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A new focus of spotted fever caused by *Rickettsia parkeri* in Brazil

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ABSTRACT

Spotted fever (SF) is a tick-borne zoonosis caused by bacteria of the genus *Rickettsia*. The disease varies in severity from mild clinical forms to severe cases. In Brazil, *Rickettsia rickettsii* SF is the most serious rickettsiosis and can result in death if not diagnosed and treated at the onset of symptoms. The SF mild form is caused by *Rickettsia parkeri* strain Atlantic Rainforest, and this etiological agent has been reported in the South, Southeast and Northeast regions of the country, in areas of preserved or little antropized Atlantic Rainforest. *Amblyomma ovale* is the proven vector and dogs are the hosts associated with the bioagent cycle. During a SF case investigation in Paraty municipality, Rio de Janeiro State, an Atlantic Rainforest biome area in Southeastern Brazil, the human pathogen *R. parkeri* strain Atlantic Rainforest was detected by PCR in a sample of human skin inoculation eschar and in a female *A. ovale* tick collected from a dog. These results expand the known area of occurrence of this mild form rickettsiosis in Brazil. In addition, the results of the present study indicate the importance of implementing programs to control canine ectoparasites and to raise awareness of the risks of infection, signs and symptoms of SF caused by *R. parkeri* strain Atlantic Rainforest.

KEYWORDS: Mild Rickettsiosis. Endemic focus. Inoculation Eschar. *Amblyomma ovale.* Atlantic rainforest biome. *Rickettsia parkeri* strain Atlantic Rainforest.

INTRODUCTION

Rickettsioses are a complex of zoonosis caused by Gram-negative, obligate intracellular, bacteria of the genus *Rickettsia*, occurring worldwide, transmitted by hematophagous ectoparasites, such as ticks, fleas, mites and lice. Among them, Spotted Fever (SF) is one of the oldest known and most important vector-borne disease with severe and mild clinical forms described: while the SF caused by *Rickettsia rickettsii* is the most lethal tick-borne rickettsiosis, occurring throughout the Americas with high case-fatality rates recorded[1-3], *Rickettsia parkeri* SF has no severe systemic manifestations or recorded deaths[1,3].

In Brazil, even though SF has been known since 1929, only in 2001 it was included in the list of compulsory notifiable diseases by the Brazilian Ministry of Health and, currently, it is considered an emerging disease due to the increased number of confirmed cases in all regions of the country[3]. The severe form of SF is characterized by high fever and severe hemorrhagic manifestations, leading to death in approximately 33% of cases. It occurs mainly in Southeast region and Northern part of the Southern region, in anthropized areas of the Cerrado and

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Atlantic Rainforest biomes. The ticks *Amblyomma sculptum* and *Amblyomma aureolatum* are the known vectors of *R. rickettsii* for humans, while capybaras, horses and dogs are the vertebrate hosts involved in the cycle of the bioagent cycle[3,4]. However, the most recent SF mild form, characterized by fever, skin rash, necrotic inoculation eschar at the site of the tick bite, lymphadenopathy, headache, myalgia, arthralgia, but no deaths[3-5], has been recorded in the South, Southeast and Northeast regions of Brazil, in preserved or little anthropized Atlantic Rainforest biome areas[5-9]. The known epidemiological scenario involves the tick *Amblyomma ovale* as the proven vector of the causal agent *R. parkeri* strain Atlantic Rainforest, and dogs as the hosts[5-9]. This strain is considered a genetic variant of *R. parkeri* sensu stricto[10], which is closely related to *Rickettsia africae* and *Rickettsia sibirica*.

The Atlantic Rainforest biome extends over 15% of the Brazilian territory, including Rio de Janeiro State, located in the Southeast region, which has one of the highest and largest urban, human population densities nationally, with rural and preserved areas in different ecoregions, in addition to the highest numbers of confirmed SF cases and deaths in the country. Accordingly, here, we report the infection by *R. parkeri* strain Atlantic Rainforest in vectors and samples of human tissue collected during field expeditions performed to investigate SF cases in the Paraty municipality, located in the region of Costa Verde, in Rio de Janeiro State.

