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Original Article

Hemoparasites in ticks of wild birds of Serra dos Órgãos National Park, state of Rio de Janeiro, Brazil

Hemoparasitos em carrapatos de aves silvestres do Parque Nacional da Serra dos Órgãos, estado do Rio de Janeiro, Brasil

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Abstract

The aim of this study is to detect the presence of tick-borne agents of genera *Rickettsia*, *Borrelia*, *Babesia*, *Ehrlichia* and *Anaplasma* in ticks collected from native wild birds in the state of Rio de Janeiro. Birds were captured and observed carefully to find the ectoparasites. DNA detection of hemoparasites was performed by means of the polymerase chain reaction (PCR). The sequences obtained were analyzed and their homologies were compared to the available isolates in the GenBank platform database. A total of 33 birds were captured from 20 different species, of which 14 were parasitized by *Amblyomma longirostre* (n = 22). There was absence of DNA from agents of the genera *Babesia*, *Anaplasma* and *Ehrlichia* in the evaluated samples. The phylogenetic analysis indicated that one sample had 100% identity with *Rickettsia bellii* (KJ534309), the other two samples showed 100% identity with *Rickettsia* sp. Aranha strain and strain AL (EU274654 and AY360216). The positive sample for *R. bellii* was also demonstrated to be positive for *Borrelia* sp., which presented a similarity of 91% with *Borrelia turcica* (KF422815). This is the first description of *Borrelia* sp. in ticks of the genus *Amblyomma* in South America.

Keywords: Conservation units, ectoparasites, rickettisiae, *Borrelia.*

Resumo

Este trabalho teve como objetivo detectar evidências moleculares da presença de agentes dos gêneros *Rickettsia, Borrelia, Babesia*, *Anaplasma* e *Ehrlichia* transmitidos por carrapatos coletados de aves silvestres no estado do Rio de Janeiro. Aves foram capturadas e observadas cuidadosamente a procura de ectoparasitos. A detecção de DNA de hemoparasitos foi realizada por meio da reação em cadeia da polimerase (PCR). As sequências obtidas foram analisadas e sua homologia comparada aos isolados disponíveis na base de dados da plataforma GenBank. Foram capturadas 33 aves, de 20 espécies diferentes das quais 14 estavam parasitadas por *Amblyomma longirostre* (n = 22). Houve ausência de DNA de agentes dos gêneros *Babesia*, *Anaplasma* e *Ehrlichia* nas amostras avaliadas. A análise filogenética indicou que uma amostra apresentou 100% de identidade com *Rickettsia bellii* (KJ534309), as outras duas amostras apresentaram 100% de identidade com *Rickettsia* sp. cepa Aranha e Cepa AL (EU274654 e AY360216.). A amostra positiva para *R. bellii* também apresentou positividade para *Borrelia* sp. que apresentou similaridade de 91% com *Borrelia turcica* (KF422815). Esta é a primeira descrição de *Borrelia* sp. em carrapatos do gênero *Amblyomma* na América do Sul.

Palavras-chaves: Unidades de conservação, ectoparasitos, rickettisiae, *Borrelia.*

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Introduction

Birds are host to a wide variety of ticks, especially when the latter are in the larva and nymph stages. Therefore, this plays an important role in the epidemiology of several important diseases for veterinary and public health (LABRUNA et al., 2007; OGRZEWALSKA et al., 2010; SANCHES et al., 2013; CAPLIGINA et al., 2014). Moreover, studies worldwide have reported the importance of wild birds in the expansion of tick-borne diseases, such as anaplasmosis, babesiosis, Lyme disease, spotted fever, and tick-borne encephalitis (PAROLA et al., 2013).

By limitations in displacement, ticks also use their hosts to overcome numerous geographical barriers. In recent years, studies of tick–bird associations have been increasing exponentially, due to their importance in the dispersal and maintenance of the different tick species, and (consequently) their passengers' pathogens (HUBÁLEK, 2004; LOSS et al., 2016; BUDACHETRI et al., 2017).

