Journal of Medical Entomology, 53(6), 2016, 1458–1466
doi: 10.1093/jme/tjw121
Advance Access Publication Date: 1 August 2016
Research article

Rickettsia (Rickettsiales: Rickettsiaceae) Vector Biodiversity in High Altitude Atlantic Forest Fragments Within a Semiarid Climate: A New Endemic Area of Spotted-Fever in Brazil

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Received 29 December 2015; Accepted 8 June 2016

Abstract

Rickettsioses are re-emerging vector-borne zoonoses with a global distribution. Recently, *Rickettsia* sp. strain Atlantic rainforest has been associated with new human spotted-fever (SF) cases in Brazil, featuring particular clinical signs: eschar formation and lymphadenopathy. These cases have been associated with the tick species, *Amblyomma ovale*. From 2010 until 2015, the Brazilian Health Department confirmed 11 human SF cases in the Maciço de Baturité region, Ceará, Brazil. The present study reports the circulation of *Rickettsia* spp. in vectors from this entirely new endemic area for SF. A total of 1,727 ectoparasites were collected in this area from the environment, humans, and wild and domestic animals. Samples ($n = 887$) were screened by polymerase chain reaction (PCR), targeting the *glt*A and *omp*A rickettsial genes. Sequencing and phylogenetic analyses of *glt*A gene amplicons were carried out for 13 samples positive for both screening PCRs. Fragments of *glt*A and *omp*A from three samples were cloned, sequenced, and analyzed further. *A. ovale* and *Rhipicephalus sanguineus* specimens, collected from dogs, were found to be infected with *Rickettsia* sp. str. Atlantic rainforest, suggesting the importance of dogs in the epidemic cycle. *Candidatus* Rickettsia andeanae, *Rickettsia felis*, and *Rickettsia bellii* were also found infecting ticks and fleas in five municipalities, demonstrating the broad diversity of rickettsiae in circulation in the studied area. This study reports, for the first time, evidence of infection with *Rickettsia* sp. strain Atlantic rainforest in *A. ovale* and *R. sanguineus* in Ceará, and *Ca.* R. andeanae in an Atlantic rainforest environment of Brazil.

Key words: spotted-fever, rickettsiae, vector-borne, disease, tick

Rickettsioses are re-emerging vector-borne zoonoses caused by Gram negative, obligate intracellular α-proteobacteria of the genus *Rickettsia*, which have a worldwide distribution (Parola et al. 2013). Different spotted-fever group (SFG) rickettsiae can coexist in the same area, involving the infection of multiple vectors, which infest various mammals, and they may, or may not, share epidemiological features (Parola et al. 2013, Szabó et al. 2013b).

Ticks can be naturally infected with *Rickettsia* sp. in different parts of the world (Parola et al. 2013). Thus, in a particular area, the presence of rickettsiae is related to the availability of tick species susceptible to infection, and vertebrate species able to sustain the tick population, factors which may vary over time and in space. In addition to this dynamic, different human activities, and their relationships with seasonal tick activity, may influence the epidemic manifestations of rickettsial diseases (Labruna et al. 2011, Szabó et al. 2013a, b).

Recent investigations have led to a better understanding of the rickettsiae epidemic and enzootic cycles, thus improving prevention and control measures. *Rickettsia rickettsii*, the most important causative agent of rickettsiosis in America, is no longer the only pathogenic species of rickettsiae reported on this continent and, in Brazil, other SFG rickettsiae species have also been reported (e.g. Labruna et al. 2011; Szabó et al. 2013a, b).

In 2010, the first human spotted-fever (SF) case was confirmed in the state of Ceará, Brazil. Until 2015, there were a total of 11 confirmed human SF cases in Ceará (Brazilian Health Department, 2016 Jul. -http://tabnet.datasus.gov.br/cgi/tabcgi.exe?sinannet/cnv/febremaculosabr.def). Recent cases were associated with *Rickettsia* sp. strain Atlantic rainforest and particular clinical symptoms, including eschar formation and lymphadenopathy (Spolidorio et al. 2010, Silva et al. 2011). In all of these cases, the foci are in the same region, Maciço de Baturité. This region features particular characteristics, including a series of areas of forest, which resemble the Atlantic rainforest, within the Caatinga biome. Thus, this region is an exceptional area in a region of semiarid climate, as its high-altitude forest, with high humidity and mild temperature conditions, differs from neighboring areas (Tabarelli and Santos 2004, Andrade-Lima 2007, Santos et al. 2012). Rohde et al. 2014 observed that the high abundance of *Drosophila* in high-altitude forest areas can be a reflection of their historical development. The authors based their observation on studies indicating the occurrence of climatic variations in the Pleistocene. These variations allowed the coastal Atlantic forest to penetrate into the Caatinga domain. Subsequently, the coastal forest returned to its place of origin, and islands of the Atlantic forest remained in places with favorable microclimates, creating high-altitude forest refuges for the Atlantic forest species in northeastern Brazil, within the Caatinga Biome.

The Caatinga biome extends over ~10% of Brazilian territory, including all states of the northeastern region and the northern part of Minas Gerais State, which is in the southeastern region of the biome (Castelletti et al. 2003). Although the Caatinga biome does not present favorable conditions for the surival of potential rickettsiae vectors, high-altitude forests occur throughout this biome at different sites in the Brazilian northeastern region, where very little is known about rickettsiae cycles and SF foci generally border forest areas and are proximal to rural and periurban environments. Hence, it is possible that there may be many additional silent SF foci in this biome.

