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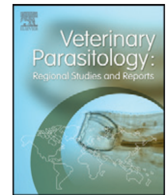

Original Article

Isolation of *Rickettsia rickettsii* from the tick *Amblyomma sculptum* from a Brazilian spotted fever-endemic area in the Pampulha Lake region, southeastern Brazil

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A B S T R A C T

Brazilian spotted fever (BSF) is a tick-borne disease caused by the bacterium *Rickettsia rickettsii*, the deadliest spotted fever of the world, transmitted in southeastern Brazil mainly by the tick *Amblyomma sculptum*, a member of the *Amblyomma cajennense* species complex. In the present study, over 5000 adults of *A. sculptum* ticks were collected by dry ice traps in the Municipal Ecological Park, alongside the Pampulha Lake region, a BSF-endemic area of Belo Horizonte city, state of Minas Gerais, southeastern Brazil. Ticks were taken alive to the laboratory, where a sample of 2100 specimens was processed for isolation of *R. rickettsii*. For this purpose, ticks were macerated and intraperitoneally inoculated into guinea pigs. Only one out of 21 inoculated guinea pigs presented high fever within 21 days post inoculation with tick homogenates. This febrile animal was euthanized and its internal organs were macerated and inoculated into additional guinea pigs (guinea pig passage). A spleen sample from a febrile guinea pig was used to inoculate Vero cells, resulting in a successful isolation and in vitro establishment of rickettsiae. *Rickettsia*-infected Vero cells were used for molecular characterization of the rickettsial isolate through PCR and DNA sequencing of fragments of three rickettsial genes (*gltA*, *ompA*, and *ompB*), which were all 100% identical to corresponding sequences of *R. rickettsii* from GenBank. The present *R. rickettsii* isolate was designated as strain Pampulha. A minimal infection rate of 0.05% *R. rickettsii*-infected ticks was estimated for *A. sculptum* population of the Pampulha Lake region. Our results, coupled with epidemiological evidences, suggest that *R. rickettsii* strain Pampulha, isolated from *A. sculptum* ticks in the present study, is the strain responsible for human clinical cases of BSF in the Pampulha Lake region of Belo Horizonte city.

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1. Introduction

Brazilian spotted fever (BSF), caused by the bacterium *Rickettsia rickettsii*, is the deadliest spotted fever of the world, with case-fatality rates over 50% in most of its occurrence area in southeastern Brazil (Szabó et al., 2013; Oliveira et al., 2016). Humans acquire the infection through the bite of a *R. rickettsii*-infected tick. In southeastern Brazil, *Amblyomma sculptum* is considered the most important vector of *R. rickettsii* to humans (Szabó et al., 2013; Martins et al., 2016). This tick species is a member of the *Amblyomma cajennense* species complex, currently composed by six distinct species distributed from southern United States to northern Argentina (Nava et al., 2014). Before the study of Nava et al. (2014), all these six tick species were treated as a single species, *A. cajennense*. Recent genetic, biological, and morphological studies have determined that this species complex is represented in southeastern Brazil solely by *A. sculptum* (Labruna et al., 2011; Beati et al., 2013; Nava et al., 2014; Martins et al., 2016); therefore, all previous reports of *A. cajennense* in this geographical area should be considered as *A. sculptum*, which is the most frequent human-biting tick species in Brazil (Guglielmone et al., 2006, Martins et al., 2016).

The Pampulha Lake region is a BSF-endemic area within the Belo Horizonte city, state of Minas Gerais, southeastern Brazil. Deadly outbreaks of BSF have been reported in that Pampulha Lake region since

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the first half of the 20th century. Most of these cases were deeply investigated during earlier studies by Prof. Octavio de Magalhães, who in 1952 stated that the Pampulha focus was known for at least 30 years, and was one of the oldest and the most feared BSF foci of Belo Horizonte (Magalhães, 1952). In an earlier publication from the 1930s, Moreira and Magalhães (1935) reported a successful isolation of *R. rickettsii* from *A. sculptum* ticks (reported as *A. cajennense*) collected from a dog in the Pampulha Lake region. While this earlier finding corresponded to the first reported isolation of *R. rickettsii* from a member of the *A. cajennense* species complex in Latin America, no further records of *R. rickettsii*-infected ticks have been reported from Belo Horizonte.

