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Survey of Ticks (Acari: Ixodidae) and Their *Rickettsia* in an Atlantic Rain Forest Reserve in the State of São Paulo, Brazil

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ABSTRACT The current study investigated the occurrence of ticks and their rickettsiae in the Serra do Mar State Park, which encompasses one of the largest Atlantic rain forest reserves of Brazil. From July 2008 to June 2009, a total of 2,439 ticks (2,196 free living and 243 collected on hosts) was collected, encompassing the following 13 species: *Amblyomma aureolatum* (Pallas), *Amblyomma brasiliense* Aragão, *Amblyomma dubitatum* Neumann, *Amblyomma fuscum* Neumann, *Amblyomma incisum* Neumann, *Amblyomma longirostre* (Koch), *Amblyomma naponense* (Packard), *Amblyomma nodosum* Neumann, *Amblyomma ovale* Koch, *Haemaphysalis juxtakochi* Cooley, *Ixodes aragaoi* Fonseca, *Ixodes loricatus* Neumann, and *Rhipicephalus sanguineus* (Latreille). Ticks were submitted to polymerase chain reaction assays targeting portions of the rickettsial genes *gltA* and *ompA*. Polymerase chain reaction products were DNA sequenced and compared with corresponding sequences available in GenBank. *Rickettsia bellii*, a rickettsia of unknown pathogenicity, was detected in one *A. aureolatum*, one *A. ovale*, and three *A. incisum* specimens. At least 8.8% (3/34) of the free-living *A. ovale* ticks, 13.6% (8/59) of the *A. ovale* ticks collected from dogs, and 1.9% (1/54) of the *R. sanguineus* (Latreille) ticks were found to be infected by *Rickettsia* sp strain Atlantic rain forest, a novel strain that has been shown to cause an eschar-associated spotted fever in the state of São Paulo. Our results suggest that *A. ovale* is the vector of *Rickettsia* sp strain Atlantic rain forest in the state of São Paulo.

KEY WORDS ticks, *Amblyomma*, *Haemaphysalis*, *Ixodes*, *Rickettsia*

The tick fauna of Brazil is currently composed of 61 tick species, divided into the families Ixodidae and Argasidae. The former family is the largest, composed of the genera *Amblyomma* (30 species), *Ixodes* (8), *Haemaphyslis* (3), *Rhipicephalus* (2), and *Dermacentor* (1) (Dantas-Torres et al. 2009). Ticks of the genus *Amblyomma* are of greater medical importance in South America, where the vast majority of human infestations refer to *Amblyomma* species (Guglielmone et al. 2006), and because several rickettsial agents pathogenic for humans are transmitted by *Amblyomma* ticks (Labruna 2009).

Bacteria within the genus *Rickettsia* are obligate intracellular short rods, 0.3–0.5 × 0.8–2.0 μm, Gram negative, primarily associated with invertebrate hosts. Pathogenic *Rickettsia* species have been classically classified into the spotted fever group (SFG) and the typhus group (Fournier and Raoult 2007). More recently, part of the SFG species was split into a new group, designated as the transitional group (Gillespie et al. 2007). Currently, all SFG species are primarily associated with ticks (Weinert et al. 2009). Whereas most of the SFG species are pathogenic for humans, to whom they are transmitted via tick bites, other SFG species are still considered of unknown pathogenicity (Fournier and Raoult 2007, Weinert et al. 2009).

The Brazilian Atlantic rain forest is characterized by species diversity higher than most of the Amazonian forests (Morellato and Haddad 2000). One of the greatest remnants of this forest is the Serra do Mar State Park, a nature reserve of 315,000 ha along the Serra do Mar mountain chain, just east to the largest metropolitan area of South America, which is composed of São Paulo city and various adjacent cities. The current study investigated the occurrence of ticks within the Serra do Mar State Park. In addition, we investigated rickettsial infection in part of the collected ticks.

