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New Epidemiological Data on Brazilian Spotted Fever in an Endemic Area of the State of São Paulo, Brazil

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Abstract

The present work evaluated rickettsial infection in dogs and their ticks in an area endemic for Brazilian spotted fever (BSF) in the metropolitan area of São Paulo, Brazil, where the tick *Amblyomma aureolatum* was presumed to be the vector of the disease. Ticks were collected on dogs from 185 houses, encompassing single infestations by *Rhipicephalus sanguineus*, *Amblyomma aureolatum*, *Amblyomma longirostre*, or *Amblyomma* sp. in dogs from 60 (32.4%), 77 (41.6%), 2 (1.1%), and 25 (13.5%) houses, respectively; 19 (10.3%) houses had dogs with mixed infestations by *R. sanguineus* and *A. aureolatum*; 1 (0.5%) house had dogs with infestations by *A. aureolatum* and *A. longirostre*; and 1 (0.5%) house had dogs with infestations by *R. sanguineus* and *Amblyomma* sp. Overall, *A. aureolatum* was present in dogs from 97 (52.4%) houses, and *R. sanguineus* in dogs from 80 (43.2%) houses. A total of 287 ticks (130 *A. aureolatum* and 157 *R. sanguineus*) infesting dogs from 98 houses were selected for testing by polymerase chain reaction (PCR) targeting rickettsial genes. Overall, 3.1% of the *A. aureolatum* ticks were infected by *Rickettsia bellii*, and 1.3% of the *R. sanguineus* were infected by *Rickettsia rickettsii*. For serology, we selected 23 dogs living in and in the vicinity of the house where the *R. rickettsii*-infected ticks were collected. The indirect fluorescent antibody (IFA) test detected antibodies reactive with *R. rickettsii* in sera from 16 (69.6%) dogs, with titers ranging from 256 to 32,768. It is established that *Amblyomma aureolatum* is a vector of *R. rickettsii* in the São Paulo metropolitan area, but our results highlight for the first time in Brazil, a possible role of *R. sanguineus* in the epidemiology of *R. rickettsii*, corroborating previous findings in Mexico and the United States, where *R. sanguineus* has been implicated in the transmission of *R. rickettsii* to humans.

Key Words: Diagnostics; Epidemiology; Field studies; *Rickettsia*; Tick(s)

Introduction

BRAZILIAN SPOTTED FEVER (BSF) IS AN ACUTE disease caused by the bacterium *Rickettsia rickettsii*. It is the most important tick-borne zoonotic agent in Brazil, being reported there since 1929 (Piza et al. 1932). In the pre-antibiotic era, lethality rates of BSF were nearly 80% (Monteiro 1931). Nowadays, its lethality is still as high as 40% in the state of São Paulo, where 155 confirmed cases of BSF were reported from 1985 to 2005 (Angerami et al. 2006). The disease is endemic in several states of southeastern Brazil, where it is vectored mainly by the tick *Amblyomma cajennense*, and also by *Amblyomma aureolatum* in the metropolitan area of São Paulo City (Pinter and Labruna 2006).

The disease caused by *R. rickettsii* is also endemic in the United States (where it is called Rocky Mountain spotted fever), Mexico, Costa Rica, Panama, and Colombia (Dumler and Walker 2005). In the United States, the ticks *Dermacentor andersoni* and *Dermacentor variabilis* are the main vectors of *R. rickettsii*, although a few studies have incriminated the brown dog tick, *Rhipicephalus sanguineus*, as an important vector in some areas (Anigstein and Bader 1943, Demma et al. 2005, Wikswa et al. 2007). In Mexico, *R. sanguineus* has been considered the most important vector, although *A. cajennense* has also been implicated there (Bustamante and Varela 1947). In Panama and Colombia, *A. cajennense* is the only tick species implicated as vector (Patino-Camargo 1941, Rodaniche 1953). In Costa Rica, the vector is still unknown,

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because the only report of *R. rickettsii* infecting ticks there was in the rabbit tick, *Haemaphysalis leporispalustris*, which is not a human-biting tick (Fuentes et al. 1985). These data indicate how complex the ecology of *R. rickettsii* can be, with the participation of different tick species in a wide variety of geographical areas.

