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Short Communication

Rickettsia rickettsii infecting *Rhipicephalus sanguineus* sensu lato (Latreille 1806), in high altitude atlantic forest fragments, Ceara State, Brazil

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ABSTRACT

In Brazil, Spotted Fever (SF) is caused by *Rickettsia rickettsii* and *Rickettsia parkeri* strain Atlantic Forest. In recent years, several human cases of a milder SF have been reported from the Maciço de Baturité region of Ceará State. Previous studies in this region found *R. parkeri* strain Atlantic Forest to be present in *Rhipicephalus sanguineus* sensu lato and *Amblyomma ovale* ticks. The present study isolated and identified the *Rickettsia* spp. present in this new endemic area in Brazil. In March 2015, *R. sanguineus* s.l. and *A. ovale* were collected in rural areas of the Maciço de Baturité region, and subjected to the isolation technique. A bacterium was isolated from one *R. sanguineus* s.l., which phylogenetic analysis clustered to the *R. rickettsii* group. In conclusion, *R. rickettsii* bacteria is circulating in the studied area and may in future have an impact on the clinical diagnoses and consequently cause changes in the profile of the disease in the region. In addition, we suggest the increase of epidemiological and environmental surveillance in the area, in order to prevent Brazilian Spotted Fever cases.

1. Introduction

In Brazil, Spotted Fever (SF) has been shown to be caused by *Rickettsia rickettsii* and *Rickettsia parkeri* strain Atlantic Forest (Labruna 2009; Oliveira et al., 2016b; Paddock et al., 2017). *Rickettsia rickettsii* is the agent of Brazilian Spotted Fever (BSF), and has been implicated as the pathogen in all SF cases in Brazil that have involved death. The highest incidence of BSF occurs in areas with high densities of *Amblyomma sculptum* ticks, a species that is part of the *Amblyomma cajennense* sensu lato species complex (Szabó et al., 2013b; Nava et al., 2014; Oliveira et al., 2016a,b). The SF caused by *R. parkeri* strain Atlantic Forest is characterized as a milder disease (Spolidorio et al., 2010; Silva et al., 2011; Krawczak et al., 2016a), and its main vector appears to be the tick *Amblyomma ovale*. This suggests that this species may be important in the epidemic cycle in different SF types in various areas of Brazil where SF occurs (Szabó et al., 2013a,b; Moerbeck et al., 2016; Vizzoni et al., 2016; Krawczak et al., 2016b). In addition, this species of bacterium has been detected in the same tick species from areas in Brazil where mild SF has yet to be recorded (Melo et al., 2016).

In the last five years, several human cases of the milder SF were reported in the Maciço de Baturite region, Ceará State, northeastern Brazil (Oliveira 2016; Oliveira et al., 2016b). This region has a set of very specific geoecological characteristics, including high altitude Atlantic Forest fragments in the Caatinga biome and a semiarid climate (Santos et al., 2012; Moerbeck et al., 2016). Previous studies have identified pathogenic rickettsiae infecting potential vectors in this region, specifically recording the presence of *R. parkeri* strain Atlantic Forest in *Rhipicephalus sanguineus* sensu lato and *A. ovale* ticks (Moerbeck et al., 2016). Indeed, it has been suggested that *R. parkeri* strain Atlantic Forest may be involved in the epidemic cycle (Moerbeck et al., 2016). However, there the identity of the circulating *Rickettsia* spp. species, essential to effective SF control, is currently unknown.

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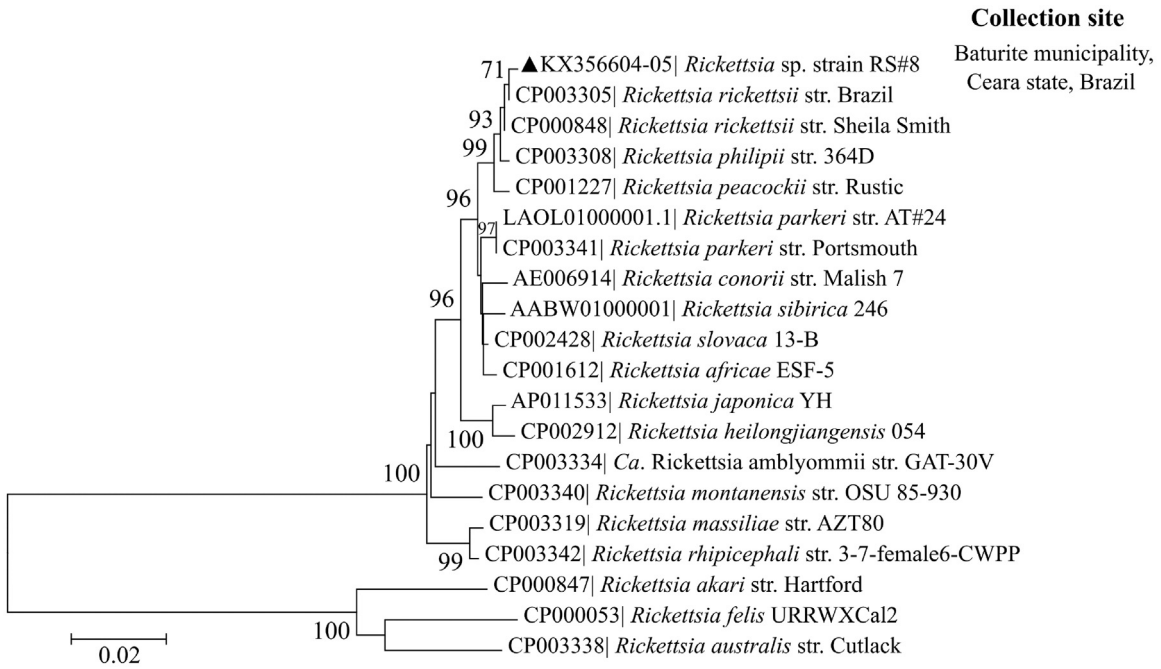

