Experimental and Applied Acarology (2022) 88:371–386
https://doi.org/10.1007/s10493-022-00754-3

Rickettsial infection in ticks from a natural area of Atlantic Forest biome in southern Brazil

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Received: 1 August 2022 / Accepted: 10 October 2022 / Published online: 7 November 2022

Abstract
From June 2013 to January 2014, blood sera samples and ticks were collected from domestic dogs and wild small mammals, and ticks from the vegetation in a preservation area of the Atlantic Forest biome (Turvo State Park), and the rural area surrounding the Park in Derrubadas municipality, state of Rio Grande do Sul, southern Brazil. Dogs were infested by *Amblyomma ovale* and *Amblyomma aureolatum* adult ticks, whereas small mammals were infested by immature stages of *A. ovale*, *Amblyomma yucumense*, *Amblyomma brasiliense*, *Ixodes loricatus*, and adults of *I. loricatus*. Ticks collected on vegetation were *A. brasiliense*, *A. ovale*, *A. yucumense*, *Amblyomma incisum*, and *Haemaphysalis juxtakochi*. Three *Rickettsia* species were molecularly detected in ticks: *Rickettsia bellii* in *I. loricatus* (also isolated through cell culture inoculation), *Rickettsia amblyommatis* in *A. brasiliense*, and *Rickettsia rhipicephali* in *A. yucumense.* The latter two are tick-rickettsia associations reported for the first time. Seroreactivity to *Rickettsia* antigens were detected in 33.5% (55/164) small mammals and 8.3% (3/36) canine sera. The present study reveals a richness of ticks and associated-rickettsiae in the largest Atlantic Forest Reserve of the state of Rio Grande do Sul, which is characterized by a rich fauna of wild mammals, typical of more preserved areas of this biome. Noteworthy, none of the detected *Rickettsia* species have been associated to human or animal diseases. This result contrasts to other areas of this biome in Brazil, which are endemic for tick-borne spotted fever caused by *Rickettsia rickettsii* or *Rickettsia parkeri.*

Keywords Ixodid ticks · IFA · PCR · *Rickettsia* · Small mammals · Dogs

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Introduction

In Brazil, the emergence and reemergence of infections caused by rickettsial agents has been evident in some regions during recent decades (Labruna 2009; Krawczak et al. 2014, 2016a; Oliveira et al. 2016). Several factors have been associated to their emergences, including changes in the diversity and geographic distribution of animals and plants, conservation measures favoring certain species and increase of the anthropization process (Ogrzewalska et al. 2011; Barros e Silva et al. 2014; Luz et al. 2019). Rickettsioses are associated with several arthropods, such as lice, fleas, ticks and other mites and, in nature, the maintenance of the rickettsiae cycle is guaranteed by the ability of ticks to act as reservoirs and vertebrates as amplifier hosts (Parola et al. 2013).

Currently, only two tick-borne *Rickettsia* species of the spotted fever group (SFG) are recognized as human pathogens in Brazil: *Rickettsia rickettsii*, the agent of Brazilian spotted fever, and *Rickettsia parkeri* strain 'Atlantic rainforest', the agent of *R. parkeri* spotted fever/rickettsiosis (Parola et al. 2013; Oliveira et al. 2016; Faccini-Martínez et al. 2018). Among the five regions of Brazil, the Southern region occupies the second position in relation to the casuistry of the tick-borne rickettsioses, behind only the Southeastern region. The state of Rio Grande do Sul, located in the Southern region, occupies the seventh place in relation to the number of confirmed cases of tick-borne SFG rickettsiosis, among the 27 federative units of Brazil (Barros e Silva et al. 2014; Brazil 2022). From 2007 to 2021, Rio Grande do Sul reported 14 laboratory confirmed cases of SFG rickettsiosis distributed in seven municipalities (Brazil 2022). Several studies from this state have reported ticks of the genus *Amblyomma* and *Haemaphysalis* infected with SFG rickettsiae, namely *R. parkeri* sensu stricto (s.s.) in *Amblyomma tigrinum* and *Haemaphysalis juxtakochi*, *R. parkeri* strain Atlantic rainforest in *Amblyomma ovale*, and *Ricketsia amblyommatis* in *Amblyomma longirostre* (Krawczak et al. 2016c; Souza et al. 2018; Weck et al. 2020). However, these studies were carried out in areas of the Pampa biome or in transition areas between this biome and the Atlantic Forest biome. Studies aiming at detecting rickettsiae in areas of natural Atlantic Forest do not exist for the state of Rio Grande do Sul, southern Brazil.

The Atlantic Forest biome originally occupied 37% of the territory of the state of Rio Grande do Sul. However, data from the Department of Environment and Sustainable Development (https://www.sema.rs.gov.br) show that only 12.9% of natural remnants remain in relation to the original vegetation cover. Recent studies have shown that the pathogen *R. parkeri* strain Atlantic rainforest has affected the human population residing in preserved areas of this biome in other Brazilian states such as São Paulo, Bahia, Santa Catarina and Espírito Santo (Spolidorio et al. 2010; Silva et al. 2011; Krawczak et al. 2016b; Faccini-Martínez et al. 2020).