Therefore, this study aimed to determine the circulation of *Rickettsia* spp. in potential vectors collected from an entirely new endemic area of SF, Maciço de Baturité, Ceará, Brazil.

Materials and Methods

From April 2011 to March 2013, potential rickettsiae vectors were collected from animals, humans, and the environment in different municipalities in Maciço de Baturité, Ceará, Brazil (Fig. 1). The map in Fig. 1 was produced using Quantum Geographic Information System software (Nanni et al. 2012).

Field campaigns were developed during surveillance and investigation of human SF cases by the 4th Regional Health Coordinating Agency of Ceará State Health Department, following the established flow-chart implemented by the National Network of Environment Monitoring of Spotted-Fever and Other Rickettsial Diseases, Brazilian Health Department.

Ticks were morphologically identified according to Amorim and Serra-Freire (1999) for larvae stages; Martins et al. (2010) for nymph stages; and Aragão and Fonseca (1961) and Barros-Battesti et al. (2006) for adult stages. Fleas, lice, and mites were taxonomically identified following Linardi and Guimarães (2000), Price et al. (2003), and Furman (1972), respectively. The specimens were

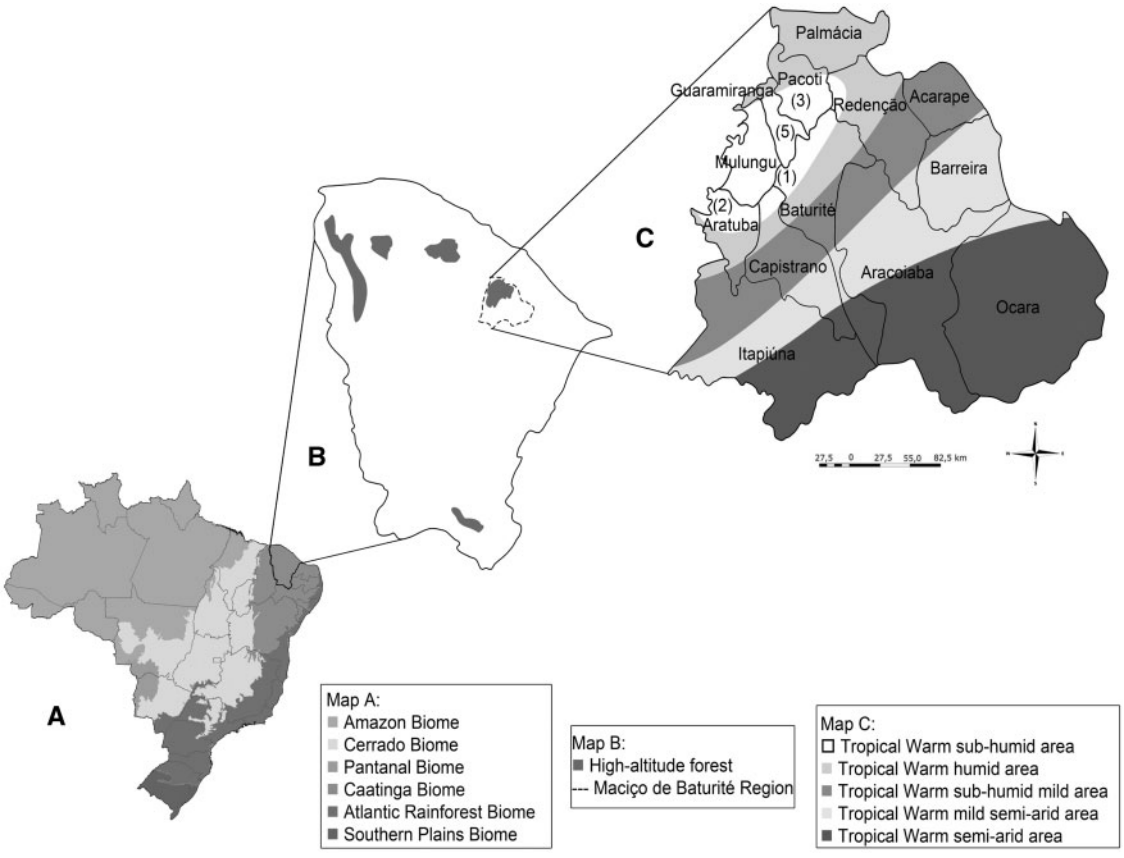

Fig. 1. (A) Map of Brazil highlighting Ceará State within the Caatinga Biome; (B) Map of Ceará State, showing high-altitude forest fragments within the Maciço de Baturité region; (C) Map of Maciço de Baturité region, showing its climatic types with all municipalities and their respective numbers of SF cases (*n*).

arranged individually or in pools for total DNA extraction, as previously described (Aljanabi and Martinez 1997).

Tick samples were initially processed by routine polymerase chain reaction (PCR), targeting two rickettsial genes; the initial portion of the *omp*A gene, using primers Rr190.70p (ATGGCGA ATATTTCTCCAAAA) and Rr190.602n (AGTGCAGCATTCGC TCCCCCT) (Regnery et al. 1991); and the CS4 portion of the *glt*A gene, using primers CS-239 (GCTCTTCTCATCCTATGGCTA TTAT) and CS-1069 (CAGGGTCTTCGTGCATTTCTT) (Labruna et al. 2004b). *Rickettsia rickettsii* DNA (Pinter and Labruna 2006) was used as positive control and DNA-free Mili-Q water was used as a negative control. Samples that yielded a fragment of the expected size for the relevant gene, as determined by agarose gel electrophoresis, were considered positive.