MATERIALS AND METHODS

In November 2016, a suspected case of SF in the Paraty municipality (23°04'19.9"S 44°42'06.5"W) was reported to the Brazilian Notifiable Diseases Information System (SINAN)[12], involving a 53-year-old male patient, resident of a rural area of Sao Roque's neighborhood, who sought the health care unit with fever, myalgia, lymphadenopathy, but without a laboratory diagnosis. The patient described having frequent contact with a forest area and with ticks, dogs and cats. Following the investigation procedures for human cases of SF, the National Network for Environment Surveillance of Spotted Fever and other Rickettsial Diseases, from the Brazilian Ministry of Health, conducted a survey for potential SF vectors in the identified neighborhood in the Paraty municipality. A total of 24 ectoparasites were collected from one human and dogs at the probable infection site (a forest area in Sao Roque's neighborhood), morphologically identified according to group-specific dichotomous keys, as previously described[11], and individually submitted to DNA extraction using the NaCl method, as described elsewhere[4,11]. Total DNA was quantified by spectrophotometry and stored at -20 ºC until they were used as template DNA in PCR amplifications.

Furthermore, in May 2017, another human case was reported to SINAN[12], involving a 35-year-old woman resident of the same rural area in Paraty (as in the previous investigation), who reported continuous fever associated with headache, myalgia, abdominal pain and inoculation eschar. The patient mentioned having visited environments of forest, river and waterfalls as well as having contact with ticks, dogs, cats and horses. In the medical unit, the crust covering the tick bite wound site was removed by a healthcare professional and stored in a sterile microtube with 70% ethanol. Then, the skin sample underwent DNA extraction using the DNeasy Blood and Tissue Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, and the DNA sample was submitted to a PCR for Rickettsiae screening.

PCR amplifications of the rickettsial genes *gltA* (CS-78/CS-323 and CS-239/CS-1069)[13,14], *ompA* (*Rr*190.70p/*Rr*190.602n)[15] and *sca4* (D1738F/D2482R)[16] were performed on the vector and human tissue samples, using molecular grade sterile water as the negative control and 300 ng of *R. rickettsii* genomic DNA as the positive control. Amplifications of mitochondrial D-loop region (D-loop3-1x/D-loop4-1x) and 12S rRNA (T1B/T2A) were also performed, as already described[17], to confirm the tick identification. The amplified DNA products were fractionated by horizontal electrophoresis on 2% agarose gels, stained with ethidium bromide and examined under UV transillumination.

Amplicons of the expected sizes were purified using the HiYield™ Gel/PCR DNA Mini Kit (RBC-Real Biotech Corporation, Taiwan) and sequenced in an automated ABI 3730xl DNA analyzer (Applied BioSystems, Carlsbad, CA, USA) by using the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems). DNA sequences were edited with SeqMan software (Lasergene DNASTAR package, DNASTAR Inc. Madison, WI, USA) and identity values obtained with the BLAST analysis of sequences published in GenBank. The Maximum-Likelihood tree was inferred using the PhyML 3.0 online software[18] and a GTR+G correction model, selected by the Smart Model Selection[19]. Tree topology reliability was evaluated using bootstrap support (1,000 repeats).

RESULTS

A total of 21 adult ticks (*A. ovale* n=5, and *Rhipicephalus sanguineus* n=16) and two fleas (*Ctenocephalides felis*) were collected when they were feeding on dogs, and one tick (*A. ovale*) was removed from a human. No rickettsial

organisms were detected in the two flea samples. Among these, one *A. ovale* female (sample code: LIC 8272A) collected from a dog was infected with a *Rickettsia* genus bacterium, identified as *Rickettsia parkeri* str. Atlantic Rainforest, and the partial sequences of *gltA, ompA* and *sca4* genes (GenBank accession MH160728-30) showed identity values of 100% (1098/1098), 100% (491/491) and 100% (684/684) respectively, with *R. parkeri* strain Atlantic Rainforest sequences (CP040325).