Here, we investigated rickettsial infection in ticks, small mammals and dogs in a natural Reserve of the Atlantic Forest biome in Rio Grande do Sul, the largest preserved area of this biome in the southernmost state of Brazil.

Fig. 1 Location of the trails where small mammals and ticks were collected in the Atlantic Forest Reserve Turvo State Park (Parque Estadual do Turvo - PET), in the state of Rio Grande do Sul, southern Brazil, from June 2013 to January 2014

Materials and methods

Study area

This study was performed in a deciduous forest of the Turvo State Park 'Parque Estadual do Turvo' (PET) (27°00'–27°20'S, 53°40'–54°10'W) and in the rural area surrounding the Park, both located within Derrubadas municipality, in the northwestern region of the Rio Grande do Sul state, Brazil. The PET was established in March 11th, 1947, as an Atlantic Forest Reserve (Law no. 2.440, of 2 October 1954), and stands out for being the largest full protection area in Rio Grande do Sul, with 17,491 ha.

The PET is located at the east bank of the Uruguay River (Fig. 1), its mean temperature during the warmest month (January) is above 22 °C, and in the coldest month (July) it ranges from −3 to 18 °C (Melo et al. 2011). In 2021, the municipality of Derrubadas had an estimated population of 2,718 habitants and the preservation area occupied by the park was approximately 50% of the municipality (https://cidades.ibge.gov.br/brasil/rs/derrubadas/panorama). The PET bears a rich fauna of medium-sized to large wild mammals, including tapirs (*Tapirus terrestris*), jaguar (*Panthera onca*), puma (*Puma concolor*), capybaras (*Hydrochoerus hydrochaeris*), collared peccaries (*Dicotyles tajacu*), and deer (*Mazama* spp.) (Kasper et al. 2007).

For the present study, domestic dogs, small mammals and ticks were collected during three field campaigns (June and October 2013, and January 2014). This study was previously approved by the Chico Mendes Institute for Biodiversity (ICMBio Permit No. 38502-1) and the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine of the University of São Paulo (protocol 2908/2013) and State Secretary for the Environment of the Rio Grande do Sul (protocol 05/2013 registration number 428).

Domestic dogs

The primary reason to sample dogs was to test them by serology for the presence of reactive antibodies to SFG rickettsiae. Thus, to determine the number of dogs to be tested, a 3.16:1 ratio (number of humans/number of dogs) for rural area was used as previously described (Soto et al. 2006), and to calculate the sample size, we considered an expected prevalence of 10% of canine seropositivity to SFG rickettsiae (Labruna et al. 2007a), and 95% accuracy according to the formula $n = [(z^2)P(1 - P)]/d^2$ of Arya et al. (2012), where n = sample size, z = z statistic for the level of confidence, P = expected prevalence, and d = allowable error. This procedure indicated a minimum sample size of 29 dogs.

During the first field campaign in the rural area of Derrubadas, in the surroundings of the PET, blood serum samples were obtained from 36 dogs, which were also examined for tick infestations. All dogs were reported to have free access to forests. In the second and third field campaigns, 18 and 15 of these dogs were re-examined for tick infestations, respectively, resulting in a total of 69 canine examinations during the three field campaigns. Each dog had its entire body examined by two observers for a period of 3–5 min for the presence of ticks, which were collected in plastic tubes and transported to the laboratory for identification. The adult ticks and engorged nymphs were held alive, whereas the remaining immature ticks were stored in absolute ethanol.

Small mammals

Attempts to capture wild small mammals were performed along three trails within the deciduous forest of the PET (Fig. 1). For this purpose, a total of 80 live-traps [75 Sherman, (n = 25 in each trail) and five Tomahawk (2 in trail A, 2 in trail B and 1 in trail C)] baited with bacon, banana, apple, peanut butter and ham were installed for four consecutive nights during each field campaign. Additionally, three pitfall station traps with five bucket of 42.5 cm diameter and 60 cm height in each station connected by a plastic fence (of at least 30 m long and 50 cm high) (Umetsu et al. 2006) were installed for the same period. The total sampling effort was 960 trap-nights for live-traps and 180 trap-nights for pitfall-traps. Trapped animals were identified to species following current literature (Bonvicino et al. 2008; Melo et al. 2011), anaesthetized with ketamine and xylazine, and carefully examined for ticks, which were collected in plastic tubes and transported to the laboratory. Blood samples for serological analysis were collected by intracardiac or tail vein venipuncture from all trapped animals. Each animal was marked with a numbered earring (fish and small animal tag size 1; National Band and Tag, Newport, KY, USA), and released at the same capture site after recovery from anesthesia.

