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Short Communication

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# Isolation of the Pathogen *Rickettsia* sp. Strain Atlantic Rainforest From Its Presumed Tick Vector, *Amblyomma ovale* (Acari: Ixodidae), From Two Areas of Brazil

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## Abstract

In this study, *Amblyomma ovale* Koch ticks were collected from domestic dogs in two localities of the Atlantic rainforest biome of Brazil: 1) the Paty Valley of the Chapada Diamantina National Park, Bahia state (northeastern Brazil), and 2) Adrianópolis, Paraná state (southern Brazil). Ticks were screened for the presence of *Rickettsia*-like structures by the hemolymph test with Giménez staining, and then processed for isolation of rickettsiae in Vero cell culture by the shell-vial technique. Rickettsiae were isolated from one *A. ovale* tick of each of the two localities. The two isolates were successfully established in the laboratory with several passages, each one reaching >90% infection of the cells. The two isolates were identified as the spotted fever group (SFG) agent *Rickettsia* sp. strain Atlantic rainforest, as their *gltA* (350 bp), *ompB* (781 bp), and *ompA* (567 bp) gene fragments were 100% equal to GenBank corresponding sequences of the original strain Atlantic rainforest, reported to be infecting a human in southeastern Brazil, and also 100% equal to the available *ompA* sequence of strain Bahia, reported to be infecting a human in Paty Valley, the same area of the present study in Bahia state. Ten dogs from Paty Valley were serologically tested against rickettsial antigens by the indirect immunofluorescence antibody test. At least 60% of them were seroreactive to SFG rickettsiae. The role of *A. ovale* as vector of *Rickettsia* sp. strain Atlantic rainforest in the Paty Valley area, as well as in other parts of Latin America, is discussed.

**Key words**: Rickettsiae, spotted fever group, Ixodidae, in vitro growth

Until 2009, the bacterium *Rickettsia rickettsii*, the etiological agent of Rocky Mountain spotted fever and Brazilian spotted fever, was the only tick-borne spotted fever group (SFG) agent known to cause human disease in Brazil (Labruna 2009). In 2010, a novel SFG agent, reported as *Rickettsia* sp. strain Atlantic rainforest, was identified as the etiological agent of spotted fever illness in a patient from an Atlantic rainforest area in the state of São Paulo, southeastern Brazil (Spolidorio et al. 2010). In this same area, an epidemiological study incriminated the tick *Amblyomma ovale* Koch as the presumed vector of *Rickettsia* sp. strain Atlantic rainforest to humans (Szabó et al. 2013a), a condition supported by a recent laboratory study (Krawczak et al. 2016).

In 2011, Silva et al. (2011) reported a case of human illness caused by *Rickettsia* sp. strain Bahia, from an Atlantic rainforest area in the state of Bahia, northeastern Brazil. Because strain Bahia shared identical DNA sequences with strain Atlantic rainforest, the case reported by Silva et al. (2011) was recognized as the second confirmed case of human illness caused by *Rickettsia* sp. strain Atlantic rainforest (Parola et al. 2013). Besides these two confirmed cases, *Rickettsia* sp. strain Atlantic rainforest has been reported infecting its presumed main vector (*A. ovale*) from Atlantic rainforest areas of southern and southeastern Brazil (Sabatini et al. 2010, Medeiros et al. 2011, Barbieri et al. 2014). More recently, this agent was reported infecting *A. ovale* ticks in the Brazilian Pantanal (Melo et al. 2016), Colombia (Londoño et al. 2014), and Belize (Lopes et al. 2016). Because of the apparent wide geographical distribution of *Rickettsia* sp. strain Atlantic rainforest in Latin America, primarily associated with the human-biting tick *A. ovale*, the occurrence of misidentified cases of human infection is very likely multiple areas of the continent.

In the present study, we performed a field study in Paty Valley, state of Bahia, the locality of the strain Bahia-caused spotted fever case reported by Silva et al. (2011). Ticks were collected from

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domestic dogs and were brought to the laboratory, where they were tested for rickettsial infection. In addition, we also evaluated ticks collected on domestic dogs from another Atlantic rainforest area in the state of Paraná, where human cases have not been reported.

## Materials and Methods

In July 2014, we visited the Paty Valley of the Chapada Diamantina National Park. While this Park is officially within the Caatinga (semi-arid) biome, the Paty Valley area (12° 47′ S, 41° 25′ W; Fig. 1) is considered to represent a fragment of the Atlantic rainforest biome (Beserra et al. 2007). Approximately 13 families used to live in the Paty Valley (Beserra et al. 2007). During our visit, we examined 10 domestic dogs from four families (the remaining families did not have dogs). When ticks were found, they were collected and brought alive to the laboratory. Under consent of dog owners, we also collected canine blood samples, from which sera were separated by centrifugation and kept frozen until being tested in the laboratory.

