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First report of a *Rickettsia asembonensis* related infecting fleas in Brazil

Arannadia Barbosa Silva[a,b], Vinicius Figueiredo Vizzoni[b], Andréa Pereira Costa[a], Francisco Borges Costa[a], Jonas Moraes-Filho[c], Marcelo Bahia Labruna[c], Gilberto Salles Gazêta[b], Rita de Maria Seabra Nogueira[a,*]

[a] Programa de Pós-graduação do Mestrado em Ciência Animal, Universidade Estadual do Maranhão, São Luís, Maranhão, MA, Brazil
[b] Fundação Oswaldo Cruz/Instituto Oswaldo Cruz (FIOCRUZ/IOC), Laboratório de Referência Nacional em Vetores das Riquetsioses (LIRN), Rio de Janeiro, RJ, Brazil
[c] Departamento de Medicina Veterinária Preventiva e Saúde Animal, Universidade de São Paulo, São Paulo, SP, Brazil

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ABSTRACT

The present study was performed in a non-endemic area for spotted fever (SF) in Imperatriz microregion, state of Maranhão, Brazil. Blood samples and ectoparasites were collected from 300 dogs of the Imperatriz microregion. Canine serum samples were tested individually by indirect immunofluorescence assay (IFA), using five *Rickettsia* isolates from Brazil. Antibodies reactive to at least one of the five species of *Rickettsia* were detected in 1.6% of the dogs (5/300). These sera were considered reactive to *Rickettsia rickettsii* and *Rickettsia amblyommatis* or very closely related species. The ticks (Acari: Ixodidae), identified as *Rhipicephalus sanguineus* sensu lato (Latreille), and the fleas, identified as *Ctenocephalides felis,* were tested by polymerase chain reaction (PCR) for detection of rickettsial DNA. More than 78% (83/106) of the *C. felis* fleas were found to be infected with *Rickettsia* species using *gltA* as rickettsial PCR targets, whereas no evidence of *Rickettsia* spp. was found in *R. sanguineus* s. l. Genetic analysis based on genes *gltA*, *htrA* and *ompB* showed that the detected strain, is most closely related to *Rickettsia asembonensis* (formerly *Candidatus* Rickettsia asemboensis). The present study is the first report of a *R. asembonensis* related infecting *C. felis* fleas in Brazil.

1. Introduction

In Brazil, Brazilian Spotted Fever (BSF) is proven to be caused by *Rickettsia rickettsii* (Angerami et al., 2006; Labruna, 2009). It is considered the major tick-borne zoonotic disease in the country (Angerami et al., 2006; Dantas-Torres, 2007; Labruna, 2009). Interestingly, until 2000 only *R. rickettsii* was known in the country, but during the last decade, other species belonging to the Spotted-fever group (SFG) have been reported, resulting in the inclusion of *Rickettsia parkeri*, *Rickettsia rhipicephali*, *Rickettsia amblyommatis* (formerly *Candidatus* Rickettsia amblyommii, *Rickettsia felis*, *Rickettsia* sp. strain Atlantic rainforest, and *Candidatus* Rickettsia andeanae) (Labruna et al., 2011; Szabó et al., 2013a,b; Nieri-Bastos et al., 2014; Karpathy et al., 2016).

R. rickettsii is considered the most pathogenic species (Parola et al., 2013), and the main responsible of BSF and Rocky Mountain Spotted Fever (RMSF) in the United States (Angerami et al., 2006; Labruna, 2009). BSF is more frequently reported in the southeastern region of the country, whose higher incidence of disease is associated with the presence of capybaras (*Hydrochoerus hydrochaeris*) and *Amblyomma sculptum* ticks (formerly named as *Amblyomma cajennense*) (Dias and Martins, 1939; Labruna, 2009; Nava et al., 2014). Another important *R. rickettsii* vector, the tick *Amblyomma aureolatum* has been reported (Gomes 1933; Pinter and Labruna, 2006). This tick species is typically found in the Atlantic rainforest. Adults of this tick species are mainly reported attached to dogs kept unrestrained in rural areas close to natural environments (Szabó et al., 2013a). Thus, at the city–forest interface, dogs can carry infected ticks to human dwellings and as a result, the transmission occurs (Ogrzewalska et al., 2012; Szabó et al., 2013b). In addition, *R. sanguineus* sensu lato ticks seem to be involved in BSF cases in the Brazil (Gehrke et al., 2009; Moraes-Filho et al., 2009; Rozental et al., 2015).