Materials and Methods

This study was conducted in the Itutinga-Pilões branch of the Serra do Mar State Park, state of São Paulo, Brazil. For this purpose, six sites of the park (trails 1–6) were visited during consecutive 6–12 mo, from July 2008 to June 2009. On each visit in each site, the vegetation on both sides of a trail was sampled for 60 min, always by four investigators, by using flagging,

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Table 1. Details of the six trails used for tick sampling in the Serra do Mar State Park, state of São Paulo, Brazil

Trails	Coordinates	Altitude (m above sea level)	Distance from trail 1 (m)
1	23°49'29.4"S 46°30'47.4"W	759	—
2	23°51'28.5"S 46°30'43.1"W	746	3,930
3	23°53'36.2"S 46°34'22.6"W	764	10,600
4	23°55'47.6"S 46°31'07.5"W	69	11,700
5	23°47'35.4"S 46°18'33.3"W	993	20,600
6	23°54'17.7"S 46°29'28.3"W	36	9,300

and also by visual search of questing ticks on the vegetation, as previously reported (Szabó et al. 2009). Geographic details of the six trails are shown in Table 1.

All free-living ticks found in the trails were collected and taken alive to the laboratory, where adults and nymphs were counted individually. Free-living larvae were not counted individually because they were always found as large groups, from dozens to thousands of individuals per cluster. For convenience, each such larval cluster was considered as a larval unit in the counting. During our monthly visits to the trails, some animals (mostly domestic dogs) were occasionally available for examination and collection of ticks. These ticks were also taken alive to the laboratory and counted. While conducting the current study, it was impossible to identify, by morphology, the immature stages of most *Amblyomma* species from Brazil to species level. For this reason, collected larvae and nymphs were brought alive to the laboratory, where attempts to rear them to the adult stage were conducted by feeding them on tick-bite naive rabbits, as previously described (Labruna et al. 2002). Adults obtained from the engorged nymphs were used for species identification of the former immature ticks, following taxonomic keys proposed by Barros-Battesti et al. (2006). The species of any adults obtained from each larval cluster collected in the environment was used for species identification of the larval cluster as a single unit. Voucher specimens of the ticks collected during this study were deposited in the tick collection Coleção Nacional de Carrapatos of the University of São Paulo (accession CNC-1151, 1195, 1196, 1361, 1553–1555).

Samples of adult ticks were individually submitted to DNA extraction by the guanidine isothiocyanate-phenol technique, as previously described (Sangioni et al. 2005). For every 10 individual ticks, a blank tube was included in the DNA extraction. Samples were tested individually by polymerase chain reaction (PCR) targeting a 401-bp fragment of the rickettsial gene *gltA*, as previously described (Labruna et al. 2004). In each set of reactions, negative control tubes containing water were included, and also a positive control tube containing DNA of the strain NOD of *Rickettsia parkeri*. Samples that yielded visible amplicons of the expected size by the *gltA*-PCR were further tested by a second PCR assay targeting a 532-bp fragment of the rickettsial gene *ompA*, as previously described (Regnery et al. 1991). All *ompA*-PCR amplicons of the expected size were submitted to direct DNA sequencing in an automated ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA), as well as the *gltA*-PCR amplicons from ticks that were negative by the *ompA*-PCR. The BLAST program (National Center for Biotechnology Information, Bethesda, MD) was used to compare appropriate similarities of the rickettsial partial sequences generated in the current study.