In August 2012, we visited a private ranch (24° 42′47″ S and 49° 08′34″ W) within Adrianópolis municipality, Paraná State, southern Brazil, where we collected ticks from six dogs that used to live in the area. These ticks were also brought alive to the laboratory.

In the laboratory, live ticks were identified to species following Barros-Battesti et al. (2006), and checked individually for the presence of *Rickettsia*-like organisms, using the hemolymph test with Giménez staining (Giménez 1964, Burgdorfer 1970), and then frozen at −80°C. Ticks that were found to be positive for *Rickettsia*-like organisms in the hemolymph test were submitted for rickettsial isolation in cell culture through the shell-vial technique, as previously described (Marrero and Raoult 1989, Labruna et al. 2004). Briefly, cultures of Vero cells were inoculated with tick-body homogenates (1 individual tick per culture) and incubated at 28°C. The percentage of Vero cells infected with rickettsiae was monitored by the use of Giménez staining of cells scraped from each inoculated monolayer. After the establishment of each isolate in the laboratory (i.e., at least three cell passages, with the prevalence of infected cells exceeding 90%), rickettsial DNA was extracted from the infected cells by using the DNeasy Blood and Tissue kit (Qiagen, Chatsworth, CA). The extracted DNA was tested in a battery of different polymerase chain reaction (PCR) protocols to amplify fragments of the rickettsial genes citrate synthase (*gltA*; primers CS-78, CS-323), and the outer membrane proteins *ompB* (primers 120-M59, 120–807), and *ompA* (primers Rr190.70p, Rr190.701), as described (Roux et al. 1996, Roux and Raoult 2000, Labruna et al. 2004). PCR products were purified using ExoSAP-IT (USB Corp., Cleveland, OH) and underwent DNA sequencing in an ABI automated sequencer (Applied Biosystems/Perkin Elmer, model ABI Prism 3500 Genetic, Foster City, CA), and the resultant sequences were compared with GenBank data by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

Canine serum samples were tested by indirect immunofluorescence antibody test using crude antigens derived from five *Rickettsia* isolates from Brazil (*Rickettsia bellii* strain Mogi, *Rickettsia amblyommii* strain Ac37, *Rickettsia rhipicephali* strain HJ5, *R. rickettsii* strain Taiaçu, and *Rickettsia parkeri* strain At24), as previously described (Labruna et al. 2007). Briefly, sera were diluted in 2-fold increments with phosphate-buffered saline, starting from the 1:64 dilution. Slides were incubated with the diluted canine sera, and thereafter, with fluorescein isothiocyanate-labeled rabbit anti-dog IgG (Sigma, St Louis, MO). For each sample, the endpoint IgG titer reacting with each of the six *Rickettsia* antigens was determined. In each slide, a serum previously shown to be nonreactive (negative control) and a known reactive serum (positive control) were tested at the 1:64 dilution.

**Fig. 1.** The Paty Valley area in the Chapada Diamantina National Park. Arrow indicates the Paty River, which is surrounded by fragments of the Atlantic rainforest biome; asterisks indicate households in which domestic dogs were sampled in the present study. (Online figure in color.)

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| **1:** | Peruíbe, São Paulo state: Infection in a human (Spolidorio et al. 2010) and *A. ovale* ticks (Szabó et al. 2013) |
| **2:** | Cubatão, São Paulo state: Infection in *A. ovale* ticks (Sabatini et al. 2010) |
| **3:** | Paty Valley, Bahia state: Infection in a human (Silva et al. 2011) and *A. ovale* tick (**PRESENT STUDY**) |
| **4:** | Several localities, Santa Catarina state: Infection in *A. ovale* ticks (Medeiros et al. 2011) |
| **5:** | Blumenau, Santa Catarina state: Infection in *A. ovale* ticks (Barbieri et al. 2014) |
| **6:** | Necocli, Antioquia: Infection in *A. ovale* ticks (Londoño et al. 2014) |
| **7:** | Poconé, Mato Grosso state: Infection in *A. ovale* ticks (Melo et al. 2015) |
| **8:** | Belize: Infection in *A. ovale* ticks (Lopes et al. 2016) |
| **9:** | Adrianópolis, Paraná state: Infection in *A. ovale* tick (**PRESENT STUDY**) |

**Fig. 2.** Localities in Latin America where *Rickettsia* sp. strain Atlantic rainforest has been reported infecting humans and *A. ovale* ticks. (Online figure in color.)