In Brazil, a rickettsial bacterium genetically related to *R. parkeri,* named *Rickettsia* sp. Atlantic rainforest, has been identified as a human pathogen (Spolidorio et al., 2010; Silva et al., 2011; Krawczak et al., 2016). Though, recently *R. parkeri* might be associated with cases of rickettsiosis in Rio Grande do Sul, Brazil (Weck et al., 2016). Both species can cause an eschar-associated rash illness less severe than BSF (Paddock et al., 2004; Spolidorio et al., 2010; Silva et al., 2011). Studies showed a close relationship between *Amblyomma ovale* and *Rickettsia* sp. strain Atlantic rainforest in different Spotted Fever (SF) endemic

* Corresponding author at: Universidade Estadual do Maranhão – UEMA, Curso de Medicina Veterinária, Laboratório de Parasitologia, Campus Universitário Paulo VI, S/N, Tirirical, São Luís, MA 65055-970, Caixa Postal 09, Brazil.
E-mail address: grita62@hotmail.com (R. de Maria Seabra Nogueira).

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areas of Brazil (Szabó et al., 2013a,b; Moerbeck et al., 2016), further suggesting the importance of dogs in the epidemic cycle (Moerbeck et al., 2016).

R. felis, a bacterium formerly belonging to the SFG rickettsiae has been reclassified into the transitional group (TRG) rickettsiae by Gillespie et al. (2007). This species has been identified as the causative agent of the flea-borne spotted fever (FBSF), an emerging human rickettsiosis that has been diagnosed in the United States (Schriefer et al., 1994), Mexico (Zavala-Velázquez et al., 2000), France and Brazil (Raoult et al., 2001), Germany (Richter et al., 2002), Thailand (Parola et al., 2003) and Spain (Bernabeu-Wittel et al., 2006). Interestingly, this species has been found quite often in *Ctenocephalides* spp. fleas in several regions of Brazil (Horta et al., 2014). In addition, the flea *Ctenocephalides felis* is the most common flea species infesting dogs in Brazil (Castro and Rafael, 2006; Costa et al., 2013).

In some scenarios of *Rickettsia* infection occurring in Brazil, it is possible to observe a direct or indirect participation of the dogs in the occurrence in the epidemic cycle or enzootic cycle, either in the maintenance of ectoparasites or rickettsiae (Gehrke et al., 2009; Moraes-Filho et al., 2009; Rozental et al., 2015). Furthermore it has been proved through experimental conditions that domestic dogs can serve as amplifier hosts of *R. rickettsii* for *R. sanguineus* s. l. ticks (Piranda et al., 2011). The results obtained by Piranda et al. (2011) indicate that dogs and *R. sanguineus* s. l. may play a role in the epidemiology of BSF in Brazil. Thus, we emphasize that dogs exposed to ticks infected with *Rickettsia* spp. pathogenic, may represent a potential risk to human transmission since carry vectors for the home environment, allowing contact of ticks to humans (Evans et al., 2000).

Several studies in Brazil have pointing circulation the rickettsiae of SFG pathogenic or unknown pathogenicity in animals or ectoparasites in some endemic and non-endemic regions for SF of Brazil (Szabó et al., 2013a; Moura-Martiniano et al., 2014; Costa et al., 2015; Minervino et al., 2015; Nunes et al., 2015; Moerbeck et al., 2016; Vizzoni et al., 2016). Up to now, the state of Maranhão is not an endemic area for SF. However, few studies on the *Rickettsia* circulation species in other areas in the state of Maranhão were performed (Costa et al., 2015). In this scenario, the present study evaluated the rickettsial infection of dogs and ectoparasites collected from the middle-west region of Maranhão, Northeast Brazil.

