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Rickettsia species infecting *Amblyomma* ticks from an area endemic for Brazilian spotted fever in Brazil

Rickettsia infectando carrapatos *Amblyomma* de uma área endêmica para febre maculosa brasileira no Brasil

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Abstract

This study reports rickettsial infection in *Amblyomma cajennense* and *Amblyomma dubitatum* ticks collected in an area of the state of Minas Gerais, Brazil, where Brazilian spotted fever is considered endemic. For this purpose, 400 adults of *A. cajenennse* and 200 adults of *A. dubitatum,* plus 2,000 larvae and 2,000 nymphs of *Amblyomma* spp. were collected from horses and from the vegetation. The ticks were tested for rickettsial infection through polymerase chain reaction (PCR) protocols targeting portions of three rickettsial genes (*gltA, ompA,* and *ompB*). Only two free-living *A. cajennense* adult ticks, and four pools of free-living *Amblyomma* spp. nymphs were shown to contain rickettsial DNA. PCR products from the two *A. cajennense* adult ticks were shown to be identical to corresponding sequences of the *Rickettsia rickettsii* strain Sheila Smith. DNA sequences of *gltA*-PCR products of the four nymph pools of *Amblyomma* spp. revealed a new genotype, which was shown to be closest (99.4%) to the corresponding sequence of *Rickettsia tamurae*. Our findings of two *R. rickettsii*-infected *A. cajennense* ticks corroborate the endemic status of the study area, where human cases of BSF were reported recently. In addition, we report for the first time a new *Rickettsia* genotype in Brazil.

Keywords: *Rickettsia rickettsii*, Brazilian spotted fever, *Amblyomma cajennense.*

Resumo

Este trabalho relata infecção por *Rickettsia* em carrapatos *Amblyomma cajennense* e *Amblyomma dubitatum*, colhidos numa área do Estado de Minas Gerais, onde a febre maculosa brasileira (FMB) é considerada endêmica. Para esse estudo, 400 adultos de *A. cajennense,* 200 adultos de *A. dubitatum,* 2.000 larvas e 2.000 ninfas de *Amblyomma* spp. foram colhidas de equinos e da vegetação. Os carrapatos foram testados para infecção por rickettsia através de reação em cadeia pela polimerase (PCR) direcionada a fragmentos de três genes de rickettsia (*gltA, ompA,* e *ompB*). Apenas 2 *A. cajennense* adultos de vida livre, e 4 grupos de ninfas de *Amblyomma* spp. continham DNA de rickettsia. Os produtos de PCR dos dois adultos de *A. cajennense* foram idênticos às sequências correspondentes de *Rickettsia rickettsii* cepa Sheila Smith. Sequências de DNA dos produtos provenientes dos quatro grupos de ninfas de *Amblyomma* spp. revelaram um novo genótipo, próximo (99,4%) à sequência correspondente de *Rickettsia tamurae*. Neste trabalho foram achados 2 carrapatos *A. cajennense* infectados por *R. rickettsii* que corroboram o caráter endêmico da área de estudo, em que casos de FMB ocorreram recentemente. Em adição, foi reportado, pela primeira vez, um novo genótipo de *Rickettsia* no Brasil.

Palavras-chave: *Rickettsia rickettsii*, febre maculosa brasileira, *Amblyomma cajennense.*

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Introduction

The ixodid ticks *Amblyomma cajennense* and *Amblyomma dubitatum* are the most frequent ectoparasites of capybaras (*Hydrochoerus hydrochaeris*) in Southeastern Brazil (VIEIRA et al., 2004; PACHECO et al., 2007). In addition, *A. cajennense* is also a common parasite of horses, dogs, and various medium to large-sized wild mammals, and is the most common biting tick affecting humans in Southeastern and Central-Western Brazil as well (VIEIRA et al., 2004; GUGLIELMONE et al., 2006). On the other hand, *A. dubitatum* is highly specific to capybaras, and is only rarely found on other host species, including humans (GUGLIELMONE et al., 2006; LABRUNA et al., 2007; NAVA et al., 2010).