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## Results

In the Paty Valley, we examined 10 dogs, which were all infested by ticks. Three tick species were collected: *A. ovale* (four males, three females), *Amblyomma sculptum* Berlese (31 nymphs), and *Rhipicephalus sanguineus* (Latreille) sensu lato (s.l.; eight males, nine females, one nymph). Canine prevalence per tick species were 30% for *A. ovale*, 90% for *A. sculptum*, and 60% for *R. sanguineus* s.l. In Adrianópolis municipality, the five dogs were infested (100% prevalence) by 17 ticks, which were all identified as *A. ovale* (7 males and 10 females). By the hemolymph test, five *A. ovale* ticks from Paty Valley and seven *A. ovale* from Adrianópolis contained *Rickettsia*-like structures within their hemocytes. Attempts to isolate rickettsiae in Vero cell culture were performed individually with three hemolymph test-positive ticks, one *A. ovale* from Paty Valley and two *A. ovale* from Adrianópolis. Rickettsiae were isolated from one tick of each of the two localities. The two isolates were successfully established in the laboratory with several passages, each one reaching >90% infection of the cells. These isolates, designated as strains Paty and Adrianópolis, have been cryopreserved and deposited at the Rickettsial Collection of the Faculty of Veterinary Medicine, University of São Paulo, São Paulo, Brazil, and at the Rickettsial Collection of the Rickettsial Zoonoses Branch of the Centers for Disease Control and Prevention, Atlanta, GA.

Rickettsial DNA samples amplified by PCR from the isolates were sequenced and submitted to BLAST analyses. The two isolates were identified as *Rickettsia* sp. strain Atlantic rainforest, as their *gltA* (350 bp), *ompB* (781 bp), and *ompA* (567 bp) gene fragments were 100% equal to corresponding sequences (GenBank GQ855235, GQ855236, and GQ855237, respectively) of the original strain Atlantic rainforest, reported to be infecting a human in southeastern Brazil (Spolidorio et al. 2010), and 100% equal to the available *ompA* sequence of strain Bahia (GQ853063), reported to be infecting a human in Paty Valley (Silva et al. 2011), the same area of the present study in the state of Bahia, northeastern Brazil.

Among the 10 dogs sampled in the Paty Valley, six (60%) reacted serologically to four rickettsial antigens (*R. rickettsii*, *R. parkeri*, *R. amblyommii*, *R. rhipicephali*). Generally, canine endpoint titers were highest against *R. parkeri*, with the six animals displaying end-point titers ≥512, and 2 dogs with endpoint titers to *R. parkeri* at least 4-fold higher than those observed for the other *Rickettsia* species.

## Discussion

After the first case of human illness caused by *Rickettsia* sp. strain Atlantic rainforest in southeastern Brazil (Spolidorio et al. 2010), Szabó et al. (2013a) conducted an epidemiological study exactly at the same site where the patient reported to have acquired the unidentified tick that caused a skin lesion (eschar) at the bite site, from which rickettsial DNA was detected during diagnostic procedures. Szabó et al. (2013a) concluded that strain Atlantic rainforest circulates at relatively high levels among *A. ovale* ticks in the focus of human rickettsiosis of southeastern Brazil, and because *A. ovale* was recognized as the most important human-biting tick in the study region, it was assigned as the presumed vector of *Rickettsia* sp. strain Atlantic rainforest (Szabó et al. 2013b). These assumptions were reinforced by a recent laboratory study that demonstrated the vector competence of *A. ovale* for *Rickettsia* sp. strain Atlantic rainforest, and that this agent can be maintained in *A. ovale* ticks through transovarial transmission and transstadial perpetuation (Krawczak et al. 2016).

Similarly, herein we performed an ecological study at the same site where the patient reported by Silva et al. (2011) acquired the unidentified tick that caused a skin lesion (eschar) at the bite site, from which *Rickettsia* sp. strain Bahia was detected through diagnostic procedures. As reported in previous studies, this clinical case caused by strain Bahia should be considered the second confirmed case of strain Atlantic rainforest, as these two strains shared identical DNA sequences (Parola et al. 2013). Our findings of viable rickettsiae infecting *A. ovale* ticks, in conjunction with serological evidence of canine infection by SFG rickettsiae, provide epidemiological support for the role of *A. ovale* as vector of *Rickettsia* sp. strain Atlantic rainforest in the Paty Valley area. These results are indeed relevant to public health, especially because hiking activities are highly intense through the Paty Valley area (M.B.L., unpublished data). In fact, the patient reported by Silva et al. (2011) acquired the infected tick during hiking activity in the area.

We also isolated *Rickettsia* sp. strain Atlantic rainforest from another area (Adrianópolis, state of Paraná, southern Brazil), where human cases of rickettsioses have never been reported. As a whole, *Rickettsia* sp. strain Atlantic rainforest has been detected in *A. ovale* ticks from nine different areas of Latin America (Fig. 2), where this tick species has a widespread distribution (Guglielmone et al. 2003). Because *A. ovale* is one of the most common human-biting ticks in the Neotropical region (Labruna et al. 2005, Guglielmone et al. 2006), it is reasonable to speculate that humans are exposed to infection by *Rickettsia* sp. strain Atlantic rainforest in a much wider area than currently recognized.

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