Similarly, the human tissue DNA analysis also detected a Rickettsiae infection, with the partial sequences of *gltA, ompA* and *sca4* genes (GenBank accession MK690469-71), showing identity values of 100% (1066/1066), 100% (350/350) and 100% (573/573) respectively, with *R. parkeri* strain Atlantic Rainforest sequences (CP040325).

The phylogenetic analysis of a concatenated sequence with 2,249 nucleotides, generated from the genes *gltA* (1,051 bp), *ompA* (514 bp) and *sca4* (684 bp), showed the samples from Paraty to be closely related to *R. parkeri* strain Atlantic Rainforest, and grouped in the *R. parkeri* cluster (Figure 1).

To confirm the tick identification, we analyzed 12S rRNA gene sequences (GenBank accession MH160731) and the *D-loop* region (GenBank accession MH160732), amplified from *A. ovale* LIC 8272A mitochondrial DNA. The BLAST analysis of the *12S* rRNA gene revealed high sequence identity values (98%, 307/312) with sequences of *A. ovale* (MF004423 and AY342273). The *D-loop* sequence obtained here was the first available in GenBank for this tick species.

DISCUSSION

Although the main hosts of *A. ovale* are small wild rodents for the immature stages and wild carnivores (such as jaguar and tapir) for adults[1,17], this tick is commonly found in domestic dogs, which transits between preserved and peridomiciliary areas. Furthermore, this species is also important as a neotropical tick, considering that it is widely distributed throughout Brazil and other Latin American countries[1,17]. Besides some recent studies that have increased the medical-veterinary importance of *A. ovale* and have shown this tick species as the main vector of *R. parkeri* strain Atlantic Rainforest, as specimens naturally infected by this bacterium[10,20] have been described, especially in SF focus areas, due to the capacity of *A. ovale* to transmit and act as reservoir of this strain[21].