After an apparent 'epidemiological silence' of BSF during the second half of the 20th century, the disease 'reemerged' during this new century in many parts of southeastern Brazil (Angerami et al., 2015), including the Pampulha Lake region. During the last 5 years, there were five Laboratory-confirmed cases of BSF; at least one of these cases, from 2016, was considered to be acquired in the Municipal Ecological Park, a public park in the Pampulha Lake region inhabited by a large population of capybaras (*Hydrochoerus hydrochaeris*) that sustain an established population of *A. sculptum* ticks (unpublished data from the Health Secretary of Minas Gerais state). In the present study, *A. sculptum* ticks were collected from the Municipal Ecological Park and brought to the laboratory, where a *R. rickettsii* strain was isolated from the ticks and was established in cell culture.

2. Materials and methods

In January 2015, over 5000 host-questing *A. sculptum* adult ticks were collected by using 20 CO_2 traps as previously described (Oliveira et al., 2000) at capybara common places in the Municipal Ecological Park (19°51′S, 43°59′W), alongside the Pampulha Lake region of Belo Horizonte city, state of Minas Gerais. Captured adult ticks were taken alive to the laboratory, where they were incubated at 35 °C and 100% relative humidity for 72–96 h, prior to being processed for rickettsial isolation, as previously described (Krawczak et al., 2014). Briefly, live adult ticks were counted, and a sample of 2100 *A. sculptum* specimens was separated into 21 pools, each pool containing 100 ticks. Ticks of each pool were disinfected for 10 min in iodine alcohol, followed by several washes in sterile water, and then macerated into brain-heart infusion broth (BHI) in a sterile mortar with the aid of a sterile alundum. The resultant tick homogenate (5–8 mL) was inoculated intraperitoneally in an adult male guinea pig. Since we had 21 tick pools, we used 21 guinea pigs, one per tick pool. Guinea pigs had their rectal temperature measured daily until the 21st day post inoculation (DPI), when they were bled through cardiac puncture, and their sera were individually tested for the presence of anti-*R. rickettsii* antibodies through the immunofluorescence assay (IFA) using *R. rickettsii* antigen, as previously described (Soares et al., 2012). If any of the tick-inoculated guinea pigs presented high fever (rectal temperature >40.0 °C) for 3 consecutive days within the 21-day period, it was euthanized at the 3rd febrile day and its liver, spleen, lungs and brain were macerated into BHI and the resultant homogenate was inoculated into additional guinea pigs (guinea pig passage). This procedure was repeated with additional guinea pigs; in each guinea pig passage, samples of the internal organs were frozen directly at -80 °C, and then at liquid nitrogen, in order to cryopreserve the isolate before it could be adapted to in vitro tissue culture.

In vitro establishment of the rickettsial isolate in Vero cells followed the procedures described by Krawczak et al. (2014). Briefly, BHI-spleen homogenate obtained from a 5th passage-febrile guinea pig was divided into two 200 μL aliquots; each one was inoculated into one shell vial containing a monolayer of confluent Vero cells. After inoculation, the shell vials were centrifuged for 1 h at 700*g* and 22 °C. Then the monolayer was washed once with Roswell Park Memorial Institute medium (RPMI; Gibco, Carlsbad, CA) containing 10% bovine calf serum (Hyclone, Logan, UT) and subsequently incubated at 28 °C. Every 3 days, the medium was switched to new medium, and the aspirated medium was checked by Giménez staining for the presence of *Rickettsia*-like organisms. If the result was positive, the monolayer of the shell vial was harvested and inoculated into a 25-cm^2 flask containing a monolayer of confluent uninfected Vero cells. Cells in the 25-cm^2 flask were checked by Giménez staining until >90% of them were infected, when they were harvested and inoculated into 75-cm^2 flasks of Vero cells. The level of infection of cells was monitored by Giménez staining of scraped cells from the inoculated monolayer. The rickettsial isolate was considered to be established in the laboratory after at least three passages through 75-cm^2 Vero cell flasks, each achieving a proportion >90% of infected cells (Krawczak et al., 2014).