The CS4 region was sequenced in samples positive for both screening PCRs using the initial amplification primers (purification and sequencing protocols are described below). It was only possible to clone and sequence the CS2 and CS4 regions (*glt*A gene) and *omp*A gene PCR products of three samples (LIC4316, LIC4317, and LIC4328). The CS2 region was amplified with primers CS-78 (GCAAGTATCGGTGAGGATGTAAT) and CS-323 (GCTTCCTT AAAATTCAATAAATCAGGAT) (Labruna et al. 2004b). Primers 120-M59 and 120-807 were used to amplify the *omp*B gene (Roux and Raoult, 2000).

Positive PCR products were cloned with a TOPO TA Cloning kit with the pCR4-TOPO TA Vector, according to the manufacturer's specifications. The resulting clones were sequenced with M13 primers.

DNA fragments used in sequencing reactions were purified using a HiYield Gel/PCR DNA Mini Kit (Real Genomics), according to the manufacturer's instructions, and sequenced in both directions on an automated ABI 3130xl genetic analyzer (Applied Biosystems) with the same primers used in PCR reactions.

Sequencing analysis was performed with Lasergene software packages (DNASTAR, Madison, WI) and multiple alignment analysis and neighbor-joining analysis were performed using MEGA 5.2 (Tamura et al. 2011). Kimura's two-parameter evolution model was used for phylogenetic analysis (Kimura, 1980). Bootstrap values for the trees were obtained from 1000 randomly generated trees. All sequences generated in this study are deposited in GenBank (accession numbers, KT153031–KT153046 and KX 130669).

Specimens not tested by PCR were deposited at the Coleção de Artrópodes Vetores Ápteros de Importância em Saúde das Comunidades (CAVAISC/FIOCRUZ), for further analysis.

Results

From 1,727 potential rickettsiae vectors collected (Table 1), 887, comprising 17 tick, flea, lice, and mite species, were tested by PCR, revealing 13 samples positive for both targeted rickettsial genes.

Based on analysis of the CS4 region of the *glt*A gene, 46% (6/13) of all samples were positive for a *Rickettsia* sp., with 100% (608/608) similarity with the *R. sibirica* 246 (NZ_AABW01000001) and *Rickettsia* sp. strain Atlantic rainforest (GQ855235) sequences, and 99% (607/608) similarity with the *Rickettsia parkeri* str. Portsmouth sequence (CP003341). This strain was found infecting *Amblyomma ovale* ($n = 5$) and *Rhipicephalus sanguineus* ($n = 1$) samples collected from dogs, in the municipalities of Aratuba (LIC4299, LIC4316, LIC4317, LIC4323) and Mulungu (LIC4275, LIC4276) (Fig. 2).

Candidatus Rickettsia andeanae was found infecting one *Amblyomma parvum* tick sample (LIC4328) in Redenção, and its CS4 sequence showed a 100% (608/608) similarity with that of *Ca.* R. andeanae str. T124 (GU169050.1) (Fig. 2).

Three samples from the *Ctenocephalides felis* flea species (LIS428, LIS429, LIS436) were collected in the municipality of Mulungu and one (LIS437) was collected in Aratuba. All were positive for a rickettsial agent, and their CS4 fragment sequences showed 100% (608/608) similarity with that of *Rickettsia felis* URRWXCal2 (CP000053) (Fig. 2).

One *A. ovale* sample (LIC4315) collected in Guaramiranga and one *Amblyomma nodosum* sample (LIC4327) collected in Baturité were positive for rickettsial agents. Their CS4 fragment sequences showed 100% similarity with that of *Rickettsia bellii* (KT153036) (608/608) and *R. bellii* strain Pontal (RML369-C) (607/607), respectively (Fig. 2).

In order to facilitate phylogenetic analyses, concatenated sequences (~1,500 bp) consisting of the CS2 and CS4 regions of *glt*A, and the *omp*A gene, were constructed for samples LIC4316, LIC4317, and LIC4328. The phylogenetic tree resulting from analysis including these sequences showed that the bacterium first classified as *Rickettsia* sp. was more closely related to *Rickettsia* sp. str. Atlantic rainforest than to *R. parkeri* (Fig. 3); however, the bootstrap value of this node was below 70 and it is, therefore, not included in the figure.

BlastN analysis of the *omp*A sequences from LIC4316 and LIC4317 demonstrated a 100% (491/491) similarity with *Rickettsia* sp. str. Aa46 (KJ855083), a strain of *Rickettsia* sp str. Atlantic rainforest, and 98% (483/491) similarity with *R. parkeri* str. Portsmouth (CP003341). The *omp*A sequence of LIC4328 showed a 99% (462/467) similarity with *Rickettsia* sp. 'Argentina' (EF451004).

The *omp*B gene could only be amplified from sample LIC4316 and phylogenetic analysis using this sequence showed the best resolution for the specific node between *Rickettsia* sp. str. Atlantic rainforest and *R. parkeri* (Fig. 4). BlastN analysis using the *omp*B gene of sample LIC4316 revealed 100% (770/770) similarity with *Rickettsia* sp. str. Aa46 (KJ855086), and 99% (759/770) similarity with *R. parkeri* str. Portsmouth (CP003341).