In Brazil, studies on the diversity of ticks on birds have been carried out in several biomes (Atlantic Forest, Amazon, Caatinga, Cerrado, and Pantanal), with the immature stages (larva and nymph) of the genus *Amblyomma* being found to be the most common (LABRUNA et al., 2007; OGRZEWALSKA et al., 2010; LUZ et al., 2012; LUGARINI et al., 2015; RAMOS et al., 2015). Moreover, studies on infectious pathogenic agents in ticks that parasitize birds in Brazil are mainly concentrated in the detection of rickettsial agents (OGRZEWALSKA et al., 2008). Other agents of the genera *Borrelia*, *Anaplasma* and *Ehrlichia* were also detected in ticks that were parasitizing wild birds. In North America and parts of Europe, the DNA of these agents was detected in these arthropods, which were collected from birds (SCOTT et al., 2010; PALOMAR et al., 2012; ERWIN et al., 2016). Moreover, the species of ticks found on birds can also parasitize humans (GUGLIELMONE et al., 2006). For these reasons, there is a need for studies that characterize organisms that infect ticks found on birds, to enable a better understanding of the diversity and ecology of the zoonoses transmitted by these arthropods. Thus, the objective of the present work is to detect (through molecular techniques) hemoparasites present in ticks from birds of the municipality of Guapimirim and Serra dos Órgãos National Park in the state of Rio de Janeiro, Brazil.

Material and Methods

The study was carried out in the municipality of Guapimirim (latitude: 22º 31' 14"/ longitude: 43º 00' 52"; altitude 256 meters) and in the Serra dos Órgãos National Park (latitude: 22º 31' 43'/ longitude: 43º 00' 12"; altitude 256 meters), both of which are located in the state of Rio de Janeiro, Brazil. Three field visits, each lasting four days, were conducted between March and September 2016 at the place of study.

The birds were captured between 06:00 and 17:00 hours, using 5–20 ornithological mist nets model (12 m long × 3 m wide, with 16 and 36 mm mesh). The birds were then photographed and identified following the recommendations based on the nomenclature approved by the Brazilian Committee of Ornithological Records (CBRO, 2014). Each bird was examined for the presence of ticks. If any were found, they were removed with forceps and placed in 1.5 mL polyethylene tubes containing absolute alcohol for later identification. Samples were initially stored at room temperature for up to four days. After collecting the ticks, the birds were released at the same collection site. All procedures were performed with live birds.

In the laboratory, the ticks were microscopically examined for identification, using a dichotomous key published by Martins et al. (2010) with the aid of a stereoscopic microscope. After identification, the arthropods were stored in RNA stabilization solution and frozen until processed for molecular analysis.

The ticks kept in RNA later were washed in distilled water three times and rehydrated in 200 µL of phosphate buffered saline (PBS). The ticks were then individually placed in 1.5 mL polyethylene tubes. Next, 2 mm zirconium oxide beads and 80 mg of 0.1 mm glass beads were added, both autoclaved, for trituration in Minibeadbeater BIOSPEC® for 1 min.

Cell lysis was performed with the addition of 250 µL of digestion solution (20 mM Tris-HCl, 20 mM EDTA, 400 mM NaCl, 1% sodium dodecyl sulphate, 10mM $CaCl_2$) with 20 µL of proteinase k (20 mg/mL) in incubation overnight at 56 °C. The DNA was extracted by a phenol treatment and another phenol-chloroform treatment followed by precipitation with isopropanol. The DNA pellet (formed after centrifugation of 16000 xg) was washed twice with 70% alcohol and re-suspended in 100 µL of elution buffer (10 mM Tris-HCl, 0,5 mM EDTA pH 9,0) in overnight at 4 °C, as according to Santolin et al. (2013).

The extracted DNA was tested by a battery of PCR assays that were targeting microorganisms of the genera *Rickettsia*, *Borrelia*, *Ehrlichia*, and *Babesia*. For this task, specific primers were used for each agent, following the original protocol of each primer (Table 1).

Reactions were carried out using reagents from the PROMEGA®, and each reaction contained 3 µL de DNA, 14,2 µL of water, 2 µL de *primers* (10 µM F+R), 2,5 µL of buffer (10X concentrado), 1,25 µL of $MgCl_2$ (50 mM) and 2 µL of dNTP's (2,5 mM), and 0,15 µL Taq DNA polimerase (PROMEGA®), in a final volume of 25 µL.