For molecular characterization of the rickettsial isolate, DNA from the third infected cell passage was extracted by using the DNAeasy Blood and Tissue Kit (Qiagen, Valencia, California), and tested by a battery of different PCR protocols, with two primer pairs (CS-78 and CS-323; CS-239 and CS-1069) targeting two overlapping fragments of the rickettsial *gltA* gene (Labruna et al., 2004), primers Rr190.70F and Rr190.701R targeting a portion of the rickettsial *ompA* gene (Roux et al., 1996), and primers 120.M59 and 120–807 targeting a portion of the rickettsial *ompB* gene (Roux and Raoult, 2000). PCR products were purified with ExoSap (USB, Cleveland, OH) and sequenced in an ABI automated sequencer (Applied Biosystems/Thermo Fisher Scientific, model ABI 3500 Genetic Analyzer, Foster City, California, USA) with the same primers used for PCR. The sequences obtained were submitted to BLAST analyses (www.ncbi.nlm.nih.gov/blast) to infer the closest similarities available in GenBank.

3. Results

Among the 21 guinea pigs inoculated with adult tick homogenate, 1 animal died in <24 h, and was discarded from the study. Among the remaining 20 guinea pigs (each inoculated with 100 *A. sculptum* ticks), 19 remained afebrile (rectal temperature <39.5 °C) until 21 DPI, when they were bled and were shown to be seronegative to *R. rickettsii*. In contrast, one guinea pig presented high fever at the 4th DPI, which persisted until the 6th DPI, when the animal was euthanized and its internal organs were used to inoculate 2 additional guinea pigs. While one of these two animals remained afebrile until 21 DPI (and seronegative to *R. rickettsii* at 21 DPI), the second guinea pig developed high fever starting at the 4th DPI. This animal was euthanized at the 3rd febrile day and its internal organs were used to inoculate 3 additional guinea pigs, which presented high fever starting at the 4th DPI and scrotal reactions (edema and marked redness) at the 5-6th DPI. From these febrile animals, new guinea pig inoculations were sequentially performed, resulting in a total of 9 guinea pig passages that encompassed 17 inoculated guinea pigs, which all but one developed high fever (>40.0 °C) that started 3 to 8 DPI. Among febrile guinea pigs, 7 individuals were not euthanized; 2 of these animals died at the 6th and 9th DPI, while 5 survived after a high fever period that lasted for 3 to 8 (mean: 6.2) consecutive days. These surviving animals were tested by serology at the 21st DPI, when their endpoint titers to *R. rickettsii* were ≥2048. In addition, two of them presented with scrotal and ear necrosis right after the febrile period.

Rickettsiae were successfully isolated in Vero cells that were inoculated with spleen homogenate derived from a 5th guinea pig passage of the *A. sculptum* rickettsial isolate. A total of five Vero cell passages has been done, always resulting in 100% infected cells 6 to 10 days after inoculation of the monolayer. DNA of rickettsia-infected cells at third passage was subjected to a battery of PCR protocols, which successfully amplified fragments of the rickettsial genes *gltA*, *ompA*, and *ompB*. We sequenced 1068, 590, and 776 nucleotides of the *gltA*, *ompA*, and *ompB* genes, respectively, which all showed 100% identity to corresponding sequences in the *R. rickettsii* genomes from Brazil (CP003305) and Colombia (CP003306). This *R. rickettsii* isolate, designated as strain Pampulha, has been cryopreserved and deposited at

the Rickettsial Collection of the Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil.

4. Discussion

Similarly to a recent study in the state of São Paulo, southeastern Brazil (Krawczak et al., 2014), we were able to isolate a viable strain of *R. rickettsii* through guinea pig inoculation of field-collected ticks. Animal inoculation is a much feasible technique for isolation of *R. rickettsii* because it allows testing a great number of ticks at the same time, in contrast to the much more laborious in vitro tests with tissue culture, which do not allow testing hundreds of ticks at the same time due to the increasing chances of contamination with other bacteria or fungi as more ticks are processed simultaneously. In this sense, we were able to test at least 2000 adult ticks at the same time, which were inoculated into 20 guinea pigs (a 21st guinea pig died within 24 h post inoculation and was discarded from our results). Only one guinea pig, inoculated with 100 *A. sculptum* adult ticks, developed rickettsiosis that was shown to be caused by *R. rickettsii*. This result indicates that at least one *R. rickettsii*-infected tick was present in the tick pool that was used to inoculate this guinea pig. Because other 19 guinea pigs, each inoculated with 100 *A. sculptum*, remained afebrile and seronegative during the study, we can infer a minimal infection rate of 0.05% (1/2000) for the *A. sculptum* population tested in the present study. Indeed, this very low infection rate is in agreement with previous reports of *A. sculptum* from BSF-endemic areas, where several studies either failed to detect *R. rickettsii*-infected ticks when testing hundreds of ticks (Sangioni et al., 2005; Estrada et al., 2006; Vianna et al., 2008; Pacheco et al., 2009; Brites-Neto et al., 2013), or reported only 0.5% *R. rickettsii* infection rates (Guedes et al., 2011; Krawczak et al., 2014).