Discussion

SF is a disease that has been recognized in Brazil since the 1920s; however, despite the knowledge accumulated since then, the characterization of its enzootic and epidemic cycles in different ecological and epidemiological conditions remains at an early stage. The majority of research efforts have focussed on the southeastern region of the country, where the majority of deaths and a larger number of reported cases occur. *Amblyomma sculptum* (= *Amblyomma cajennense*, Nava et al. 2014) is the most important tick species in the SF epidemic cycle and *A. aureolatum* appears to be almost as important, as both species transmit *R. rickettsii*, the most lethal SF agent (Sabatini et al. 2010, Medeiros et al. 2011, Szabó et al. 2013b, Barbieri et al. 2014, Moura-Martiniano et al. 2014). However, both of these tick species were identified at a very low frequency in our research and were not infected by *Rickettsia* (Table 1). This result is likely to be attributable to the eco-epidemiological conditions of the region studied, which has extensive preserved forest areas and an economy based on small farm agricultural production. Therefore, our results support the suggestion of Szabó et al. (2009) that the distribution of *A. cajennense* increases in direct correlation with the

Table 1. Absolute frequency of collected vectors (*N*) and absolute frequency of samples tested by PCR (including pools) (*n*) of vectors collected in the high-altitude forest, Ceará, Brazil

VECTOR/HOST	Environment		*Canis familiaris*		*Felis catus*		*Bos taurus*		*Equus caballus*		*Equus asinus*		*Homo sapiens*	
			$N = 188$	72.31%	$N = 3$	1.15%	$N = 28$	10.77%	$N = 6$	2.31%	$N = 18$	6.92%	$N = 4$	1.55%
Ixodida	***N***	***n***	***N***	***n***	***N***	***n***	***N***	***n***	***N***	***n***	***N***	***n***	***N***	***n***
Amblyomma aureolatum	0	0	1[h]	1[h]	0	0	0	0	0	0	0	0	0	0
Amblyomma longirostre	1[b]	1[b]	0	0	0	0	0	0	0	0	0	0	0	0
Amblyomma nodosum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amblyomma ovale	0	0	170[b–e,g-i]	109[b–e,g–i]	0	0	2[b]	2[b]	0	0	0	0	2[b,e]	2[b,e]
Amblyomma parvum	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Amblyomma sp.	68[b]	68[b]	0	0	0	0	0	0	0	0	4[e]	4[e]	0	0
Amblyomma sculptum	0	0	3[b,d]	3[b,d]	0	0	0	0	0	0	0	0	0	0
Amblyomma tigrinum	0	0	0	0	0	0	0	0	0	0	0	0	1[i]	1[i]
Dermacentor nitens	0	0	78[a,b,e,f]	29[a,b,e,f]	0	0	15[b,c]	6[b,c]	58[f,g,i]	22[f,g,i]	283[a,b,d,e,g–i]	133[a,b,d,e,g–i]	0	0
Rhipicephalus microplus	0	0	6[b,g]	6[b,g]	0	0	298[a–e,g,i]	84[a–e,g,i]	42[b,i]	14[b,i]	0	0	0	0
Rhipicephalus sanguineus	2[b]	1[b]	490[a–d,f–i]	280[a–d,f–i]	0	0	16[b,e,f]	7[b,e,f]	0	0	2[d]	2[d]	1[c]	1[c]
Siphonaptera														
Ctenocephalides felis	5[f]	5[f]	59[a,b,e,g,h]	51[a,b,e,g,h]	4[b]	4[b]	0	0	0	0	0	0	0	0
Polygenes bohlsi bohlsi	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Pulex irritans	0	0	1[b]	0	0	0	0	0	0	0	0	0	0	0
Xenopsylla cheopis	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Phtiraptera														
Heterodoxus spiniger	0	0	5[b]	3[b]	0	0	0	0	0	0	0	0	0	0
Mesostigmata														
Gigantolaelaps tiptoni	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Sub-total	76[b,f]	75[b,f]	813[a–i]	481[a–i]	4[b]	4[b]	331[a–g,i]	99[a–g,i]	100[b,f,g,i]	36[b,f,g,i]	289[a,b,d,e,g–i]	139[a,b,d,e,g–i]	4[b,c,e,i]	4[b,c,e,i]

	Myrmecophagidae		Procyonidae		Dasypodidae		Cervidae		*Cerradomys* sp.		*Oligoryzomys* sp.		TOTAL	
Ixodida	$N = 1$	0.38%	$N = 1$	0.38%	$N = 1$	0.38%	$N = 1$	0.38%	$N = 5$	1.92%	$N = 4$	1.55%	260	100%
Amblyomma aureolatum	0	0	0	0	0	0	0	0	0	0	0	0	1[h]	1[h]
Amblyomma longirostre	0	0	0	0	0	0	0	0	0	0	0	0	1[b]	1[b]
Amblyomma nodosum	7[c]	3[c]	0	0	0	0	0	0	0	0	0	0	7[c]	3[c]
Amblyomma ovale	0	0	2[e]	1[e]	0	0	0	0	0	0	0	0	176[b–e,g–i]	114[b–e,g–i]
Amblyomma parvum	0	0	0	0	13[i]	7[i]	2[i]	2[i]	0	0	0	0	15[i]	9[i]
Amblyomma sp.	0	0	0	0	0	0	0	0	0	0	0	0	72[b,e]	72[b,e]
Amblyomma sculptum	0	0	0	0	0	0	0	0	0	0	0	0	3[b,d]	3[b,d]
Amblyomma tigrinum	0	0	0	0	0	0	0	0	0	0	0	0	1[i]	1[i]
Dermacentor nitens	0	0	0	0	0	0	5[i]	3[i]	0	0	0	0	439[a–i]	193[a–i]
Rhipicephalus microplus	0	0	0	0	0	0	0	0	0	0	0	0	346[a–e,g,i]	104[a–e,g,i]
Rhipicephalus sanguineus	0	0	0	0	0	0	0	0	1[b]	1[b]	2[b]	2[b]	514[a–i]	294[a–i]

