ribosomal RNA gene (487 bp) sequences of *Theileria, Cytauxzoon, Babesia,* and *Hepatozoon* using Maximum Likelihood method (PhyML). Numbers >70% above the branches indicate bootstrap values. The scale bars indicate an evolutionary distance of 0.06 substitutions per position in the sequence and the branch labels include GenBank accession numbers. The Tamura 3-parameter model with gamma-distributed heterogeneity (T92 + G) was selected as the best-fit evolutionary model.

Although no morphological data are available, the present study opens the possibility that the isolate *Theileria* sp. strain GR8 involves the same species as that reported by Regendanz and Kikuth (1928), Reichenow (1953), and Sampaio and Massard (2003). This survey was conducted in marsupials in a location near the one chosen by Sampaio and Massard (2003) for their research. However, a novel and more detailed analysis related to morphological data, compared with those described previously and those described in this molecular study, is necessary for a consistent definition.

Didelphis spp. are recognized as reservoirs of major diseases within veterinary medicine, such as Equine Protozoal Myeloencephalitis, *Sarcocystis neurona* (Dubey, 2001). Silva et al. (2017) reported that the infection of *Hepatozoon canis* suggests the participation of this species in the epidemiological cycle of this hemoparasite. However, little data exist regarding tick-borne diseases in the scientific literature, particularly in relation to zoonoses, because they are animals with synanthropic habits, although it is suspected that these species act as essential amplifiers of bacteria of the genus *Rickettsia* (Horta et al., 2009, 2010).

In summary, *Ehrlichia* spp., *Babesia* spp., *Anaplasma* spp., and *Borrelia* spp. were not detected in the analyzed samples. However, the detection of *Theileria* sp. in *D. aurita* challenges us to pursue more in-depth studies of marsupial piroplasmosids and to evaluate the morphological aspects of the findings and their possible involvement in zoonoses.

Acknowledgments

The authors offer special thanks to the Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq) and Fundação Carlos Chagas Filho de Amparo à Pesquisa do Estado do Rio de Janeiro (FAPERJ).

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