Host-seeking ticks

Host-seeking ticks were collected from vegetation in each field campaign using the cloth dragging technique and the visual search method according to Oliveira et al. (2000) and Terassini et al. (2010). Dragging was conducted by passing a cotton flannel (75 × 100 cm) over the ground level vegetation by one person through a 50–100 m trail in each campaign. The same trail was used for the visual search method, in parallel to dragging. Collected ticks were put in plastic tubes and transported to the laboratory.

Tick identification

Collected ticks were morphologically identified to species based on Marques et al. (2004), Barros-Battesti et al. (2006) and Martins et al. (2010). The morphological identification of some *Amblyomma* larvae was confirmed through molecular analysis. In this case, individual larval DNA was extracted by boiling (Horta et al. 2007) and tested by polymerase chain reaction (PCR) with primers targeting an approximately 460 bp fragment of the tick 16 S rDNA mitochondrial gene (Mangold et al. 1998). PCR products were DNA sequenced in an automatic sequencer (Model ABI 3500 Genetic Analyzer; Applied Biosystems/Thermo Fisher Scientific, Foster City, CA, USA) according to manufacturer's instructions. Generated sequences were submitted to BLAST analysis to determine the closest identities available in GenBank.

Rickettsial infection in ticks

Adult ticks that arrived alive at the laboratory were stored in a −80 °C freezer until tested for isolation of rickettsiae in Vero cell culture. For this purpose, ticks were thawed at room temperature and processed by the shell vial technique, as previously described (Labruna et al. 2004). A rickettsial isolate was considered to be established in Vero cells after at least three passages at 28 °C with the prevalence of infected cells exceeding 95%. A sample of 4th passage-infected cells was submitted to DNA extraction by the DNeasy Tissue Kit (Qiagen, Chatsworth, CA, USA), and tested by a PCR protocol with primers CS-78 and CS-323, which amplify a 401-bp fragment of the rickettsial citrate synthase gene (*gltA*) (Labruna et al. 2004). PCR products were DNA sequenced; generated sequences were submitted to BLAST analyses, as cited above.

DNA extraction using the guanidine isothiocyanate and phenol/chloroform technique (Sangioni et al. 2005) was applied to the remnants of ticks processed by the shell vial technique, as well as other frozen or alcohol-preserved adults. Larval and nymphal ticks were processed individually by boiling (Horta et al. 2007). Tick DNA samples were tested by a TaqMan real-time PCR assay targeting a 147-bp fragment of the rickettsial *gltA* gene (Labruna et al. 2004; Guedes et al. 2005). Once a tick was demonstrated by real-time PCR to contain rickettsial DNA, amplification of a larger fragment of the *gltA* gene was attempted by two conventional PCR protocols. One used primers CS-78 and CS-323 for the *gltA* gene (Labruna et al. 2004), and the second protocol used primers Rr190.70 F and Rr190.701R, which amplify an approximately 632 bp fragment of the 190 kDa outer membrane protein gene (*ompA*) of SFG rickettsiae (Roux et al. 1996). The PCR products were sequenced and submitted to BLAST analysis to determine their closest similarities to *Rickettsia* sequences available in GenBank.

All real-time PCR negative tick samples were evaluated by conventional PCR, aiming to amplify an approximately 460-bp fragment of the mitochondrial 16 S rDNA gene from ticks (Mangold et al. 1998), in order to validate the presence of viable DNA in the extractions protocol. If the tick sample yielded no product by this PCR, it was considered that DNA extraction was not successful, and the individual tick was discarded from the study.

Serology

Sera samples from dogs, rodents and marsupials were tested by immunofluorescence assay (IFA) against six *Rickettsia* antigens isolated from Brazil, namely: *Rickettsia rickettsii* strain Taiaçu, *R. parkeri* s.s. strain At24, *R. amblyommatis* strain Ac37, *Rickettsia rhipicephali* strain HJ5, *Rickettsia felis* strain Pedreira, and *Rickettsia bellii* strain Mogi, as previously described (Labruna et al. 2007a). Briefly, sera were diluted in twofold increments with phosphate buffered saline (PBS) from an initial dilution 1:64. Slides were incubated with fluorescein isothiocyanate-labelled rabbit anti-dog IgG (Sigma, St Louis, MO, USA), goat anti-mouse IgG (Sigma) and sheep anti-opossum IgG (CCZ, São Paulo, Brazil) for canine, rodent and marsupial sera, respectively. For each sample, the endpoint IgG titer reacting with each of the six *Rickettsia* antigens was determined. An endpoint titer at least 4-fold higher for a *Rickettsia* species than that observed for any other *Rickettsia* species was considered probably homologous to the first *Rickettsia* species or to a very closely related species (Labruna et al. 2007a; Szabó et al. 2013). In each slide, a serum previously shown to be non-reactive (negative control) and a known reactive serum (positive control), both from the studies of Szabó et al. (2013) and Krawczak et al. (2016c), were tested at the 1:64 dilution.