(continued)

Table 1. Continued

VECTOR/HOST	Environment		*Canis familiaris*		*Felis catus*		*Bos taurus*		*Equus caballus*		*Equus asinus*		*Homo sapiens*	
			$N = 188$	72.31%	$N = 3$	1.15%	$N = 28$	10.77%	$N = 6$	2.31%	$N = 18$	6.92%	$N = 4$	1.55%
Ixodida	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*	*N*	*n*
Siphonaptera														
Ctenocephalides felis	0	0	0	0	0	0	0	0	0	0	0	0	$68^{a,b,e,f,g,h}$	$60^{a,b,e,f,g,h}$
Polygenes bohlsi bohlsi	0	0	0	0	0	0	0	0	1^{b}	0	0	0	1^{b}	0
Pulex irritans	0	0	0	0	0	0	0	0	0	0	0	0	1^{b}	0
Xenopsylla cheopis	0	0	0	0	0	0	0	0	0	0	4^{b}	3^{b}	4^{b}	3^{b}
Phtiraptera														
Heterodoxus spiniger	0	0	0	0	0	0	0	0	0	0	0	0	5^{b}	3^{b}
Mesostigmata														
Gigantolaelaps tiptoni	0	0	0	0	0	0	0	0	46^{b}	16^{b}	27^{b}	10^{b}	73^{b}	26^{b}
TOTAL													$1,727^{a-i}$	887^{a-i}

Maciço de Baturité municipalities: [a]Aracoiaba; [b]Aratuba; [c]Baturité; [d]Capistrano; [e]Guaramiranga; [f]Itapiuna; [g]Mulungu; [h]Pacoti; [i]Redenção.

level of degradation of the Atlantic forest biome. However, we found that *A. ovale* collected from dogs was infected with *Rickettsia* sp. strain Atlantic rainforest in different areas, including in the municipality of Aratuba, where there are confirmed SF cases. The clinical symptoms in these areas, including eschar formation and lymphadenopathy, are quite similar to those reported in other areas where *Rickettsia* sp. strain Atlantic rainforest is the etiological agent (Spolidorio et al. 2010, Silva et al. 2011).

Although *Rickettsia* sp. strain Atlantic rainforest has been detected in different tick species (Sabatini et al. 2010, Medeiros et al. 2011, Szabó et al. 2013b, Monje et al. 2015), previous studies showed a close relationship between *A. ovale* and this SFG *Rickettsia* species in different SF endemic areas of Brazil (Parola et al. 2013, Szabó et al. 2013a, b), particularly the Atlantic rainforest. In the context of our study, the high altitude forests of Maciço de Baturité can be considered equivalent to the Atlantic rainforest, and the forest edge is implicated as the main source of parasitic SF vectors. These hot spots are characterized by easy access for humans, whether for work purposes (livestock or agriculture) or leisure, and their pets. This is particularly true in the case of dogs, which have free access to the forest and show the highest frequency of parasitism among all vertebrate hosts in the present study (Table 1), as well as samples positive for the presence of rickettsial DNA fragments (Fig. 2). Hence the eco-epidemiological situation favors dog parasitism (Szabó et al. 2013a, b) and human infestation (Guglielmone et al. 2006, Serra-Freire et al. 2011) by *A. ovale*, allowing the emergence of the epidemic cycle in the transition areas between anthropogenic and natural environments.

Rickettsia sp. strain Atlantic rainforest was also detected in *R. sanguineus*, collected from dogs in Aratuba, where there are confirmed SF cases. Hence both *A. ovale* and *R. sanguineus* isolated from dogs were infected by this *Rickettsia* species, supporting previous studies that have shown that *A. aureolatum* and *R. sanguineus* can be infected by *Rickettsia* sp. strain Atlantic rainforest, in the Atlantic rainforest biome; however, in those studies, only *A. ovale* was found among the canine population (Sabatini et al. 2010, Medeiros et al. 2011, Szabó et al. 2013b), underlining the importance of *A. ovale* in the rickettsiae cycle in the endemic areas of high altitude forest.

Furthermore, although *R. sanguineus* can bite humans and do participate in the cycle of pathogenic rickettsiae (e.g., Parola et al. 2013, Szabó et al. 2013b), in Brazil, this species is mostly found on dogs, with occasional parasitization of humans (Szabó et al. 2013b). In addition, there is no confirmation that this tick species participates in the SF epidemic cycle; it is more likely to participate in the enzootic cycle. However human parasitism by *R. sanguineus* in Brazil may be underreported (Serra-Freire 2010; Dantas-Torres et al. 2006), and their potential for transmiting pathogens should be investigated further.