Amblyomma aureolatum has been found to be restricted to the Neotropical region, covering the eastern area of South America, from Uruguay to Surinam, including northeastern Argentina, eastern Paraguay, southeastern Brazil, and French Guiana (Guglielmone et al. 2003a). Notably, cases of BSF transmitted by *A. aureolatum* have been reported solely in the metropolitan area of São Paulo City (Fonseca 1935, Pinter and Labruna 2006). This tick species is typical of the Atlantic rain forest, where optimal conditions of high humidity and cool temperatures are provided throughout the year (Pinter et al. 2004). Main hosts for *A. aureolatum* are canids (including domestic dogs) for adult ticks, and a few rodent and bird species for the subadult tick stages (Guglielmone et al. 2003a). Only the adult stage is known to bite humans (Guglielmone et al. 2003a), and it is the tick stage implicated in the transmission of BSF (Pinter et al. 2004).

The brown dog tick, *R. sanguineus*, has a cosmopolitan distribution and has been reported in almost all countries of the American continent (Guglielmone et al. 2003b). In Brazil, it is distributed in all regions, with large populations in most of the urban areas. It is a typical nidicolous tick that has adapted to living indoors. In contrast to *A. aureolatum*, *R. sanguineus* is not found in forest areas. Despite the close proximity of *R. sanguineus* to humans (because it colonizes homes), the tick is seldom reported biting humans. In fact, in Brazil, there have been only two reports of *R. sanguineus* biting humans (Dantas-Torres et al. 2006, Louly et al. 2006). While *R. sanguineus* is a known vector of *R. rickettsii* in Mexico and United States, its role in the ecology of *R. rickettsii* has never been highlighted in South America.

The present work evaluated the rickettsial infection in ticks and dogs in a BSF-endemic area in metropolitan São Paulo, where *A. aureolatum* is thought to be the main vector of the disease.

Materials and Methods

This study was performed in the “Recreio da Borda do Campo” community, which occupies an area of 3.52 km² in the Santo André Municipality, within the São Paulo Metropolitan area (23°44'07" S, 46°28'36" W). The community was formed about 40 years ago as a result of irregular occupation in an Atlantic rain forest reserve. For this reason, the community is surrounded by dense forest on three sides, and by the Billings Dam to the southeast (Fig. 1). Approximately 6,700 people live in the community, mostly under poor sanitary conditions. Almost all families have domestic dogs, mostly reared unrestrained with free access to outdoors and to the neighboring forests. At the time of our study the estimated canine population in the community was 1,732 dogs (unpublished data from the Health Secretary of Santo André Municipality). During the years 2005 and 2006, there were five laboratory-confirmed human cases of BSF in the study area (Fig. 1) (data available from the São Paulo Health Secretary website: <http://www.cve.saude.sp.gov.br>). The pres-

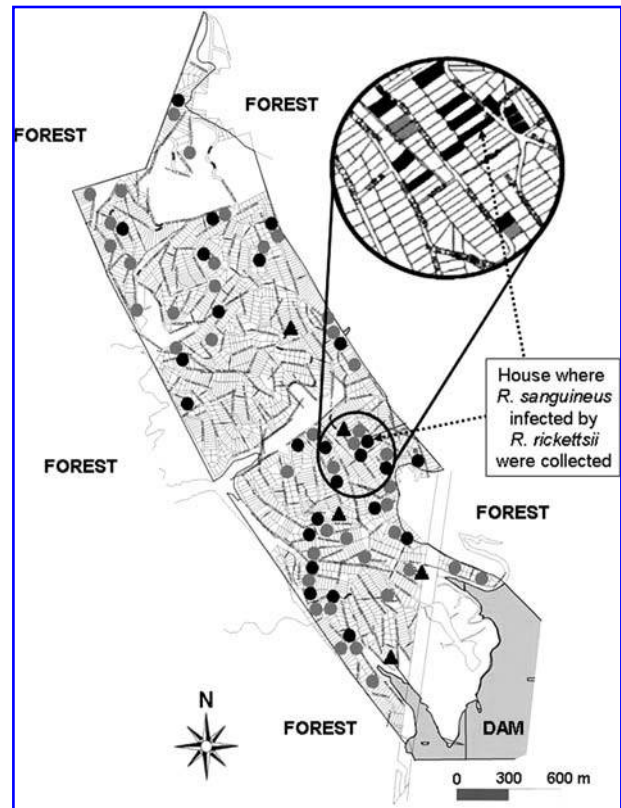


FIG. 1. Diagram of residential lots of the “Recreio da Borda do Campo” community, where the present study was performed. Black circles indicate the streets containing houses with dogs infested by *Amblyomma aureolatum*; gray circles indicate the streets containing houses with dogs infested by *Rhipicephalus sanguineus*; black triangles indicate the houses of the persons affected by Brazilian spotted fever during the years 2005–2006. Inset circle illustrates the 16 houses where dogs were tested for serology: 13 black rectangles represent the houses where at least one *Rickettsia rickettsii*-reactive dog serum was collected (it includes the house where *R. rickettsii*-infected *R. sanguineus* ticks were collected), whereas gray rectangles represent three houses where all dog sera were nonreactive for *R. rickettsii*.