Results

Ticks from animals

During the period from June 2013 to January 2014, three field campaigns were carried out, encompassed a study period of 6 months; for this reason, all data were pooled for presentation. From a total of 69 canine examinations, 17% (12/69) revealed tick infestations, which were identified as adults of *Amblyomma aureolatum* (one male on one dog; 1.5% infestation rate) or *A. ovale* (17 males and 17 females on 11 dogs; 16%).

A total of 164 small mammals of nine species (two marsupials and seven rodents) were captured, of which 23 (14%) were infested by ticks. Considering the host species that yielded > 1 captured specimen, tick infestations were detected on 100% (7 infested/7 captured) of the marsupial *Didelphis aurita* (black-eared opossum), 57% (4/7) of the rodent *Oxymycterus quaestor* = *Oxymycterus judex* (quaestor hocicudo), 50% (2/4) of the rodent *Sooretamys angouya* (Paraguayan rice rat), 11% (1/9) of the rodent *Oligoryzomys nigripes* (pygmy rice rat), 8% (1/13) of the rodent *Brucepattersonius iheringi* (Ihering's hocicudo), 5% (6/117) of the rodent *Akodon montensis* (montane grass mouse), and 0% (0/5) of the rodent *Thaptomys nigrita* (blackish grass mouse). The single captured specimen of the rodent *Euryoryzomys russatus* (big-headed rice rat) and the marsupial *Cryptonanus guahybae* (Guahiba gracile opossum) were both infested by ticks.

Four tick species were found infesting small mammals (Table 1). The most frequent and abundant tick species was *A. yucumense*, of which larvae and nymphs were collected on the rodent *O. quaestor* (22 larvae, 11 nymphs) and the marsupial *D. aurita* (56 larvae, 53 nymphs), and only nymphs were collected on the rodents *A. montensis* (one nymph), *E. russatus* (one nymph) and *S. angouya* (two nymphs). Although most of these records of *A. yucumense* were previously reported by Krawczak et al. (2015), the following specimens are here reported for the first time: 22 larvae and three nymphs from *O. quaestor*, 23 larvae

Table 1 Infestation by ticks on wild small mammals in Derrubadas municipality, state of Rio Grande do Sul, Brazil, from June 2013 to January 2014

Small mammal species (no. captured specimens)	No. tick specimens according to species and feeding stage (no. infested animals)[a]							
	A.bra	A.ova	A.yuc		I.lor			A.sp
	N	N	L	N	L	N	Ad.	L
Rodents								
Akodon montensis (117)				1 (1)	3 (2)	2[d] (2)		1 (1)
Brucepattersonius iheringi (13)								1 (1)
Euryoryzomys russatus (1)				1 (1)				
Oligoryzomys nigripes (9)					1 (1)			
Oxymycterus quaestor (7)			22[b] (2)	11 (2)				86 (3)
Thaptomys nigrita (5)								
Sooretamys angouya (4)		1 (1)		2 (2)				
Marsupials								
Didelphis aurita (7)	8 (3)		56[b] (2)	53[c] (4)			25 (7)	1 (1)
Cryptonanus guahybae (1)								1 (1)
TOTAL (164)	8 (3)	1 (1)	78 (4)	68 (10)	4 (3)	2 (2)	25 (7)	90 (7)

[a] A.bra: *Amblyomma brasiliense*; A.ova: *A. ovale*; A.yuc: *A. yucumense*; I.lor: *Ixodes loricatus*; A.sp: *Amblyomma* sp.; N: nymphs; L: larvae; Ad.: adults

[b] The 22 larvae from *O. quaestor* and 23 larvae from *D. aurita* were identified to species by analysis of their 16 S rDNA partial sequences; the remaining 33 larvae from *D. aurita* molted to nymphs, which were identified to species based on their morphology

[c] Among the 53 nymphs, four molted to the adult stage in the laboratory

[d] One of these nymphs molted to the adult stage in the laboratory

from *D. aurita*, and one nymph from *S. angouya*; these larvae were identified to species level by generating 16 S rDNA partial sequences, which were 99–100% identical to available sequences of *A. yucumense* in GenBank (KJ914670, MH282856).

Ixodes loricatus was the second most abundant tick species, with immature stages on two rodent species [*A. montensis* (three larvae, two nymphs) and *O. nigripes* (one larva)], and adults (eight males, 17 females) on the marsupial *D. aurita.* Here we report for the first-time larvae and nymphs of *I. loricatus* on *A. montensis*. These four larvae were also identified in the molecular level, generating 16 S rDNA partial sequences that were 99% identical to sequences of *I. loricatus* from GenBank (AF549840, KX137895). Two other tick species were each found on a single host species: *Amblyomma brasiliense* (eight nymphs) on *D. aurita* and *A. ovale* (one nymph) on *S. angouya.* Finally, 90 larvae could not be identified to species level and were retained as *Amblyomma* sp.