In addition to *Rickettsia* sp. strain Atlantic forest, another pathogenic rickettsiae species, *R. felis* (e.g., Raoult et al. 2001, Choi et al. 2005, Znazen et al. 2006, Gillespie et al. 2009), was found infecting *C. felis* in Aratuba and Mulungu (Fig. 2). This appears to be common in Brazil, as *R. felis* has been detected throughout the six major biomes in Brazil: Amazon, Caatinga, Cerrado, Atlantic forest, Pampa (Southern Plains), and Pantanal (Gehrke et al. 2009, Horta et al. 2014; Fig. 1A). It is interesting to observe that Mulungu shows a diversity of ectoparasites infected with rickettsial agents (Fig. 2); however, no SF cases have been reported in this municipality, which is bordered to the south by Aratuba, to the north by Guaramiranga, and to the east by Baturité (Fig. 1), and hence shares the eco-epidemiological conditions of these municipalities, which are

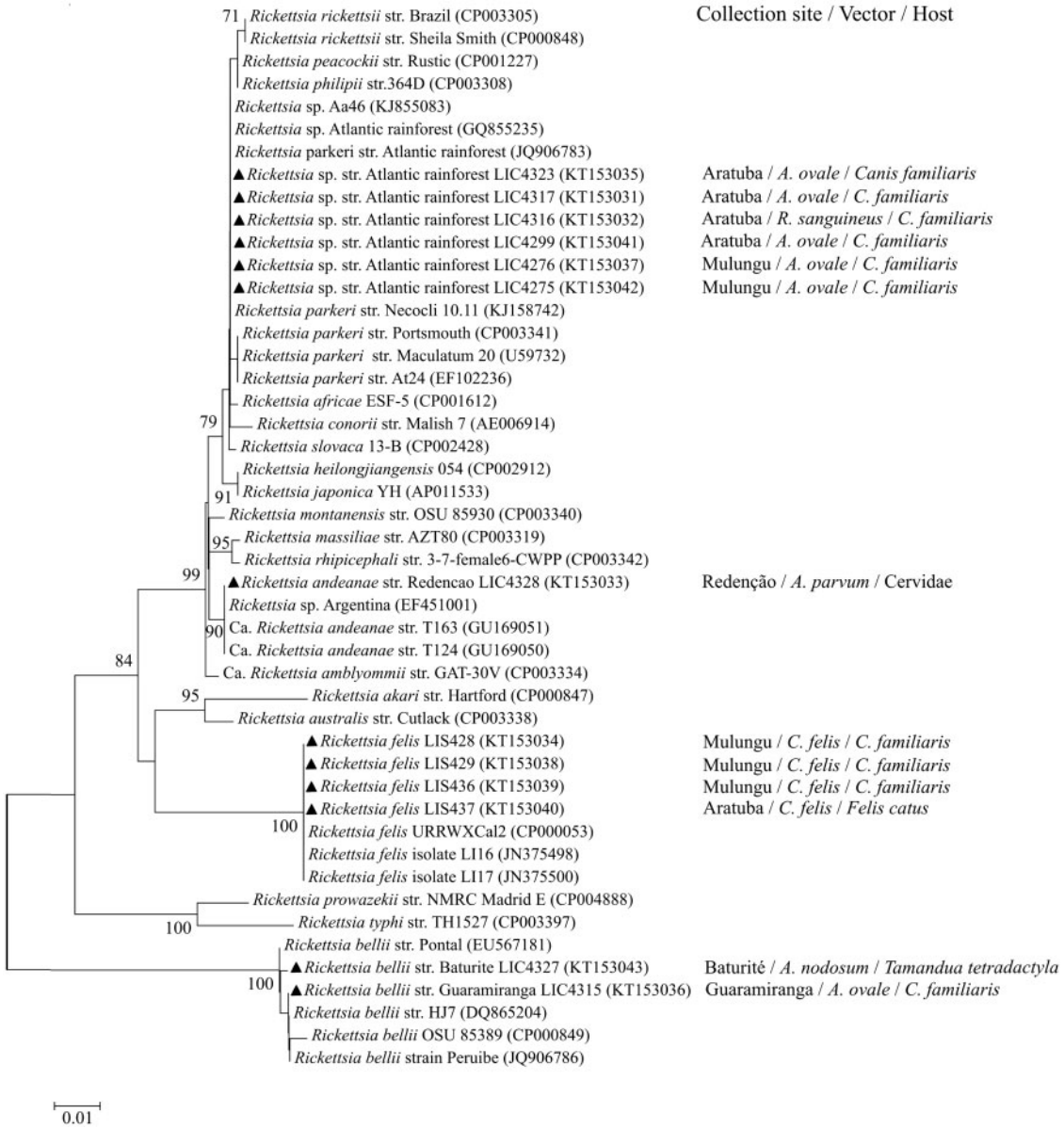

Fig. 2. Phylogenetic tree based on CS4 nucleotide sequences of the *glt*A gene constructed using the neighbor-joining method and Kimura's 2-parameter evolution model. The GenBank accession codes of included sequences are presented in parenthesis. The numbers at nodes are the bootstrap values obtained from 1,000 re-samplings. Bootstrap values below 70% are not presented. Triangles indicate sequences obtained in this study.

characterized as SF endemic areas. In addition, two *A. ovale* samples collected from dogs in Mulungu were infected by *Rickettsia* sp. strain Atlantic rainforest, showing a silent circulation of different bioagents in this municipality (Fig. 2), and allowing us to identify it as silent focus, since there are no confirmed human SF cases in Mulungu to date.