In Brazil, *A. cajennense* is the main vector of the bacterium *Rickettsia rickettsii*, the etiological agent of Brazilian spotted fever (BSF), which is the most lethal rickettsiosis in the world (LABRUNA, 2009). Recent studies have reported that most of the BSF-endemic areas contain large populations of free-living capybaras, which act as primary hosts for both *A. cajennense* and *A. dubitatum*, and are exposed to rickettsial infection (HORTA et al., 2007; GUEDES; LEITE, 2008; PEREZ et al., 2008; SOUZA et al., 2008). In addition, it has been demonstrated that capybaras play an important role in the epidemiology of BSF, since they serve as a rickettsial source for creating new lineages of *R. rickettsii*-infected *A. cajennense* ticks (SOUZA et al., 2009).

The role of *A. dubitatum* in the epidemiology of BSF remains unknown. Regardless of being a common tick species in many BSF-endemic areas, *A. dubitatum* has never been found infected with *R. rickettsii.* Nevertheless, 6 to 45% of *A. dubitatum* ticks have been found to be infected by *Rickettsia bellii* (a rickettsia of unknown pathogenicity for humans) in many tick populations, including some from BSF-endemic areas (LABRUNA et al., 2004; HORTA et al., 2007; PACHECO et al., 2009). In addition, one study reported that 7.5% of the *A. dubitatum* ticks from a BSF-endemic area of São Paulo were infected by the *Rickettsia* sp. strain Cooperi, which is possibly a new strain of the human pathogen *Rickettsia parkeri* (LABRUNA et al., 2004; LABRUNA, 2009).

The goal of the present study was to investigate rickettsial infection in *A. cajennense* and *A. dubitatum* ticks collected in a BSF-endemic area of the state of Minas Gerais, Southeastern Brazil. In this same area, Guedes et al. (2005) reported that 1.28% of the *A. cajennense* ticks were infected by *R. rickettsii,* whereas no rickettsial infection was found in *A. dubitatum* ticks.

Material and Methods

From May 2006 to April 2008, ticks were collected from naturally infested horses, and from the pasture (free-living ticks), by using CO_2-baited traps in the Embrapa Gado de Leite (Embrapa Dairy Cattle) experimental farm, located at Coronel Pacheco (21°35' S and 43°15' W), state of Minas Gerais, Brazil, as part of another study that evaluated the seasonal dynamics of *Amblyomma* spp. ticks. Details about the study area and tick collections have been described by Guedes and Leite (2008). This area is inhabited by large groups of free-living capybaras (*Hydrochoerus hydrochaeris*), and is considered to be an endemic area for BSF, since human cases of the disease were confirmed recently (GUEDES; LEITE, 2008). In addition, a previous study in the same area reported that an *A. cajennense* specimen was naturally infected by *R. rickettsii* (GUEDES et al., 2005).

For the present study, the following numbers of free-living ticks were tested for rickettsial infection: 200 adults of *A. cajennense;* 200 adults of *A. dubitatum;* 1,000 larvae; and 1,000 nymphs of *Amblyomma* spp. These immature stages were not identified to species level at the time of the study. At the same time, ticks were collected from horses and tested for rickettsial infection: these included 200 adults of *A. cajennense,* and 1,000 larvae and 1,000 nymphs of *Amblyomma* spp., also not identified to species level. While adult ticks were processed individually, larvae and nymphs were processed in pools of 10 and 5 individuals, respectively.