Host-seeking ticks

A total of 319 ticks were collected on vegetation and were identified as *A. brasiliense* (20 larvae, 61 nymphs, six males, 10 females), *Amblyomma incisum* (62 nymphs, 15 males, 19 females), *A. ovale* (three males), *A. yucumense* (eight nymphs, 13 males, nine females),

Haemaphysalis juxtakochi (seven nymphs, two females), and *Amblyomma* sp. (83 larvae). The 20 *A. brasiliense* larvae derived from a cluster found on vegetation, and their identification to species relied on a 16 S rRNA gene partial sequence that was generated from this larval pool, which was 100% identical to a corresponding sequence of *A. brasiliense* from GenBank (FJ424399). Although not foreseen, taxonomic identification of a *H. juxtakochi* nymph was also confirmed by molecular analysis, generating a 16 S rRNA partial sequence 99% identical to *H. juxtakochi* from GenBank (AY762323). The above-mentioned host-seeking specimens of *A. yucumense* were previously reported by Krawczak et al. (2015).

Rickettsial infection in ticks

Attempts to isolate rickettsiae in Vero cell culture were performed individually with the following adult ticks: two *A. brasiliense* from vegetation, two *A. incisum* from vegetation, five *A. ovale* from dogs, two *A. yucumense* from vegetation, two *H. juxtakochi* from vegetation and two *I. loricatus* from marsupial *D. aurita*. Rickettsiae were successfully established in Vero cell culture only from one *I. loricatus* tick. By PCR, the infected Vero cells generated a 350 bp fragment (excluding primer sequences) of the rickettsial *gltA*, which was 100% identical to a corresponding sequence of *R. bellii* from GenBank (DQ146481). This isolate was further molecularly characterized as *R. bellii* strain IL-RS1, in another study that performed broader genotypic characterization of *R. bellii* isolates (Krawczak et al. 2018).

From a total of 266 tick specimens tested by real-time PCR, rickettsial DNA was detected in 22 samples (Table 2), which were further tested by conventional PCR targeting fragments of two rickettsial genes, *gltA* and *ompA*. PCR products were DNA sequenced from the 22 ticks, and when submitted to BLAST analyses, the *gltA* (350 bp) and *ompA* (588 bp) fragments amplified from *A. brasiliense* (larvae, nymphs and adults) were 100% identical to available sequences of *R. amblyommatis* in GenBank (CP015012 and KX434739, respectively). The *gltA* (350 bp) and *ompA* (488 bp) fragments amplified from *A. yucumense* (larvae, nymphs and adults) and *H. juxtakochi* (nymphs) were 100% identical to available sequences of *R. rhipicephali* in GenBank (CP013133). The *gltA* (350 bp) fragment amplified from *I. loricatus* (adults) and *H. juxtakochi* nymphs were 100% identical to available sequences of *R. bellii* in GenBank (CP000849).

Serology

Serum samples were obtained from 198 individuals (36 dogs and 162 small mammals) and tested by IFA against six rickettsial antigens. Overall, the proportions of seroreactive animals to SFG antigens were 24% for *R. parkeri*, 23% for *R. rickettsii*, 21% for *R. amblyommatis* and 17% for *R. rhipicephali*. Then, only 9% of the sera reacted to *R. bellii* and none to *R. felis* antigens (Table 3). Among 36 dogs, only 2 (5.5%) reacted to *R. rhipicephali* (endpoint titers: 64 and 256) and 1 (2.8%) to *R. bellii* (endpoint titer: 256). Regarding the small mammals, a total of 116 sera of *A. montensis* were tested, of which 34 (29%) were reactive to *R. parkeri* (endpoint titers: 64 to 512) and *R. rickettsii* (64 to 512), 30 (27%) to *R. amblyommatis* (64 to 512), 21 (18%) to *R. rhipicephali* (64 to 512), and 15 (13%) to *R. bellii* (64 to 1024). Among 12 tested sera of *B. iheringi*, six (50%) were reactive to *R. rickettsii* (endpoint titers: 256 to 4096) and *R. rhipicephali* (128 to 2048), and five (42%) were reactive to *R. parkeri* (128 to 1024) and *R. amblyommatis* (128 to 2048). Among seven

Table 2 Molecular detection of rickettsial DNA in ticks collected in Derrubadas municipality, state of Rio Grande do Sul, Brazil, from June 2013 to January 2014