ent study was divided into two phases, one dealing with ticks collected from dogs, and another focused on canine blood samples.

Ticks

From September to November 2005, a total of 481 ticks were collected from dogs living in 185 houses in the Recreio da Borda do Campo community. Tick collection was performed by workers from the Health Secretary of Santo André Municipality, and sent to our laboratory with no additional data but the address of the house. For this reason, we do not have data about the total number of dogs sampled per house. Although it can be said that at least 185 dogs (minimum of one per house) were sampled, this number was certainly higher, because houses usually had two or more dogs. Ticks were sent in dry glass tubes, and some of them arrived dead in the laboratory. All ticks were taxonomically identified and then 287 live ticks were selected for testing by poly-

merase chain reaction (PCR) for rickettsial DNA. Ticks were subjected to DNA extraction by the guanidine isothiocyanate-phenol technique as described elsewhere (Sangioni et al. 2005). For this purpose, 80 ticks were processed individually, and the other 207 ticks were processed in 69 pools, each containing 3 ticks. Care was taken to include ticks from the same house in each pool. DNA samples from all ticks was tested by PCR using the primers CS-78 (forward) and CS-323 (reverse), which amplify a 401-bp fragment of the citrate synthase (*gltA*) rickettsial gene of possibly all *Rickettsia* species (Labruna et al. 2004). If DNA from an individual tick or from a tick pool generated an expected amplicon in this PCR, it was further tested by a battery of PCRs with the following primer pairs: primers CS-239 and CS-1069, which amplify a 834-bp fragment of the *gltA* gene (Labruna et al. 2004); primers 17k-5 and 17k-3, which amplify a 549-bp fragment of the 17-kDa protein gene (*htrA*) (Labruna et al. 2004); primers Rr 190-70p and Rr 190-620n, which amplify a 530-bp fragment of the 190-kDa outer membrane protein gene (*ompA*) (Regnery et al. 1991); and primers 120-M59 and 120-807, which amplify a 856-bp fragment of the 135-kDa outer membrane protein gene (*ompB*) (Roux and Raoult 2000). Reactions were performed as described elsewhere (Labruna et al. 2004). In each set of reactions, three negative controls (water) and a positive control (DNA extracted from *A. cajennense* ticks experimentally infected with *R. parkeri*, using the same DNA extraction protocol used in the present study [Sangioni et al. 2005]) were included. All PCR products of the expected size obtained were purified with ExoSap (USB) and sequenced in an automatic sequencer (Applied Biosystems/PerkinElmer, model ABI Prism 310 Genetic, Foster City, CA) according to the manufacturer's protocol. Partial sequences obtained were submitted to BLAST analysis (Altschul et al. 1990) to determine the closest similarities to other *Rickettsia* species.

If a rickettsial genotype was found in a tick species not previously reported in Brazil, the tick DNA was tested by PCR targeting the 16S rRNA gene of the tick, as previously described (Mangold et al. 1998). The PCR product was processed to DNA sequencing as described above, and the resulting sequence was submitted to BLAST analysis in order to certify the taxonomic identification of the tick.

Blood serum samples

Blood samples were collected from 23 dogs from 16 houses on 14 November 2006 (approximately 1 year after the collection of ticks). Dogs selected for blood collection were those living in the vicinity of a house where *R. rickettsii*-infected ticks had been detected (as shown below), as well as the dogs living in that house. Blood samples were identified according to the dog name and house address, and they were transported at room temperature to the laboratory, where samples were centrifuged (1,500 g, 10 min), and the sera were aliquoted into labeled microtubes and stored at -20°C until tested by indirect immunofluorescence assay (IFA) using 12-well slides containing crude antigens derived from *R. rickettsii* strain Taiacu, as described elsewhere (Labruna et al. 2007). Canine sera were diluted in twofold increments with PBS starting from a 1:64 dilution. For each sample, the endpoint titer reacting with *R. rickettsii* antigen was determined. Slides were read in an ultraviolet microscope (Olympus

BX60, Japan) at $400\times$ magnification. In each slide, a serum previously shown to be nonreactive (negative control) and a known reactive serum (positive control) were tested.