In the laboratory, ticks were subjected to DNA extraction by means of the guanidine isothiocyanate-phenol technique, as previously described (SANGIONI et al., 2005). For this purpose, each adult tick or a larval or nymphal pool was placed in a 1.5 mL microtube containing 150 µL of TE buffer (Tris HCl 10 mmol.L^{-1}, EDTA 1 mmol.L^{-1}, pH 7.4), and was homogenized by using a sterile micropestle. Microtubes containing the homogenized, triturated ticks were then vortexed vigorously. Next, 450 µL of guanidine thiocyanate 5 mol.L^{-1}) were added to the tube, which was vortexed again and incubated for 10 minutes at room temperature with brief vortexing every 2 minutes. Following this, 100 µL of chloroform was added to the tube, which was inverted several times and left resting for 2 minutes. The tube was centrifuged at 12,000 x *g* for 5 minutes to separate the aqueous phase, which was transferred to a clean 1.5 mL microtube. Next, 600 µL of isopropanol was added to the aqueous phase (400 µL), which was homogenized by inverting the tube several times and then incubated at –20 °C for 2 to 18 hours. The tube was then centrifuged at 12,000 x *g* for 15 minutes; the supernatant was discarded, and the pellet was resuspended with 800 µL of 70% ethanol, and then centrifuged at 12,000 x *g* for 5 minutes; the supernatant was discarded, and the pellet was dried at room temperature and then resuspended with 30 µL of buffer TE. Finally, the microtubes were incubated at 56 °C for 15 minutes to facilitate DNA homogenization and then stored at –20 °C until tested using the polymerase chain reaction (PCR). For every 10 tubes containing ticks, a blank tube was included in the DNA extraction.

Samples were tested individually by means of PCR targeting a 401-bp fragment of the rickettsial gene *gltA*, using primers CS2-78 and CS2-323 as previously described (LABRUNA et al., 2004). This PCR protocol has been shown to be 100% effective in detecting *R. rickettsii*-infected ticks (LABRUNA et al., 2008; 2011). In each set of reactions, negative control tubes containing water were included, and also a positive control tube containing DNA of the *Rickettsia parkeri* strain NOD. Ten microliters of the PCR product underwent electrophoresis in 1.5% agarose gel, stained with ethidium bromide, and examined under UV transillumination. Samples that yielded visible amplicons of the expected size from the *gltA*-PCR were further tested using a second PCR assay targeting a 532-bp fragment of the rickettsial gene *ompA,* using the primers Rr-190.70p and Rr-190.602n, as previously described (REGNERY et al., 1991), and using a third

PCR assay targeting a 862-bp fragment of the rickettsial gene *ompB*, using the primers 120-M59 and 120-807, as previously described (ROUX; RAOULT, 2000). All PCR amplicons of the expected size were subjected to direct DNA sequencing in an automated ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The BLAST program (National Center for Biotechnology Information, Bethesda, MD, USA) was used to compare appropriate similarities among the rickettsial partial sequences generated in the current study.

Results

None of the ticks collected from horses or the *A. dubitatum* free-living adult ticks yielded rickettsial DNA by means of PCR (Table 1). However, two free-living *A. cajennense* adult ticks (1 male, 1 female), both collected in March 2007, and four pools of free-living *Amblyomma* spp. nymphs (collected in November 2006 and May, October and November 2007) were shown to contain rickettsial DNA using the PCR protocol targeting the rickettsial gene *gltA.* While the two adult ticks also yielded visible amplicons from both the *ompA* and the *ompB* PCR protocol, the four nymphal pools yielded no visible amplicon from the *ompA*-PCR, and only a weakly visible amplicon of the expected size was yielded from the *ompB*-PCR. DNA sequences of 321, 490 and 760-bp of the *gltA*, *ompA* and *ompB* products, respectively, from the two *A. cajennense* adult ticks, were shown to be identical to each other. Moreover, after Blast analysis, they were shown to be all 100% identical to corresponding sequences in the genome of the *R. rickettsii* strain Sheila Smith (GenBank accession number CP000766). DNA sequences of 350-bp obtained from the *gltA*-PCR products of the four nymphal pools of *Amblyomma* spp. were identical to each other, and after Blast analysis, were shown to be closest (99.4%; 348/350) to the corresponding sequence of *R. tamurae* (AF394896), and second closest (99.1%; 347/350) to *Rickettsia monacencis* (DQ100163). No DNA sequence of quality was obtained from the *ompB*-PCR performed on these four *Amblyomma* spp. nymphal pools.