The amplified products were visualized in 1.5% agarose gel, which were stained with ethidium bromide and visualized in a UV-Transilluminator.

Polymerase chain reaction positive samples were submitted to sequencing and phylogenetic analyses. Multiple sequence alignments were performed with the sequences obtained from this study and sequences from GenBank using MUSCLE, using the SeaView v.4 software program (GOUY et al., 2010). The best-fit evolutionary model was determined using MEGA version 7, using the Bayesian information criterion (KUMAR et al., 2016). Phylogenetic relationships were estimated using (a) Maximum likelihood (ML) phylogenetic inference that used PhyML, which was implemented in SeaView (GOUY et al., 2010) and (b) a Bayesian Markov chain Monte Carlo (MCMC) method implemented in MrBayes v.3.2.6 (SOARES et al., 2015). The MCMC settings consisted of two simultaneous independent runs with four chains each that were run for 10 million generations and sampled every 100th generation, thus yielding 100,000 trees. After eliminating 25% of the samples as burn-in, a consensus tree was built. Statistical

Table 1. Sequences of the oligonucleotide primers used, along with their respective target genes and the size of the amplified fragment.

Primer	Gen	Organism	Nucleotide sequence (5'-3')	Fragment size	Reference
CS239 F	*glt*A	*Rickettsia* spp.	GCTCTTCTCATCCTATGGCTATTAT	834 bp	(LABRUNA et al., 2004)
CS1069 R			CAGGGTCTTCGTGCATTTCTT		
BorFlaF1	*fla*B	*Borrelia* spp.	TACATCAGCTATTAATGCTTCAAGAA	740 pb	(BLANCO et al., 2017)
BorFlaR1			GCAATCATWGCCATTGCRGATTG		
BorFlaF2			CTGATGATGCTGCTGGWATGG		
BorFlaR2			TCATCTGTCATTRTWGCATCTT		
Hptf	*hpt*	*Borrelia* spp.	GCAGAYATTACAAGAGARATGG	433 pb	(MCCOY et al., 2014)
HptR			CYTCRTCACCCCATTGAGTTCC		
glpQ + 1	*glpQ*	*Borrelia* spp.	GGGGTTCTGTTACTGCTAGTGCCATTAC	817 pb	(SCHWAN et al., 2005)
Glpq – 1			CAATTTTAGATATGTCTTTACCTTGTTGTTTATGCC		
BT-F3	18S rRNA	Piroplasmida Order	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		

support of the clades was measured by a heuristic search, with 1000 bootstrap replicates and the Bayesian posterior probabilities.

The study was evaluated and approved by the Animal Experimentation Ethics Committee of the Federal Rural University of Rio de Janeiro and was conducted with the permission of IBAMA; process num. 43917/3/2505369.

Results and Discussion

Detailed data of the tick–bird association that was reported in the current study can be found in Table 2. In total, 33 birds were captured, representing three orders, seven families, and 20 species, of which 14 (42%) of 9 species (45%) were parasitized by 22 immature forms of *Amblyomma* spp. Birds of the order Passeriformes were the most frequent, with 27 (82%) specimens captured, divided into 5 families and 15 species, corroborating with the data published in previous literature (LUZ & FACCINI, 2013; OGRZEWALSKA & PINTER, 2016). No ticks were recorded on the following bird species: Passeriformes - *Myiozetetes similis*, *Coereba flaveola*, *Sicalis flaveola*, *Tangara cayana*, *Tangara sayaca*, *Troglodytes musculus*; Columbiformes - *Leptotila rufaxilla*, *Geotrygon montana*, *Columbina talpacoti*; Cuculiformes - *Piaya cayana*. However, there are numerous records of these species in association with ticks in different biomes in Brazil (LABRUNA et al., 2007; LUGARINI et al., 2015; LUZ et al., 2016; OGRZEWALSKA & PINTER, 2016).