Fig. 1. Phylogenetic tree of concatenated rickettsiae *htrA* and *sca4* genes constructed by the neighbor-joining method, using 2-Kimura-parameters evolutionary model with MEGA 5.2 software. Bootstrap values below 70% were not shown. The black triangle indicates sequences obtained in the current study, as a result of molecular analyzes carried out on an *Rickettsia* spp. found infecting a *Rhipicephalus sanguineus* sensu lato tick removed from a dog in Baturité municipality, Ceará State, Brazil.

Consequently, in the current study we isolated and identified the *Rickettsia* spp. circulating in this new endemic area of a mild SF in Brazil.

2. Materials and methods

2.1. Study area

In March 2015, a total of 125 adult ticks were collected from 25 domestic dogs in rural areas of six municipalities (Baturité, Guaramiranga, Mulungu, Aratuba, Pacoti, and Redenção), in the Maciço de Baturite region of Ceará State, Brazil. Collections were carried out in collaboration with 4th. Regional Health Coordinating Agency of Ceará State Health Department. Subsequently, the ticks were transported alive to the biosafety level 3 laboratory of Divisão de Epidemiologia e Controle de Doenças (DECD) at Fundação Ezequiel Dias – FUNED, Belo Horizonte, Minas Gerais, Brazil.

2.2. Tick identification

The ticks were identified as *R. sanguineus* sensu lato and *A. ovale* using taxonomic keys (Barros-Battesti et al., 2006). Forty eight ticks (9 *R. sanguineus* s.l. and 39 *A. ovale*) were selected and subjected to the Rickettsiae isolation technique previously described by Kelly et al. (1991) and Labruna et al. (2004), but with some modifications developed during this study.

2.3. Isolation of Rickettsiae

Ticks were individually disinfected for 10 min in iodine alcohol and then washed several times in sterile Milli-Q water. Then, with a sterile scalpel, a section was made at the base of the capitulum. After being separated from the body, this was macerated with a drop of MEM (Sigma-Aldrich) containing 10% bovine calf serum (BCS), iron enriched (Hyclone) and antibiotics (10U penicillin/mL and 10 μg streptomycin/mL). Each homogenate was inoculated into a well, giving in total two 24-well plates containing a confluent monolayer of Vero cells. After inoculation, the plates were centrifuged for 1 h at 400*g* at room temperature. 1 mL of the same culture medium was added and incubated at 28 °C without CO_2. The level of infection was monitored every 7 days using Gimenez staining (Giménez, 1964). If *Rickettsia*-like organisms were seen, the monolayer containing the bacteria was harvested and inoculated in a new well of a 24-well plate or 25 cm^2 flasks containing a monolayer of confluent uninfected Vero cells.

2.4. Molecular characterization of the rickettsial isolates

For the molecular characterization of isolated bacteria, samples of infected cells from the eleventh Vero cell passage were subjected to DNA extraction using a QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany), as recommended by the manufacturer, and tested by PCR using previously described primer pairs that targeted fragments of the rickettsial genes *htrA* (17 kDa) and *sca4* (*geneD*) (Webb et al., 1990; Sekeyova et al., 2001; Labruna et al., 2004).

Amplified products were purified using NucleoSpin® Gel and PCR Clean-up kit (Macherey-Nagel, Düren, Germany) according to manufacturer's instructions and sequenced using an automated ABI 3730xl DNA analyzer (Applied Biosystems®, Foster City, CA, USA), following the protocol given by Otto et al. (2008). Sequence editing was performed with Lasergene software packages (DNASTAR, Madison, WI). To perform phylogenetic analysis, the neighbor-joining method (Tamura et al., 2011) was applied with Kimura two-parameter as the correction model (Kimura 1980), with bootstrap values obtained from 1000 randomly generated trees. Sequences generated in this study were deposited in GenBank with accession numbers KX356605 and KX356604, for *htrA* and *sca4* genes, respectively.

3. Results

Coccobacillary organisms were found on the 21st day in two isolates from *R. sanguineus* s.l. and *A. ovale* ticks. Theses isolates were contaminated by extracellular bacteria and discarded. All isolates with null growth were also discarded. *Rickettsia*-like organisms were visualized on the 28th day and were successfully isolated from a one well (1/48). The isolate, designated as RS#8, was obtained from a *R. sanguineus* s.l. collected in Baturité municipality and was maintained with twelve