Tick species	Tick stage	Source	No. ticks with rickettsial DNA / no. tested ticks (%)	*Rickettsia* species identified by DNA sequencing
Amblyomma brasiliense	Larva	Vegetation	1/20 (5)[a]	*R. amblyommatis*
	Nymph	Vegetation	5/48 (10)	*R. amblyommatis*
	Adult	Vegetation	1/15 (7)	*R. amblyommatis*
A. ovale	Nymph	*Sooretamys angouya*	0/1 (0)	
	Adult	Dogs	0/34 (0)	
A. incisum	Nymph	Vegetation	0/44 (0)	
	Adult	Vegetation	0/33 (0)	
A. yucumense	Larva	*Didelphis aurita*	4/6 (67)	*R. rhipicephali*
	Nymph	*D. aurita*	3/16 (19)	*R. rhipicephali*
	Adult	Vegetation	1/4 (25)	*R. rhipicephali*
Haemaphysalis juxtakochi	Nymph	Vegetation	3/5 (60)	*R. rhipicephali*
	Adult	Vegetation	0/2 (0)	
Ixodes loricatus	Larva	*Akodon montensis*, *Oligoryzomys nigripes*	0/4 (0)	
	Nymph	*A. montensis*	0/2 (0)	
	Adult	*D. aurita*	4/22 (18)	*R. bellii*
Amblyomma sp.	Larva	Vegetation	0/10 (0)	
TOTAL			22/266 (8)	

[a] Refers to a pool of 20 larvae; thus, 5% is the minimal infection rate (at least one infected larva among 20 larvae)

tested sera of *O. quaestor*, six (86%) were reactive to *R. parkeri* (endpoint titers: 64 to 256), *R. rickettsii* (256 to 512) and *R. amblyommatis* (128 to 512), five (71%) were reactive to *R. rhipicephali* (128 to 2048), and one (14%) to *R. bellii* (128). Only two (29%) out of *D. aurita* were reactive to *R. parkeri* (endpoint titers: 128), whereas none of nine *O. nigripes*, five *T. nigrita*, four *S. angouya*, one *C. guahybae*, or one *E. russatus* was reactive to any rickettsial antigen (Table 3).

One dog had endpoint titers to *R. rhipicephali* at least 4-fold higher than those observed for the other five *Rickettsia* species, indicating a possible homologous reaction to *R. rhipicephali* or a closely related species. Using the same criterion, one dog and three *A. montensis* were exposed to *R. bellii* or a closely related species, two *D. aurita* were exposed to *R. parkeri* or a closely related species, and one *O. nigrita* was exposed to *R. rickettsii* or a closely related species (Table 3).

Accession numbers

GenBank accession numbers for the DNA partial sequences generated in the present study are KX434748–KX434754 for the 16 S rRNA gene of *A. yucumense*, *A. brasiliense*, *H. juxtakochi*, *I. loricatus*; KX434741, KX434739 for the *gltA* and *ompA* genes of *R. amblyommatis*, KX434744, KX434745 for the *gltA* gene of *R. rhipicephali*, KX434735, KX434736 for the *ompA* gene of *R. rhipicephali*; and KX434740 for the *gltA* gene of *R. bellii*. Voucher

Table 3 Seroreactivity to six *Rickettsia* species of animals from Derrubadas municipality, state of Rio Grande do Sul, a non-endemic area for Brazilian Spotted Fever, from June 2013 to January 2014

Dogs and small mammal species (no. tested specimens)	No. seroreactive animals to each *Rickettsia* species (% seroreactivity)[a]						No. animals with PAIHR [b]
	R.pa	R.ri	R.am	R.rh	R.fe	R.be	
Dogs (36)	0	0	0	2 (6)	0	1 (3)	1 R.rh, 1 R.be
Akodon montensis (116)	34 (29)	34 (29)	30 (27)	21 (18)	0	15 (13)	3 R.be
Brucepattersonius iheringi (12)	5 (42)	6 (50)	5 (42)	6 (50)	0	0	
Cryptonanus guahybae (1)	0	0	0	0	0	0	
Didelphis aurita (7)	2 (29)	0	0	0	0	0	2 R.pa
Euryoryzomys russatus (1)	0	0	0	0	0	0	
Oligoryzomys nigripes (9)	0	0	0	0	0	0	
Oxymycterus quaestor (7)	6 (86)	6 (86)	6 (86)	5 (71)	0	1 (14)	1 R.ri
Sooretamys angouya (4)	0	0	0	0	0	0	
Thaptomys nigrita (5)	0	0	0	0	0	0	
TOTAL (198)	47 (24)	46 (23)	41 (21)	34 (17)	0 (0)	17 (9)	

[a] R.pa: *Rickettsia parkeri*; R.ri: *R. rickettsii*; R.am: *R. amblyommatis*; R.rh: *R. rhipicephali*; R.fe: *R. felis*; R.be: *R. bellii*

[b] PAIHR: possible antigen involved in a homologous reaction. A homologous reaction was determined if the endpoint titer to a *Rickettsia* species was at least 4-fold higher than those observed for the other *Rickettsia* species. In this case, the *Rickettsia* species (or a very closely related species) involved in the highest endpoint titer was considered the PAIHR.

specimens of the tick species collected in the present study have been deposited in the tick collection 'Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva' (CNC) of the University of São Paulo, under accession numbers CNC 3323–3331.