After the laboratory analysis, all ticks were identified as nymphs of *Amblyomma longirostre*. In general, the infestations found in the birds were low, and were not exceeding the average intensity of 2.5/tick per bird. All the ticks were collected in the head and neck regions, and similar parasitic intensities have also been reported in the Atlantic Forest (LUZ & FACCINI, 2013). The immature parasitism of *A. longirostre* in wild birds has been reported throughout the neo-tropical region, especially on birds from the Passeriformes sub-group (LABRUNA et al., 2007; NAVA et al., 2010; LUZ & FACCINI, 2013, 2016). There are also reports of this species of tick on birds in the neartic region, but these arthropods do not have populations established in this region (GUGLIELMONE et al., 2014). These findings reinforce the importance of wild birds in the maintenance and dispersion of this ectoparasite, as they are the main group of hosts for immature forms of *A. longirostre* in the wild environment. On the other hand, the adult stage of *A. longirostre* has been recorded mainly to be on neo-tropical porcupines of the family Erethizontidae, followed by occasional records on a variety of wild and domestic mammals of the families Cervidae, Canidae, Mustelidae, Phyllostomidae, Equidae, Bradypodidae and Sciuridae (BARROS-BATTESTI et al., 2006; GUGLIELMONE et al., 2014).

The arboreal habits of its primary hosts may justify the presence of the immature forms on birds that tend to share similar habits, suggesting an arboreal cycle for *A. longirostre* (LABRUNA et al., 2007).

Molecular analysis by the CS239/CS1069 primers revealed a 838 bp amplification of the gltA gene of *Rickettsia* spp. in three samples of *A. longirostre* that were collected on birds of three different species: *Saltator similis*, *Turdus leucomelas*, and *Tangara seledon*. The products sequenced from one sample had a 100% identity rate with the *Rickettsia bellii* isolated H3 (access in the GenBank: KJ534309). With the other two, the identity was 100% with *Rickettsia* sp. strain AL and the *Rickettsia* sp. Aranha strain (access in the GenBank: EU274654 and AY360216, respectively) both currently correlated with *Rickettsia amblyommatis* (OGRZEWALSKA et al., 2011; KARPATHY et al., 2016), as shown in Figure 1. These agents have already been described

Table 2. Prevalence and mean intensity of infestation by ticks collected in birds in the Serra dos Órgãos National Park and Guapimirim.

ORDER	FAMILY	SPECIES	BC	IB	PI (%)	NTC	MII	STI
Passeriformes	**Tyrannidae**	*Pitangus sulphuratus*	1	1	100 (%)	1	1	*Amblyomma longirostre*
		Mionectes oleagineus	1	1	100 (%)	2	2	*Amblyomma longirostre*
		Myiozetetes similis	1	0				
	Pipridae	*Manacus manacus*	3	3	100 (%)	5	1.6	*Amblyomma longirostre*
	Turdidade	*Turdus rufiventris*	1	1	100 (%)	1	1	*Amblyomma longirostre*
		Turdus leucomelas	4	2	50 (%)	5	2.5	*Amblyomma longirostre*
		Turdus amaurochalinus	2	1	50 (%)	1	1	*Amblyomma longirostre*
	Thraupidae	*Coereba flaveola*	1	0				
		Sicalis flaveola	1	0				
		Tachyphomnus coronatus	4	2	50 (%)	3	1.5	*Amblyomma longirostre*
		Tangara seledon	3	2	66 (%)	2	1	*Amblyomma longirostre*
		Tangara cayana	2	0				
		Tangara sayaca	1	0				
		Saltator similis	1	1	100 (%)	2	2	*Amblyomma longirostre*
	Troglodytidae	*Troglodytes musculus*	1	0				
Columbiformes	**Columbidae**	*Leptotila rufaxilla*	2	0				
		Geotrygon montana	1	0				
		Columbina talpacoti	2	0				
Cuculiformes	**Cuculidae**	*Piaya cayana*	1	0				

BC: number of birds captured; **IB**: infested birds; **PI**: prevalence of infestation; **NTC**: number of ticks collected; **MII**: mean intensity of infestation; **STI**: Species of tick identified.