Indeed, guinea pig inoculation remains as the most feasible rickettsial isolation method for *R. rickettsii*, but not for other *Rickettsia* species, due to two main reasons: (i) *R. rickettsii* is the only *Rickettsia* species that is shown to be highly pathogenic to guinea pigs, which are the animal model for the disease caused by *R. rickettsii* (Yu and Walker, 2005); (ii) in contrast to the very low *R. rickettsii* infection rates in natural tick populations, other *Rickettsia* species are usually found at much higher (usually > 10%) infection rates (Parola et al., 2013), facilitating rickettsial isolation by in vitro methods with much lower samples of field-collected ticks. For these reasons, all reported isolations of *R. rickettsii* from *A. sculptum* ticks (reported as *A. cajennense*) were achieved only through guinea pig inoculations, either during earlies studies in the last century (Moreira and Magalhães, 1935; Dias et al., 1937; Vallejo-Freire, 1946; Magalhães, 1952) or during this current century (Krawczak et al., 2014).

The *A. sculptum* population of the Pampulha Lake region is sustained primarily by capybaras. Recent studies have confirmed that capybaras play a major role in the epidemiology of BSF in many endemic areas of southeastern Brazil, where this vertebrate animal acts as an amplifier host, which develops rickettsemia and creates new lines of *R. rickettsii* infected ticks (Souza et al., 2009; Labruna, 2013). Capybaras are a large rodent species that has its population growth regulated by its habitat's carry capacity, primarily controlled by food availability through the year (Moreira et al., 2013). It has been proposed that any human interference that raises the reproduction rate of a capybara population in a BSF-endemic area would also increase the number of susceptible animals, which are prone to develop rickettsemia. Consequently, the *R. rickettsii*-infection rate of the *A. sculptum* population could increase in the area, increasing the risks of human disease (Labruna, 2009, 2013; Soares et al., 2012). Interestingly, because of a two fatal BSF-confirmed cases in the Pampulha Lake region during 2013–2014, in 2014 government authorities decided to remove a great part of the capybara population from the area, which on the other hand remained offering the same food availability for remaining animals (unpublished data). Based on the above concepts, this procedure could have resulted in higher food availability for resident capybaras, resulting in an increased reproduction rate. Consequently, more susceptible animals could have been generated, resulting in more rickettsemic animals that could have generated more *R. rickettsii*-infected *A. sculptum* ticks. While this theoretical assumption needs to be confirmed by field quantitative experiments, this sequence of facts could have facilitated our finding of at least one *R. rickettsii*-infected tick in the present study.

After 80 years from a report of isolation of *R. rickettsii* from *A. sculptum* ticks in the Pampulha Lake region (Moreira and Magalhães, 1935), we provide a new isolation of this tick-borne pathogen in the laboratory. Molecular characterization based on partial sequences of three rickettsial genes (*gltA*, *ompA*, *ompB*) revealed no polymorphism of the new isolate when compared with other Latin American isolates. This result is quite expected since recent studies demonstrated that while there is a substantial genetic polymorphism among geographically distinct *R. rickettsii* isolates from North America, there is virtually no polymorphism among human and tick isolates from Central and South America (Paddock et al., 2014; Labruna et al., 2014). These statements, coupled the notorious aggressiveness of *A. sculptum* ticks toward humans, suggest that *R. rickettsii* strain Pampulha, isolated from *A. sculptum* ticks in the present study, is the strain responsible for human clinical cases of BSF in the Pampulha Lake region of Belo Horizonte city.

Conflict of interest

The authors declare that they have no conflict of interest.

Ethical approval

This work was approved by the Ethical Committee of Animal Use of the Faculty of Veterinary Medicine of the University of São Paulo (protocol no. 5948070314). Tick collections were authorized by Instituto Chico Mendes de Conservação da Biodiversidade (Sisbio License #11459-1).

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