Discussion

Through the investigation of ticks and rickettsial infection/exposure in a natural Reserve of the Atlantic Forest biome and its surroundings in the Rio Grande do Sul, we found a richness of seven tick species harboring three *Rickettsia* species, and serological evidence of rickettsial exposure in domestic dogs and small mammals.

The two tick species found on dogs in the present study, *A. aureolatum* and *A. ovale*, have been reported in another area of the Atlantic Forest biome in southern Brazil (Barbieri et al. 2014). In both studies, there was a predominance of *A. ovale*, which is implicated as the most important vector of *R. parkeri* strain Atlantic rainforest in southern Brazil (Barbieri et al. 2014; Krawczak et al. 2016b; Voizzoni et al. 2016). In endemic areas for tick-borne spotted fever of southern Brazil, 20–60% of the dogs were reported to be seroreactive to SFG rickettsiae, with highest endpoint titers to *R. parkeri* (Barbieri et al. 2014; Krawczak et al. 2016b). Herein, only three (8%) out of 36 rural dogs, with free access to forest, were reactive to rickettsiae; however, none of them was reactive to *R. parkeri* or *R. rickettsii*, the agents that cause tick-borne SFG rickettsiosis in Brazil (Parola et al. 2013). Moreover, we

found no SFG agent infecting *A. ovale* ticks in the present study. These results indicate that the surroundings of the PET are not endemic for *R. parkeri* rickettsiosis, in contrast to the transition area of Atlantic Forest and Pampa biomes of Rio Grande do Sul, where human cases of rickettsiosis have been reported and 15% of the *A. ovale* ticks from dogs were infected by *R. parkeri* strain Atlantic rainforest (Krawczak et al. 2016b). In other *R. parkeri* rickettsiosis-endemic areas of Brazil, 8–15% infection rates by *R. parkeri* strain Atlantic rainforest have been reported in *A. ovale* ticks (Szabó et al. 2013; Barbieri et al. 2014).

Immature stages of *A. yucumense* were found on four rodent species (*A. montensis*, *E. russatus*, *O. quaestor*, *S. angouya*) and one marsupial (*D. aurita*). Moreover, > 70% of the larvae and nymphs were collected from *D. aurita*, suggesting a more important role as host for immature stages of *A. yucumense*. Contrastingly, hosts for adults of *A. yucumense* have never been reported; however, their host-seeking behavior inside the forest is compatible with ticks of tapirs (Krawczak et al. 2015). Regarding *I. loricatus*, our records of immature stages parasitizing two rodent species (*A. montensis*, *O. nigripes*) and the adult stage on *D. aurita* agrees with the typical host pattern reported for *I. loricatus* in South America, i.e., immature stages on Cricetidae rodents, and adult ticks on marsupials, chiefly *Didelphis* spp. (Nava et al. 2017).

Our findings of nymphs of *A. brasiliense* on *D. aurita* is supported by a recent study in the Argentinean Atlantic Forest, where *D. aurita* was reported as occasional hosts for immature stages of *A. brasiliense* (Lamattina et al. 2018). While a broad range of mammal hosts has been reported for *A. brasiliense* (Guglielmone et al. 2021), all records of have been from areas inhabited by peccaries (*D. tajacu* and/or *Tayassu pecari*), suggesting that these mammals are primary hosts for this tick species (Szabó et al. 2009). The present record of a nymph of *A. ovale* on the rodent *S. angouya* also agrees with current literature, which includes a vast list of Cricetidae species as hosts for immature stages of *A. ovale*, whose adults are primarily associated with Carnivora (Martins et al. 2016; Guglielmone et al. 2021).

Among the five tick species collected from vegetation, *A. brasiliense*, *A. ovale*, *A. yucumense*, *A. incisum*, and *H. juxtakochi*, only the latter two were not collected from small mammals. This result is expected for *A. incisum*, as one study in another Atlantic Forest Reserve showed that larvae, nymphs and adults of this tick species quest for hosts on the vegetation at heights usually above 30 cm, suggesting that they are primarily associated to large mammals, i.e., tapirs and peccaries (Szabó et al. 2009). In fact, *A. incisum* has never been reported on small rodents (Guglielmone et al. 2021). Although *H. juxtakochi* is primarily associated to deer (*Mazama* spp.), there have been a few records of larvae and nymphs on small mammals (Guglielmone et al. 2021). Therefore, the absence of *H. juxtakochi* on small mammals of the present study could be a result of low tick density, as this tick represented < 3% of the collected host-seeking ticks.