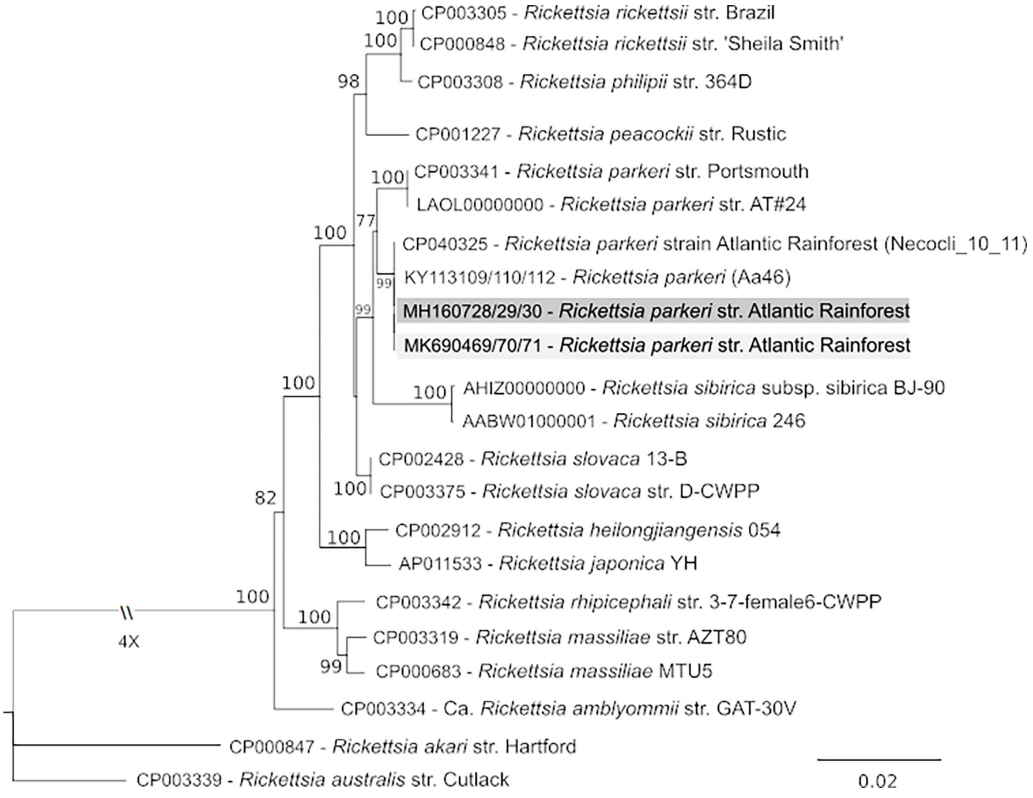

Figure 1 - Phylogenetic inferences by the Maximum-Likelihood method from 1,000 replicated trees based on concatenated nucleotide sequence of Spotted Fever Group Rickettsiae *gltA, ompA* and *sca4* genes. Evolutionary distances were estimated by the GTR+G model. Bootstrap values > 70% are shown. Sequences obtained are highlighted in dark grey (total DNA from *A. ovale*) and light grey (DNA from an eschar sample). GenBank accession numbers precede the sequence names. The scale bar indicates nucleotide substitutions per site.

To date, a single larva of *A. ovale* was reported as being infected with *R. parkeri* strain Atlantic Rainforest in Rio de Janeiro State[20] and this tick was collected while parasitizing a bird (*Turdus rufiventris*) from the Itatiaia National Park, located in Resende municipality, a conservation unit on the borders of Rio de Janeiro and Minas Gerais States, in Southeastern Brazil. However, in the present study we analyzed ectoparasites collected in a SF focus area, indicating this tick species as the presumable vector of the detected pathogen.

In terms of the epidemic cycle, four clinical *R. parkeri* SF cases have been reported from widely-spread different Brazilian regions: Southeast, Sao Paulo State[6], Northeast, Bahia State[7,9] and South, Santa Catarina State[8]. However, the present study provides the first report of human infection by *R. parkeri* in a focus area in Rio de Janeiro State, Atlantic Rainforest biome, therefore expanding the occurrence area of the mild form of SF in Brazil.

Both SF eco-epidemiological scenarios occur in Rio de Janeiro State. However, in some localities, the circulating Rickettsiae, and the tick involved in the cycle remain unknown, thus enhancing the importance of the public awareness programs and continuous training processes conducted by the National Network for Environment Surveillance of Spotted Fever and other Rickettsial Diseases throughout the full range of Brazilian biomes[3]. In this context, our results highlight the medical-veterinary importance of Costa Verde region, since Paraty is one of the five municipalities with the highest numbers of SF cases in Rio de Janeiro State[4], with 57 human cases reported in the last 11 years[12] (of these, six cases were confirmed by laboratory diagnosis). In addition, the area includes both urban and conserved Atlantic Rainforest areas, and is subjected to intense human transit due to a variety of purposes (*e.g.* tourism, livestock, agriculture, fishing). In addition, the geographic characteristics of the site allow dogs to freely access the forest, stimulating canine parasitism by *A. ovale* and the emergence of an epidemic cycle of *R. parkeri* strain Atlantic Rainforest, the only pathogenic Rickettsiae found in the region. Therefore, Paraty is considered to be a new focus for *R. parkeri* SF in Brazil.

CONCLUSION

Due to the new site, there is an epidemiological scenario of mild cases of SF similar to other Brazilian areas. Similarities include: (i) report of the presence of inoculation eschar and lymphadenopathy symptoms in human cases (Municipal Health Department, unpublished data), with a patient tissue positive for *R. parkeri* infection; (ii) the preserved characteristics of the environment; and (iii) the discovery of *A. ovale* parasitizing dogs and humans. The detection, for the first time, of a strain phylogenetically related to *R. parkeri* in SF focus area of Rio de Janeiro State highlights the importance of implementing an orientation program for the control of canine ectoparasites, as well as a campaign of public awareness of the risks of infection and the signs and symptoms of SF caused by *R. parkeri* strain Atlantic Rainforest.

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