The gene partial sequences obtained in the present study have been deposited in GenBank under the accession numbers JF690667 (*gltA*), JF690668 (*ompA*) and JF690669 (*ompB*) for *R. rickettsii*, and JF690670 for the *gltA* partial sequence generated from the four *Amblyomma* spp. nymphal pools.

Discussion

The area of the present study has been considered to be endemic for BSF because of the recent history of BSF cases in humans (GUEDES; LEITE, 2008). Therefore, our findings of two *R. rickettsii*-infected *A. cajennense* ticks corroborate the endemic status. Guedes et al. (2005) found that 1.28% of the *A. cajennense* ticks collected from this same area during 2003-2004 were infected by *R. rickettsii.* In the present study, the *R. rickettsii*-infected ticks were collected in March 2007; thus, it is likely that *R. rickettsii* infection had been established in this *A. cajennense* population for at least three years. Considering that a total of 400 adults of *A. cajennense* were tested (200 from horses and 200 free-living) for rickettsial infection, we found an overall *R. rickettsii* infection rate of 0.5% in this tick species. Although this rate is lower than what was reported by Guedes et al. (2005), it is within the values reported for other tick vectors of *R. rickettsii*, which ranged from 0.05 to 1.3% (BURGDORFER, 1988; PINTER; LABRUNA, 2006).

No rickettsial infection was detected in *A. dubitatum* adult ticks. Although this result was in accordance with Guedes et al. (2005), who also found no rickettsial infection in *A. dubitatum* from the same area of this study, it contrasts with other studies, especially in the state of São Paulo, where the majority of the *A. dubitatum* populations are infected with *R. bellii* (HORTA et al., 2007; PACHECO et al. 2009).

We found four *Amblyomma* nymphal pools to be infected by a new rickettsial strain sharing closest identity (99.4%) with *R. tamurae*, based on a partial fragment of the *gltA* gene. Since we detected this rickettsial strain in nymphal pools, we suppose that the agent was infecting nymphs of one of the two *Amblyomma* species found at the study site, namely *A. cajennense* or *A. dubitatum.* Interestingly, this new rickettsial strain has never been reported before, even though hundreds of *A. cajennense* or *A. dubitatum* ticks have been tested for rickettsial infection in other areas (GUEDES et al. 2005; SANGIONI et al., 2005; HORTA et al., 2007; PACHECO et al., 2009). Currently, *R. tamurae* is known to occur in Japan, where it infects the tick *Amblyomma testudinarium* (FOURNIER et al., 2006). Its role as a human or animal pathogen remains unknown. Unfortunately, we could not amplify fragments of other rickettsial genes of the new rickettsial strain found in *Amblyomma* nymphs, thus precluding any further taxonomic inference from our findings.

Table 1. *Rickettsia* infection, as determined using molecular methods applied to *Amblyomma* spp. ticks that were collected from horses and vegetation (free-living) in Coronel Pacheco, State of Minas Gerais, Brazil.

Ticks	Stage	Source	Nº tested ticks	Nº infected (%)	*Rickettsia* species
A. cajennense	Adult	Horse	200	0 (0)	
Amblyomma sp.	Nymph	Horse	1,000	0 (0)	
Amblyomma sp.	Larvae	Horse	1,000	0 (0)	
A. dubitatum	Adult	Free-living	200	0 (0)	
A. cajennense	Adult	Free-living	200	2 (1.0)	*R. rickettsii*
Amblyomma spp.	Nymph	Free-living	1,000	4 (0.4)*	*Rickettsia* sp.**
Amblyomma spp.	Larvae	Free-living	1,000	0 (0)	

*Nymphs were tested in 200 pools, each containing 5 ticks. Four pools were found to be infected, which represents a minimal infection rate of 0.4%, assuming that each PCR-positive pool contained only one infected tick. **This *Rickettsia* sp. was genetically closest to *Rickettsia tamurae.*

Further studies are needed to determine the tick species that carry this new rickettsial strain, which should be isolated from ticks in order to provide suitable molecular characterization and thus reach conclusions regarding its taxonomic position.

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