Results

A total of 2,439 ticks (2,196 free living and 243 collected on hosts) encompassing 13 different species was collected during the current study, as shown in Table 2. Two *Rickettsia* species were found infecting ticks (Table 3). *Rickettsia bellii* was detected in one *Amblyomma aureolatum* (Pallas), one *Amblyomma*

Table 2. Ticks found in the Serra do Mar State Park, state of São Paulo, Brazil, 2008–2009

Tick species	Trails[a]	No. ticks collected on vegetation			Animals (no. parasitized)–no. ticks collected	
		Adults	Nymphs	Larval clusters	Adults	Nymphs
Amblyomma aureolatum	1,2,5	7	—	—	Dogs (70) – 114	—
Amblyomma brasiliense	3,4	36	—	—	—	Dog (1) – 1
Amblyomma dubitatum	3	—	11	—	—	—
Amblyomma fuscum	4	—	—	—	—	*Didelphis aurita* (1) – 2
Amblyomma incisum	1,2,3,4,6	101	482	9	—	—
Amblyomma longirostre	1	1	—	—	—	—
Amblyomma naponense	4	2	—	—	Dog (1) – 1	—
Amblyomma nodosum	4	—	—	—	Dogs (2) – 2	—
Amblyomma ovale	4,5,6	34	—	—	Dogs (35) – 61	—
Haemaphysalis juxtakochi	1,2,3,4	34	146	11	—	—
Ixodes aragaoi	5	1	—	—	—	—
Ixodes loricatus	4	—	—	—	*D. aurita* (1) – 2	—
Rhipicephalus sanguineus	4	—	—	—	Dogs (35) – 59	—
Amblyomma spp[b]	1,2,3,4,5,6	—	1,295	26	—	Dog (1) – 1
Total		216	1,934	46	239	4

[a] See trail geographic information in Table 1.
[b] Subadult ticks that died before reaching the adult stage in the laboratory, precluding their identification to species.

Table 3. Rickettsial infection in adult ticks collected from the vegetation and from dogs in the Serra do Mar State Park, state of São Paulo, Brazil, 2008–2009

Tick species	Vegetation			Dogs		
	No. tested	No. infected (%)	*Rickettsia* species (no. ticks)	No. tested	No. infected (%)	*Rickettsia* species (no. ticks)
A. aureolatum	6	0 (0)	—	75	1 (1.3)	*R. bellii* (1)
A. brasiliense	35	0 (0)	—	—	—	—
A. incisum	101	3 (2.9)	*R. bellii* (3)	—	—	—
A. longirostre	1	0 (0)	—	—	—	—
A. naponense	1	0 (0)	—	1	0 (0)	—
A. nodosum	—	—	—	2	0 (0)	—
A. ovale	34	4 (11.7)	*Rickettsia* sp[a] (3), *R. bellii* (1)	59	8 (13.6)	*Rickettsia* sp[a] (8)
H. juxtakochi	25	0 (0)	—	—	—	—
R. sanguineus	—	—	—	54	1 (1.9)	*Rickettsia* sp[a] (1)

[a] *Rickettsia* sp refers to strain Atlantic rain forest reported by Spolidorio et al. (2010).

ovale Koch, and three *Amblyomma incisum* Neumann specimens through DNA sequencing of *gltA*-PCR products, which showed to be 100% identical (350/350) to corresponding sequences of *R. bellii* in GenBank (CP000087, DQ865204, EU567181). However, 8.8% (3/34) of the free-living *A. ovale* ticks, 13.6% (8/59) of the *A. ovale* ticks collected from dogs, and 1.9% (1/54) of the *Rhipicephalus sanguineus* (Latreille) ticks were found to be infected by a SFG rickettsia. Through DNA sequencing of the *ompA*-PCR products, a 463-bp fragment from each of these 12 ticks was shown to be identical to each other, and 100% identical to the corresponding sequence of *Rickettsia* sp strain Atlantic rain forest (GQ855237).