Ca. R. andeanae belongs to the SFG; however, its pathogenicity to humans remains unknown. In Brazil, this *Rickettsia* species has been reported in *Amblyomma auricularium* in the Caatinga ecosystem (Lugarini et al. 2015) and in *A. parvum* in the Cerrado and Pantanal ecosystem, as well as in Paraguay and Argentina (Pacheco et al. 2007b, Nieri-Bastos et al. 2014, Ogrzewalska et al. 2014).

In the high altitude forest, one female *A. parvum* specimen was infected by *Ca.* R. andeanae, collected from a Cervidae (Fig. 2) and other adult specimens were found parasitizing armadillo (Table 1). These findings corroborate those of a previous study (Guglielmone and Nava 2006), which verified the preference of *A. parvum* adults parasiting medium to large mammals, while its larvae and nymphs stages are more common in small mammals, mostly rodents (Nava et al. 2008).

Although *A. parvum* is a potential vector in South America with a predilection for humans (Guglielmone et al. 2006), in the present study, this tick species was limited to Redenção, and only found parasitizing wild animals (Table 1), suggesting a wild cycle for this SFG Rickettsiae in the Atlantic forest environment. Ours is the first report of this rickettsiae in the Atlantic forest environment in Brazil.

R. bellii has been found infecting different tick species (Tomassone *et al.*, 2010, Parola *et al.*, 2013). This species has been characterized as a rickettsiae agent which is nonpathogenic to humans, although there are records of mild intradermal inflammatory reactions at sites of inoculation, with the subsequent appearance of necrotic scabs in rabbits and guinea pigs (Ogata et al. 2006), as well as serological evidence of infection in vertebrates (Pacheco et al.

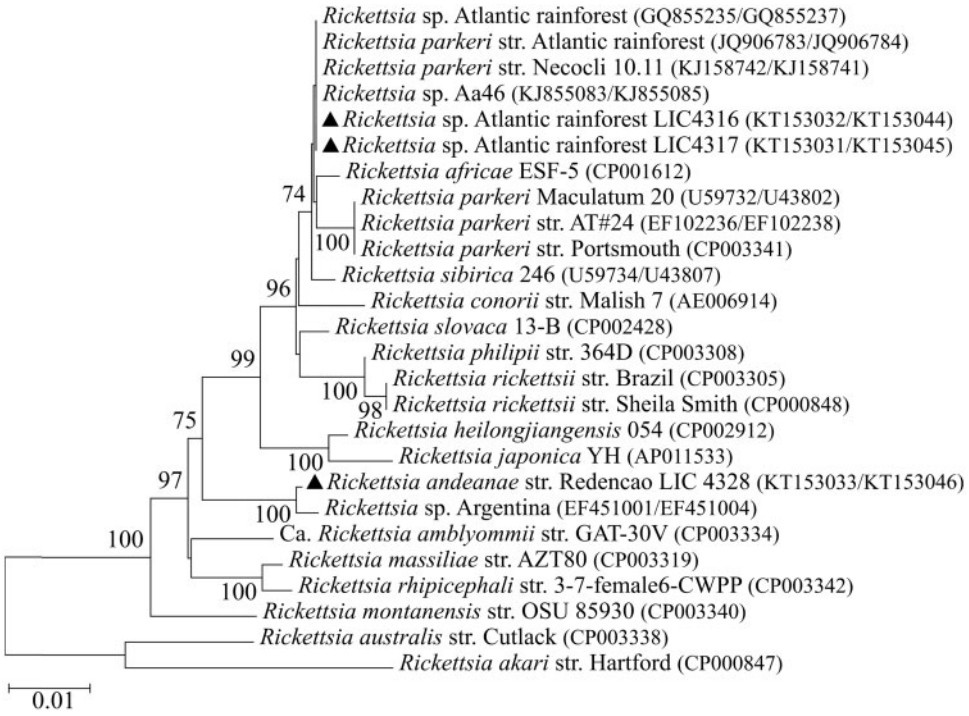

Fig. 3. Phylogenetic tree based on *glt*A CS2 and CS4 fragment, and *omp*A rickettsial gene nucleotide sequences concatenated to produce a 1,500 bp DNA fragment. The tree was constructed using the neighbor-joining method and Kimura's 2-parameter evolution model. GenBank accession codes of included sequences are presented in parenthesis. The numbers at nodes are the bootstrap values obtained from 1,000 re-samplings. Bootstrap values below 70% are not presented. Triangles indicate sequences obtained in this study.