Regarding the *Rickettsia* species detected in ticks of the present study, the presence of *R. bellii* in *I. loricatus* is corroborated by several studies in southeastern and southern Brazil (Horta et al. 2007; Szabó et al. 2013; Krawczak et al. 2016b), suggesting a widespread tick-rickettsia association among *I. loricatus* populations. Similarly, our findings of *R. rhipicephali* in *H. juxtakochi* is corroborated by previous studies in southeastern, midwestern and northern Brazil (Labruna et al. 2007b; Soares et al. 2015; Acosta et al. 2016). On the other hand, we report two tick-rickettsia association for the first time, *R. rhipicephali* in *A. yucumense*, and *R. amblyommatis* in *A. brasiliense.* Indeed, the latter adds *A. brasiliense*

to the broad list of *Amblyomma* species that have been found infected by *R. amblyommatis* in Central and South America (Parola et al. 2013; Soares et al. 2015; Binetruy et al. 2020; Bermúdez et al. 2021).

Serological analyses revealed that a few dogs, opossums (*D. aurita*) and some individuals of three rodent species (*A. montensis*, *B. iheringi*, *O. quaestor*) were reactive to rickettsial antigens, indicating previous exposure to *Rickettsia* spp. Since there are serological cross-reactions between different *Rickettsia* species notably within the SFG (Parola et al. 2013), most of the seroreactive animals reacted to two or more *Rickettsia* antigens, although this result could also be strengthened by the exposure to multiple species of *Rickettsia*. The fact that two *D. aurita* reacted solely to *R. parkeri* antigens, and a single *O. quaestor* reacting to *R. rickettsii* with endpoint titers ≥ 4-fold higher than those observed for the other five *Rickettsia* species do not necessarily indicate exposure to these specific rickettsial pathogens, as it could be exposure to closely related agents, pathogenic or not, yet to be investigated in the study area. On the other hand, the ≥ 4-fold higher antibody titers to *R. rhipicephali* in a dog or to *R. bellii* in three *A. montensis* and one dog suggest previous exposure to these specific agents because they were shown to be present in the ticks of the study area.

None of the tested dogs or small mammals were reactive to *R. felis*, an agent primarily associated with fleas of the genus *Ctenocephalides* infesting dogs in all regions of Brazil (Horta et al. 2014). It is noteworthy that some of the sampled dogs of the present study were infested by fleas (*Ctenocephalides* sp.), which were collected from two dogs and shown in our laboratory to harbor '*Candidatus* Rickettsia asemboensis' (GenBank acc. nr. KX533943) (data not shown). Indeed, '*Ca.* R. asemboensis' is an agent very closely related to *R. felis* (Jiang et al. 2013); therefore, if this agent had infected the sampled dogs, we would have detected some serological reactiveness to *R. felis.*

The present study reveals a richness of ticks and associated-rickettsiae in the largest Atlantic Forest Reserve of the state of Rio Grande do Sul, which is characterized by a rich fauna of wild mammals (tapirs, peccaries, jaguar), typical of more preserved areas of this biome. Noteworthy, none of the detected *Rickettsia* species have been associated to human or animal diseases. This result contrasts to other areas of this biome in Brazil, which are endemic for tick-borne spotted fever caused by *R. rickettsii* (Ogrzewalska et al. 2012; Luz et al. 2019) or *R. parkeri* (Barbieri et al. 2014; Krawczak et al. 2016b). These spotted fever-endemic areas have in common a notable anthropization process, reflected by more fragmented areas of the Atlantic Forest, devoid of a rich fauna of wild large mammals. Similarly, to the present study, studies in the largest Atlantic Forest Reserve of the state of São Paulo (southeastern Brazil) also revealed a richness of ticks and associated-rickettsiae, albeit not *R. rickettsii* or *R. parkeri* (Labruna et al. 2007b; Pacheco et al. 2008, 2011; Szabó et al. 2009). Future studies are warranted to investigate key elements that could trigger the emergence of tick-borne spotted fever in areas of the Atlantic Forest biome.

Author contribution FSK and MBL conceived and designed the study, and critically revised the manuscript. FSK, CS, FBC, FG, GLM, GP, GTP, JS and LCB performed the experiment, analyzed the data, and drafted the manuscript. FSK, CS, FBC, LCB, JS, MBL and TFM helped in the implementation and execution of the study. FSK, LCB, MBL, FG and TFM performed and interpreted the laboratory analyses. All authors read and approved the final manuscript.

Funding This work was financially supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP process 2012/21915-4), Conselho Nacional de Desenvolvimento Científico e Tecnológico

(CNPq process 474307/2013-1), and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES Finance Code 001).

Data Availability The data presented in this study are available within the article.

Declarations

Ethics approval This study was previously approved by the Chico Mendes Institute for biodiversity (ICMBio Permit No. 38502-1) and the Institutional Animal Care and Use Committee (IACUC) of the Faculty of Veterinary Medicine of the University of São Paulo (protocol 2908/2013) and State Secretary for the Environment of the Rio Grande do Sul (protocol 05/2013 registration number 428).

Consent for publication All authors consent to publication of this manuscript.

Conflict of interest The authors declare that they have no conflict of interest relevant to the content of this article.

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