Results

From the 481 ticks collected on dogs, 454 were identified into three species: *R. sanguineus*, *A. aureolatum*, and *Amblyomma longirostre* (Table 1). The remaining 27 ticks were identified to the generic level as *Amblyomma* sp. nymphs due to the absence of specific taxonomic literature about *Amblyomma* nymphs from Brazil. Ticks were collected from dogs from 185 houses, encompassing single infestations by *R. sanguineus*, *A. aureolatum*, *A. longirostre*, or *Amblyomma* sp. in 60 (32.4%), 77 (41.6%), 2 (1.1%), and 25 (13.5%) houses, respectively; 19 (10.3%) houses had dogs with mixed infestations by *R. sanguineus* and *A. aureolatum*; 1 (0.5%) house had dogs with infestation by *A. aureolatum* and *A. longirostre*; and 1 (0.5%) house had dogs with infestation by *R. sanguineus* and *Amblyomma* sp. Overall, *A. aureolatum* was present in dogs from 97 (52.4%) houses, whereas *R. sanguineus* was found in dogs from 80 (43.2%) houses.

A total of 287 ticks (130 *A. aureolatum* and 157 *R. sanguineus*) collected from dogs from 98 houses distributed in 60 different streets were selected for testing by PCR. For each species, 40 ticks were tested individually, and the remaining were tested in pools of 3 ticks each, for a total of 30 and 39 pools for *A. aureolatum* and *R. sanguineus*, respectively. The streets of the houses where the *A. aureolatum* and *R. sanguineus* ticks tested by PCR were collected are shown in Figure 1.

Among the *A. aureolatum*, 3 individual adult male ticks (from 3 different houses) and a single tick pool of 3 males generated the expected PCR products with the initial *gltA* primers. After DNA sequencing, these products were all identical to the corresponding sequence of *Rickettsia bellii* available in GenBank (U59716). Because these ticks were negative in the PCR targeting fragments of the rickettsial genes *ompA* and *ompB*, no further tests were performed on them. Considering that at least one *R. bellii*-infected tick was present in the single PCR-positive tick pool, the overall frequency of *R. bellii*-infected *A. aureolatum* ticks was 4/130 (3.1%) (Table 1).

Among *R. sanguineus*, 2 individual adult ticks (1 male, 1 female) from the same house generated the expected PCR products with the initial *gltA* primers. These same individual ticks generated PCR products with all the other primer pairs. All PCR products were sequenced. Generated sequences were identical for the two ticks considering each gene, and after BLAST analysis they gave the following results: a 1,075-bp fragment of the *gltA* gene 100% identical to the corresponding sequence of *R. rickettsii* (DQ115890); a 497-bp fragment of the *htrA* gene 100% identical to the corresponding sequence of *R. rickettsii* (AY281069); a 439-bp fragment of the *ompA* gene 100% identical to the corresponding sequence of *R. rickettsii* (DQ002504); a 767-bp fragment of the *ompB* gene 100% identical to the corresponding sequence of *R. rickettsii* (X16353). The overall frequency of *R. rickettsii*-infected *R. sanguineus* ticks was 2/157 (1.3%) (Table 1).

To confirm the taxonomic identification of the two *R. sanguineus* ticks that generated *R. rickettsii* PCR products, DNA from each of those ticks was subjected to PCR targeting a

TABLE 1. TICKS COLLECTED FROM DOGS LIVING IN 185 HOUSES IN THE RECREIO DA BORDA DO CAMPO COMMUNITY, SANTO ANDRÉ MUNICIPALITY, STATE OF SÃO PAULO, BRAZIL

Tick species	No. of ticks collected			No. of houses containing dogs infested by each of the tick species (%)	No. of ticks infected by <i>Rickettsia</i> /No. of ticks tested (%)
	Males	Females	Nymphs		
<i>Rhipicephalus sanguineus</i>	139	171	6	80 (43.2)	2/157 (1.3) ^a
<i>Amblyomma aureolatum</i>	56	79	—	97 (52.4)	4/130 (3.1) ^b
<i>Amblyomma longirostre</i>	1	2	—	3 (1.6)	Not tested
<i>Amblyomma</i> sp.	—	—	27	26 (14.0)	Not tested

^aSome ticks were tested for rickettsial infection by polymerase chain reaction (PCR). DNA sequencing of PCR products showed that ticks were infected by *Rickettsia rickettsii*.