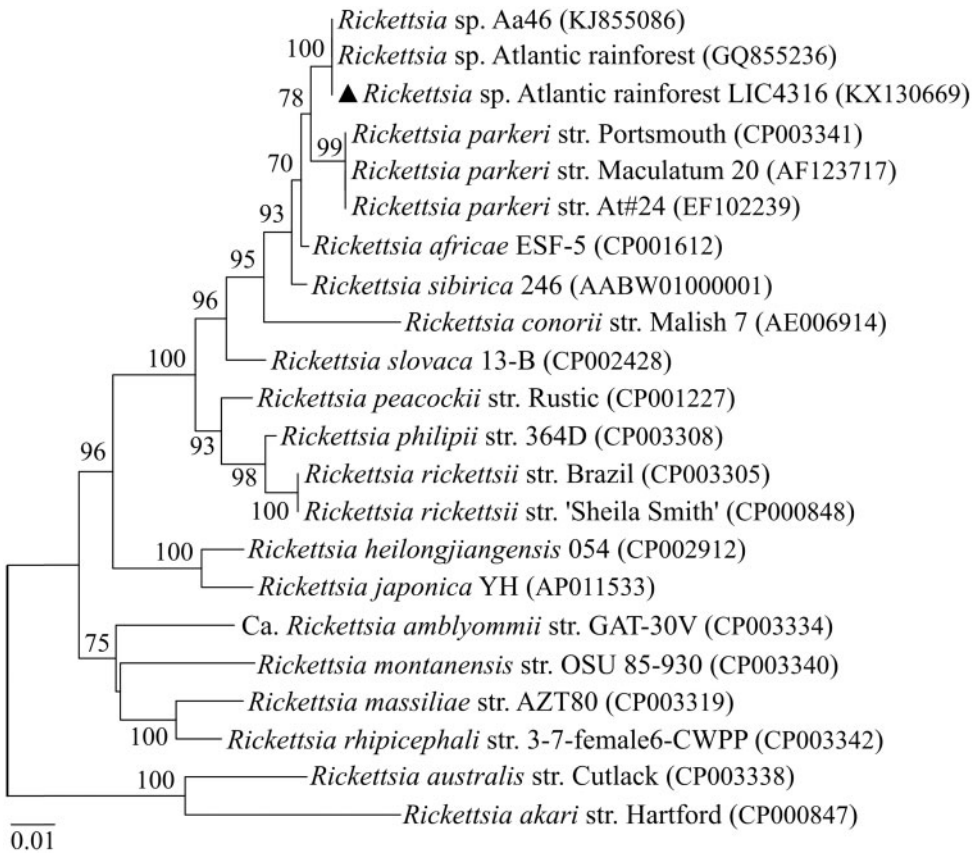

Fig. 4. Phylogenetic tree of *omp*B gene nucleotide sequences constructed using the neighbor-joining method and Kimura's 2-parameter evolution model. The GenBank accession codes of included sequences are presented in parenthesis. The numbers at nodes are the bootstrap values obtained from 1,000 re-samplings. Bootstrap values below 70% are not presented. Triangles indicate sequences obtained in this study.

2007a, Labruna et al. 2011). In Brazil, *Amblyomma* ticks appear to be the most important invertebrate vector for *Rickettsia* spp.(Labruna et al. 2004a, Labruna et al. 2004b, Labruna et al. 2011, Barros-Lopes et al. 2014, Moura-Martiniano et al. 2014, McIntosh et al. 2015). This assertion is supported by the results of the present study in which infections of *A. nodosum* and *A. ovale* with *R. bellii* were detected (Fig. 2), corroborating previous reports of infection of this tick species with this *Rickettsia* species in the Atlantic forest area (Ogrzewalska et al. 2009, Szabó et al. 2013a). This study provides the first record of *R. bellii* in the State of Ceará.

Ectoparasites collected from dogs have been found to be involved in the transmission of pathogenic rickettsia species in different epidemiological scenarios (Raoult and Roux 1997, Rydkina et al. 1999, Parola et al. 2013, Szabó et al. 2013b). In the high-altitude forest, we found that dogs were infested with a great diversity of ectoparasites (Table 1) maintaining different vector species populations, with the potential to transmit pathogenic rickettsiae to humans. This appears to be common in Atlantic forest foci (Ogrzewalska et al. 2012; Parola et al. 2013; Szabó et al. 2013a, b; Barbieri et al. 2014), where dogs can circulate between wild and anthropic environments carrying infected ticks. Therefore, dogs appear to act as the primary vertebrate host to the epidemic cycle of pathogenic *Rickettsia* in the high-altitude forest, and our results emphasize the need for a program to control tick species in dogs, as well as implementation of a program to monitor tick infestations in humans, focussed on *A. ovale*. In addition, guidance should be issued to the population indicating the risks of infestation by this tick species, and the signs and symptoms of disease.

In the context of the absence of severe cases of rickettsioses and the deficiency of studies of rickettsiae circulation in these areas, the high-altitude forests of northeastern Brazil can be considered a natural and silent focus of SF.

In conclusion, in Ceará, Brazil, *A. ovale* acts as primary vector of *Rickettsia* sp. strain Atlantic rainforest and has a key role in maintaining the epidemic cycle in the region; this is the first report of *Rickettsia* sp. strain Atlantic rainforest in the state of Ceará. We also identified *C. felis* as a potential vector able to support the *R. felis* cycle in the region studied and provide the first report of *Ca.* R. andeanae in the Atlantic forest environment in Brazil and of *R. bellii* in an SF endemic area in the state of Ceará.

Our results suggest that dogs act as the primary vertebrate host of the epidemic cycle of *Rickettsia* sp. strain Atlantic rainforest, and the enzootic cycle of *R. felis*, in the Maciço de Baturité region.

Acknowledgments

We would like to express our appreciation to our mentor Nicolau Maués da Serra-Freire (*in memoriam*), who is always in our hearts, and to Ingrid A. Costa for her help. This work was supported by Comissão de Aperfeiçoamento de Pessoal do Nível Superior (CAPES) and by 4ª Coordenadoria Regional de Saúde da Secretaria Estadual de Saúde do Estado do Ceará (4ª CRES/SES/CE).

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