2. Materials and methods

2.1. Study area

In March 2011, domestic dogs were sampled in countryside and urban areas of three municipalities, within the Imperatriz microregion, middle-west region of Maranhão, northeastern Brazil. Of these, 100 dogs (50 urban and 50 countryside) were in the municipality of Imperatriz (5°31′33″S and 47°28′33″W), 100 (50 urban and 50 countryside) in Governador Edison Lobão (5° 44′56″S and 47°21′39″W), and 100 (50 urban and 50 countryside) in Davinópolis (5°33′28″S and 47°25′33″W). A total of 300 dogs were sampled, comprising 173 males and 127 females; 59 dogs were < 1 year old, 153 dogs were 1–3 years old, and 88 dogs were > 3 years old. The present study was approved by the Bioethics Committee for Animal Experimentation of the State University of Maranhão (n° 29/2010), Brazil.

2.2. Indirect immunofluorescence assay

Canine serum samples were tested individually by indirect immunofluorescence assay (IFA), using five *Rickettsia* isolates from Brazil: *R. rickettsii* strain Taiaçu, *R. parkeri* strain At24, *R. amblyommatis* strain Ac37, *R. rhipicephali* strain HJ5 and *Rickettsia bellii* strain Mogi, as previously described (Labruna et al., 2007). Samples that reacted at the screening dilution (1:64) were then titrated using serial two-fold dilutions to determine endpoint titers. Reactions were performed using fluorescein-conjugated anti-dog IgG (Sigma-Aldrich, St. Louis, MO, USA). For all reactions, negative and positive controls were included on each slide.

Ticks and fleas were collected from the dogs and were stored in 70% ethanol at room temperature. The ectoparasites were taken to the laboratory for taxonomic identification by keys Aragão and Fonseca (1961) and Barros-Battesti et al. (2006) for ticks and Linardi and Guimarães (2000) for fleas.

2.3. Dna extraction and PCR reactions

A total of 369 ticks and 106 fleas were collected from dogs. 235 ticks and 106 fleas individually subjected to DNA extraction applying the guanidine isothiocyanatephenol technique (Sangioni et al., 2005). *Rickettsia* infected samples were identified by PCR screening using the primers CS-78/CS-323 and 17k3/17k5, targeting the *gltA* gene (CS2 regions) and *htrA* gene (17-kD antigen) of *Rickettsia* spp., respectively (Labruna et al., 2004). Some positive flea samples were subjected to novel PCR reactions using the primers CS-239/CS-1069 and 120.M59/120.807 for the rickettsial genes *gltA* (CS4 regions) and *ompB*, respectively (Roux and Raoult 2000; Labruna et al., 2004).

2.4. DNA sequencing

PCR products were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions. Purified PCR products were sequenced in both directions making use of an automated ABI 3730×l DNA analyzer (Applied Biosystems®, Foster City, CA, USA) using the protocol previously described (Otto et al., 2008). Sequence edition was performed with Lasergene software packages (DNASTAR, Madison, WI).

2.5. Phylogenetic analysis

Sequences of *gltA* and *ompB* genes were generated for phylogenetic reconstruction. Neighbor-joining method (MEGA 5.2 – Tamura et al., 2011) was applied with Kimura two-parameter as the correction model (Kimura, 1980) and bootstrap values JN315968 randomly generated trees.

2.6. Statistical analyses

Statistical associations of seropositivity to *Rickettsia* spp. with potential risk factors with the variables area of collection (urban and countryside), gender and age were evaluated. Data were tested by means of the chi-square or Fischer's exact test (both 95% confidence interval), when it necessary. All analyses were performed using the Epi Info software, version 6.04d (CDC, Atlanta, GA, USA).

3. Results

The serological results from the dogs demonstrated seropositive titers ranged from 128 to 512 (Table 1). Table 1 summarizes the serological results of IFA for the municipalities studied. Of five positive sera (1.6%), one had *R. rickettsii* titers at least four-fold higher than *R. parkeri* titers. This serum was considered reactive to *R. rickettsii* or very closely related species (Table 1). In addition, two sera were reactive to only *R. amblyommatis* and two sera were not possible to determine the closely related species (Table 1). There was no seropositive animal to the municipality of Governador Edison Lobão, only the municipalities of Imperatriz and Davinopólis. There was no significant difference between urban and countryside dogs ($p = 0.1853$), gender ($p = 0.6424$) and age ($p = 0.5727$) with seroreactive to *Rickettsia* spp.

A total of 369 *R. sanguineus* s. l. ticks and 106*C. felis* fleas were collected from 150 and 31 dogs, respectively. These, 235 ticks and 106 fleas were submitted for research of rickettsial DNA by PCR. 78.30%