Discussion

The 13 tick species found in the current study have been previously reported in Atlantic rain forest areas of the state of São Paulo (Aragão and Fonseca 1961, Barros-Battesti and Knysak 1999, Barros-Battesti et al. 2005, Szabó et al. 2009). Adults of *A. aureolatum* and *A. ovale* were collected in relatively large amounts, both on the vegetation and on dogs during the current study. Dogs have been reported to be one of the main hosts for the adult stage of these two tick species, whereas immature stages seem to feed primarily on birds and small rodents (Guglielmone et al. 2003, Labruna et al. 2005). Interestingly, these two species were not found sympatric in the current study; i.e., while free-living *A. aureolatum* was found only in trails 1, 2, and 5 (high altitude trails; >700 m above sea level [Table 1]), free-living *A. ovale* was found only in trails 4 and 6 (low altitude trails; <100 m above sea level). In addition, no *A. aureolatum* was found on dogs in low altitude trails. In trail 5 (high altitude), where *A. aureolatum* predominated on the dogs, we found 10 dogs also infested by *A. ovale;* however, each of these 10 dogs had visited low altitude areas in the previous days, as their owners had informed us at the moment of tick collection. Although very similar in vegetation cover and vertebrate fauna, the different altitude between these trials could have provided different microclimatic conditions, resulting in this marked distribution of *A. aureolatum* and *A. ovale* in the Serra do Mar State Park.

Two different rickettsiae, *R. bellii* and the SFG agent *Rickettsia* sp strain Atlantic rain forest, were found infecting ticks in the current study (Table 3). *R. bellii* is of unknown pathogenicity, and has been previously reported infecting *A. aureolatum*, *A. incisum*, and *A. ovale* ticks from other Atlantic rain forest areas in the state of São Paulo (Pinter and Labruna 2006, Pacheco et al. 2008). However, strain Atlantic rain forest is the etiological agent of a novel SFG rickettsiosis recently reported in a human patient in the state of São Paulo, Brazil (Spolidorio et al. 2010), and is the subject of much speculation as to its taxonomic status (see Walker and Ismail 2008 and Goddard 2009 for further discussion). According to the original case-report description, infection by the strain Atlantic rain forest was acquired through the bite of an infected tick in Barra do Una, an Atlantic rain forest area of low altitude (<100 m above sea level), ≈80 Km southwest of trails 1–6 of the current study. In fact, this Barra do Una locality belongs to the Serra do Mar State Park along the Atlantic coast in the state of São Paulo, where *A. ovale* is found throughout low altitude areas (unpublished data from the Coleção Nacional de Carrapatos). Unfortunately, the tick specimen that transmitted rickettsia to the patient in Barra do Una was not saved for taxonomic identification. *A. ovale* is an important human-biting tick in Brazil (Labruna et al. 2005, Guglielmone et al. 2006). Thus, our results suggest that *A. ovale* is the main vector of *Rickettsia* sp strain Atlantic rain forest in the state of São Paulo, especially at low altitude areas among the Serra do Mar State Park, where thousands of tourists visit annually.

We also found strain Atlantic rain forest in the tick *R. sanguineus*, which is highly specific to dogs, and has been seldom reported biting humans in South America (Guglielmone et al. 2006). Because the *R. sanguineus* specimens of the current study were collected on dogs together with *A. ovale* specimens in trail 4 (data not shown), it is possible that the rickettsial infection found in this single *R. sanguineus* specimen was acquired through cofeeding with an infected *A. ovale* tick on a dog. Cofeeding transmission might also be the reason for the higher infection rate of *A. ovale* collected from dogs than from vegetation (Table 3).

Finally, intense serologic cross-reactions were shown between strain Atlantic rain forest and *Rick-*

ettsia rickettsii (Spolidorio et al. 2010). This later agent is the etiological agent of Brazilian spotted fever (BSF), an acute tick-borne spotted fever endemic in southeastern Brazil, including the state of São Paulo. Because *R. rickettsii* comprises the sole antigen employed for serological diagnosis of BSF in Brazil (Labruna 2009), it is likely that more cases of clinical infection by *Rickettsia* sp strain Atlantic rain forest have been misdiagnosed with BSF, because relatively high infection rates by strain Atlantic rain forest were found in the human-biting tick *A. ovale.*

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