^bSome ticks were tested for rickettsial infection by polymerase chain reaction (PCR) DNA sequencing of PCR products showed that ticks were infected by *Rickettsia bellii*.

fragment of tick 16S rRNA mitochondrial gene. The PCR products, after DNA sequencing, were identical to each other and 100% (393/393) identical to the corresponding sequence of *R. sanguineus* in GenBank (DQ016293).

For serology, we selected 23 dogs living in and in the vicinity of the house where the 2 *R. rickettsii*-infected ticks were collected. This sample encompassed dogs from 16 houses. During blood collection, infestations by both *A. aureolatum* and *R. sanguineus* ticks were observed on the dogs, but no tick was collected at this time. The IFA test detected antibodies reactive with *R. rickettsii* in sera from 16 (69.6%) dogs living in 13 houses (Fig. 1). Seropositive dogs had titers varying from 256 to 32,768 (Table 2). Three dogs that lived in the house where the two *R. rickettsii*-infected *R. sanguineus* had been collected were included in this serosurvey. Two of them had titers of 8,192 and 32,768, respectively, and the third one was seronegative.

Discussion

The present study in a BSF-endemic area of the São Paulo metropolitan area reported *A. aureolatum* infesting 52.4% of the dogs studied. At the same time, 43.2% of those dogs were infested by *R. sanguineus*. These two tick species were widespread in the community, as indicated by their presence in dogs from numerous streets (Fig. 1). Both *A. aureolatum* and *R. sanguineus* use dogs as their primary host, but in two distinct situations. All parasitic stages of *R. sanguineus* feed on dogs, and the free-living life stages are found mostly in walls, fences, and ceilings of the houses themselves and other constructions surrounding the houses (Labruna and Pereira 2001). On the other hand, only the adult stage of *A. aureolatum* feeds on dogs (larvae and nymphs feed on birds and small rodents), and the free-living stages are found mostly inside humid forest areas, such as the Atlantic rain forest

(Guglielmo et al. 2003a, Pinter et al. 2004). Based on these statements, we infer that in the area of the present study, dogs became infested by *R. sanguineus* while resting in and near the houses, and/or by *A. aureolatum* while visiting the neighboring forest.

The classic transmission cycle of BSF found in most of the BSF-endemic areas in southeastern Brazil involves the participation of the tick *A. cajennense*, and some of its main hosts such as horses and/or capybaras (*Hydrochoerus hydrochaeris*) (Lemos et al. 1997, Horta et al. 2004, Guedes et al. 2005, Sangioni et al. 2005). In addition, however, a distinct transmission cycle involving *A. aureolatum* and dogs has been suggested for the metropolitan area of São Paulo since the 1930s (Gomes 1933, Fonseca 1935, Pinter et al. 2004, Pinter and Labruna 2006). The conditions found in the present study are compatible with this known *A. aureolatum* transmission cycle. In fact, during our field work, two *A. aureolatum* adult ticks were found biting humans (data not shown), suggesting that this tick has been the vector of *R. rickettsii* in the study area. However, we also found the tick *R. sanguineus* nearly as common as *A. aureolatum* in the study area. Previous studies have reported *R. sanguineus* to be common in other BSF-endemic areas where *A. cajennense* has been implicated to be the vector (Lemos et al. 1997, Cardoso et al. 2006).

After testing ticks for rickettsial infection, we found *R. bellii* infecting 3.1% of the 130 *A. aureolatum* ticks and *R. rickettsii* infecting 1.3% of the 157 *R. sanguineus* ticks. In a previous study in Mogi das Cruzes (another BSF-endemic area in the São Paulo Metropolitan area), Pinter and Labruna (2006) reported that 6 (0.9%) and 10 (1.5%) of 669 *A. aureolatum* ticks were infected by *R. rickettsii* and *R. bellii*, respectively. Based on their results and our own, because we worked with a much smaller sample size, we cannot discard the possibility that the *A. aureolatum* population in the study area was infected by *R. rickettsii*. Nevertheless, we found 2