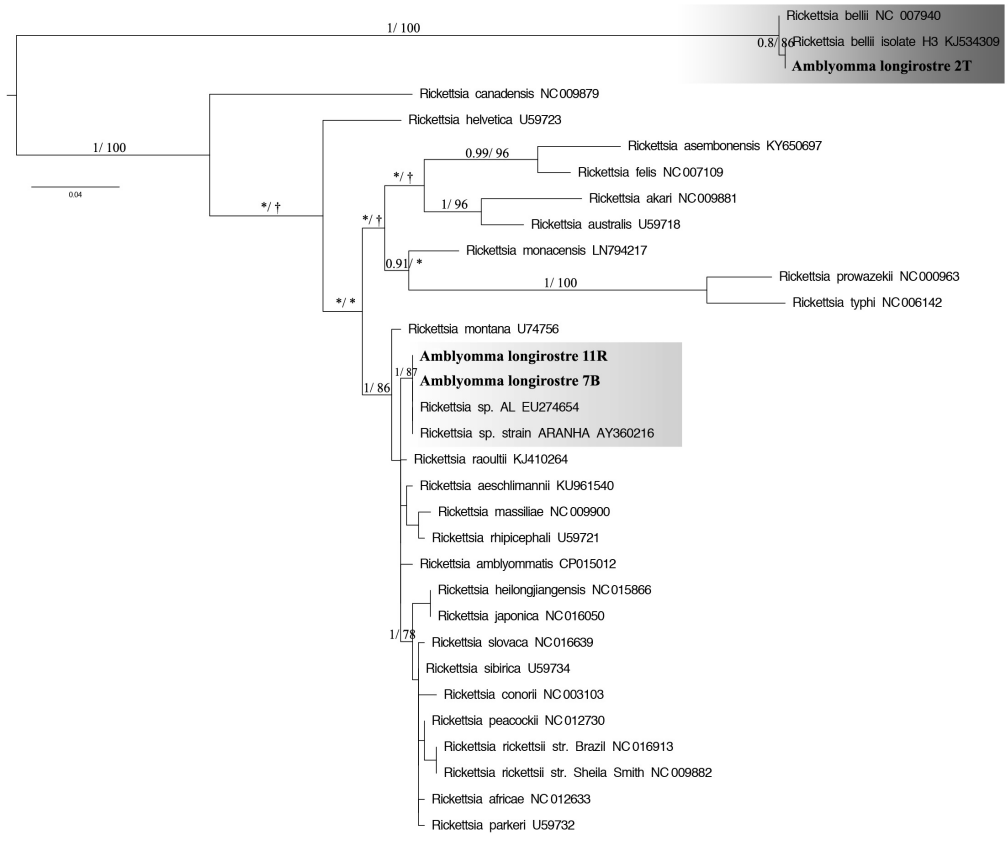

Figure 1. Phylogenetic tree based on the gltA gene (734nt) sequences of *Rickettsia*, using ML and Bayesian methods. Numbers (>0.7/>70%) above the branches indicate posterior node probabilities or bootstrap values (MrBayes/ML). *Indicate values below 0.7/70. †Exhibited difference between ML and MrBayes tree-building method topology. The scale bars indicate an evolutionary distance of 0.04 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.