TABLE 2. ENDPOINT TITERS OF INDIRECT IMMUNOFLUORESCENCE ASSAY (IFA) FOR RICKETTSIA RICKETTSII ANTIGEN IN 23 DOGS FROM THE "RECREIO DA BORDA DO CAMPO" COMMUNITY, SANTO ANDRÉ MUNICIPALITY, STATE OF SÃO PAULO, BRAZIL

Number of dogs according to IFA titer									
Titer	<64 ^a	256	512	1,024	2,048	4,096	8,192	32,768	Total
No. dogs	7	1	1	1	1	1	6	5	23

Dogs living in a specific house where *R. rickettsii*-infected ticks were detected (Fig. 1) were sampled, as were dogs living in the vicinity of that house.

^aConsidered to be nonreactive.

R. rickettsii-infected *R. sanguineus* ticks collected from a single house. Previous studies in Brazil and the United States have shown that *R. sanguineus* is a competent vector of *R. rickettsii* under laboratory conditions (Parker et al. 1933, Regendans and Muniz 1936). However, a study in the United States demonstrated that *R. rickettsii*-infected dogs were not able to infect as much as 1% of the *R. sanguineus* ticks that fed on them during the rickettsemic period (Normant and Burgdorfer 1984). In contrast, an ongoing study in our laboratory has shown that at least 20% of *R. sanguineus* nymphs became infected by *R. rickettsii* after fed as larvae on experimentally infected dogs, and were capable of transmitting *R. rickettsii* to susceptible guinea pigs (Piranda et al. 2006). These results suggest that *R. sanguineus* could also play a role in the epidemiology of *R. rickettsii* in the study area, with the potential to act as a vector to humans, as reported in Mexico and the United States (Bustamante and Varela 1947, Demma et al. 2005). In addition, it is possible that dogs could play a significant role in the ecology of *R. rickettsii*, serving as the infection source for ticks.

The *R. rickettsii* sequences obtained from the two *R. sanguineus* individuals in the present study were 100% identical to the corresponding sequences of *R. rickettsii* strain Taiaçu, previously isolated from *A. aureolatum* in Mogi das Cruzes (Pinter and Labruna 2006). Interestingly, *R. rickettsii* strains from South America have been shown to differ from most strains in the United States by an indel of the codon CCG within the *gltA* gene (Eremeeva et al. 2003, Guedes et al. 2005, Pinter and Labruna 2006). Other gene partial sequences (*htrA*, *ompA*, *ompB*) have been shown to be identical between strains from North and South America, although only relatively small fragments of *ompA* and *ompB* genes from South American strains have been sequenced (Guedes et al. 2005, Pinter and Labruna 2006).

In the second phase of the present study, canine blood was collected for serosurvey in November 2006, nearly 1 year after the *R. rickettsii*-infected ticks were collected. Because very high titers to *R. rickettsii* were found on dogs in 2006, it is possible that the agent remained circulating in the area during this 1-year interval, indicating endemicity. This possibility is supported by the occurrence of BSF cases among humans from the area during both 2005 and 2006.

The tick *R. sanguineus* originated in the Old World and was introduced into the Americas, possibly during European colonization (Guglielmone et al. 2003b). At present, it is widespread in Brazil, especially in urban areas, which have numerous dogs and houses, the main conditions necessary for its establishment (Labruna and Pereira 2001). It is not known if the presence of dogs and *R. sanguineus* alone is sufficient for maintenance of *R. rickettsii* in a given area. The BSF-endemic area of the present study was surrounded by forest, which provides conditions for infestations by *A. aureolatum* on dogs, and occasionally on humans. Certainly, the forest harbors a variety of other animal species (i.e., birds and small rodents) that act as hosts for the immature stages of *A. aureolatum* and a variety of other tick species (e.g., *A. longirostre* and *Amblyomma* sp, found in the present study). However, nothing is known about the role of these animals in the ecology of BSF. For this reason, the results of the present study cannot be extrapolated to other places within the large distribution area of *R. sanguineus* throughout Brazil and other American countries. In contrast to the huge distribu-

tion area of *R. sanguineus* in Brazil, there are only a few known areas of BSF occurrence. Similar conditions are found for *A. aureolatum* and *A. cajennense*, which have large distributions in Brazil but for which there are only few areas where they are implicated in transmitting *R. rickettsii* to humans. These facts reinforce the conclusion that defining the ecology of *R. rickettsii* is a highly complex issue, with much yet to be explored.

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