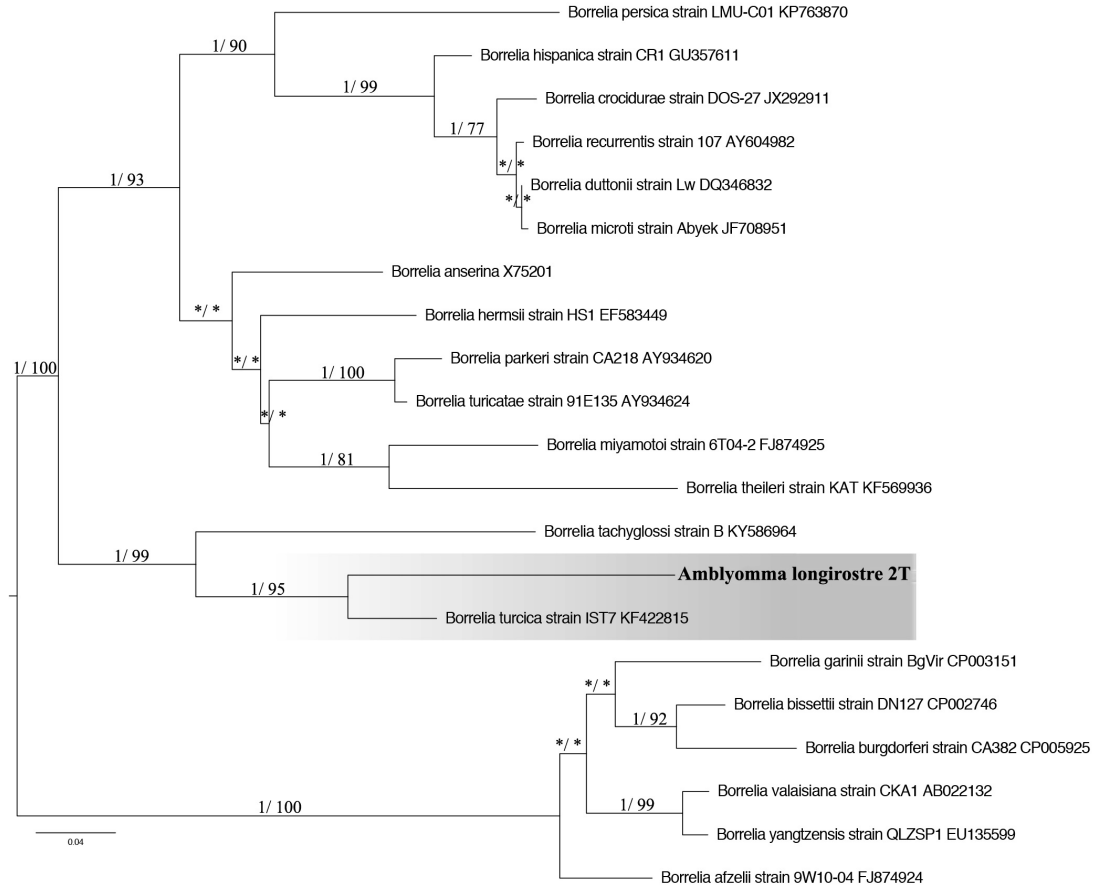

Figure 2. Phylogenetic tree based on the flaB gene (652nt) sequences of *Borrelia*, using ML and Bayesian methods. Numbers (>0.7/>70%) above the branches indicate posterior node probabilities or bootstrap values (MrBayes/ML). *Indicate values below 0.7/70. †Exhibited difference between ML and MrBayes tree-building method topology. The scale bars indicate an evolutionary distance of 0.04 substitutions per position in the sequence. 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.

in Brazil by Ogrzewalska et al. (2008) in the same tick species, *A. longirostre*. However, it was on birds of different species.

A sample of *A. longirostre* from *Tangara seledon* was positive for *Borrelia* spp. when using primers BorFlaF1/BorFlaR1 and BorFlaF2/BorFlaR2, which amplifies 740 bp of the flagellin B gene.

This sample presented a 91% identity rate with *Borrelia turcica* IST7 (access in the genbank: KF422815) (Figure 2), found in *Hyalomma aegyptium*. The partial sequence of the flaB gene also showed 99% similarity (coverage of 47% e 41%) with *Borrelia* sp. TX-Amac2 and *Borrelia* sp. F3 (access number KP861337 and KF395231), and both were found in *A. maculatum* that infested humans in the United States of America (MITCHELL et al., 2016).

The primers that target the hpt and glpQ genes of *Borrelia* spp. did not amplify products, probably because it is a conventional PCR and the sample does not have DNA in concentration sufficient for the amplification. Although *A. longirostre* has been described to infest humans in its immature stages (GUGLIELMONE et al., 2006), it cannot be said that the transmission may occur, because it is not aware of the pathogenic potential of the bacteria. This finding is of great importance for the literature, because it is the first time a report has pertained to the presence of *Borrelia* sp. in *A. longirostre.* There was no DNA from *Anaplasma* sp., *Ehrlichia* sp., and protozoa of the order Piroplasmida infecting the ticks in the present study. Therefore, bacteria of the genera *Borrelia* and *Rickettsia* can be found in *A. longirostre* that parasitize birds in the state of Rio de Janeiro.

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