Short Communication

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

CrossMark

Arannadia Barbosa Silva[a,b,1], Myrian Morato Duarte[c,1], Robson da Costa Cavalcante[d], Stefan Vilges de Oliveira[e], Vinicius Figueiredo Vizzoni[b], Ana Íris de Lima Duré[c], Felipe Campos de Melo Iani[c], Erik Machado-Ferreira[f], Gilberto Salles Gazêta[a,b,*]

[a] Fundação Oswaldo Cruz/Instituto Oswaldo Cruz/(FIOCRUZ/IOC), Programa de Pós-Graduação em Biodiversidade e Saúde, Rio de Janeiro, RJ, Brazil, Brazil
[b] Fundação Oswaldo Cruz/Instituto Oswaldo Cruz/(FIOCRUZ/IOC), Laboratório de Referência Nacional em Vetores das Riquetsioses (LIRN), Rio de Janeiro, RJ, Brazil, Brazil
[c] Fundação Ezequiel Dias (FUNED), Serviço de Virologia e Riquetsioses, Belo Horizonte, MG, Brazil, Brazil
[d] Secretaria Estadual de Saúde do Estado do Ceará, Baturité, Brazil, Brazil
[e] Secretaria de Vigilância em Saúde, Ministério da Saúde, Brasília-DF, Brazil, Brazil
[f] Universidade Federal do Rio de Janeiro (UFRJ), Laboratório de Genética Molecular de Eucariontes e Simbiontes, Rio de Janeiro, RJ, Brazil, Brazil

ARTICLE INFO

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.

Collection site

Baturite municipality,
Ceara state, Brazil

71 ┌ ▲KX356604-05| *Rickettsia* sp. strain RS#8
   └ CP003305| *Rickettsia rickettsii* str. Brazil
93 ┌ CP000848| *Rickettsia rickettsii* str. Sheila Smith
99 └ CP003308| *Rickettsia philipii* str. 364D
   └ CP001227| *Rickettsia peacockii* str. Rustic
96 ┌ LAOL01000001.1| *Rickettsia parkeri* str. AT#24
97 └ CP003341| *Rickettsia parkeri* str. Portsmouth
   └ AE006914| *Rickettsia conorii* str. Malish 7
96 ┌ AABW01000001| *Rickettsia sibirica* 246
   ├ CP002428| *Rickettsia slovaca* 13-B
   └ CP001612| *Rickettsia africae* ESF-5
   ┌ AP011533| *Rickettsia japonica* YH
100 └ CP002912| *Rickettsia heilongjiangensis* 054
100 ┌ CP003334| *Ca*. Rickettsia amblyommii str. GAT-30V
    ├ CP003340| *Rickettsia montanensis* str. OSU 85-930
    ├ CP003319| *Rickettsia massiliae* str. AZT80
99 └ CP003342| *Rickettsia rhipicephali* str. 3-7-female6-CWPP
    ┌ CP000847| *Rickettsia akari* str. Hartford
100 ├ CP000053| *Rickettsia felis* URRWXCal2
    └ CP003338| *Rickettsia australis* str. Cutlack
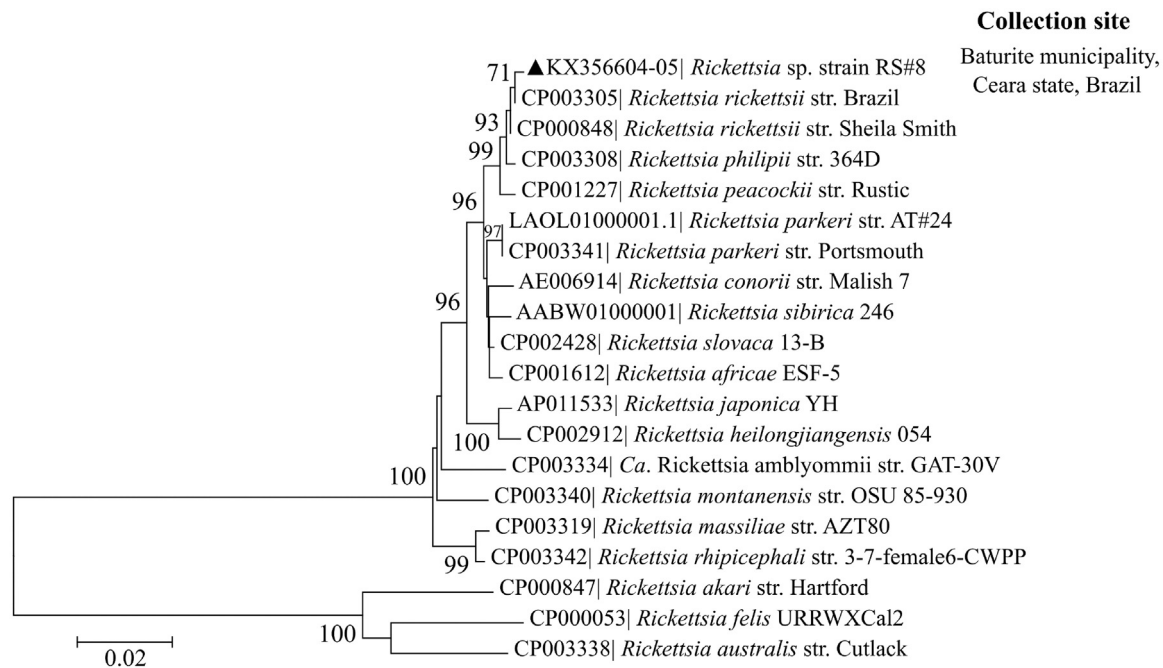
├─── 0.02 ───┤

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
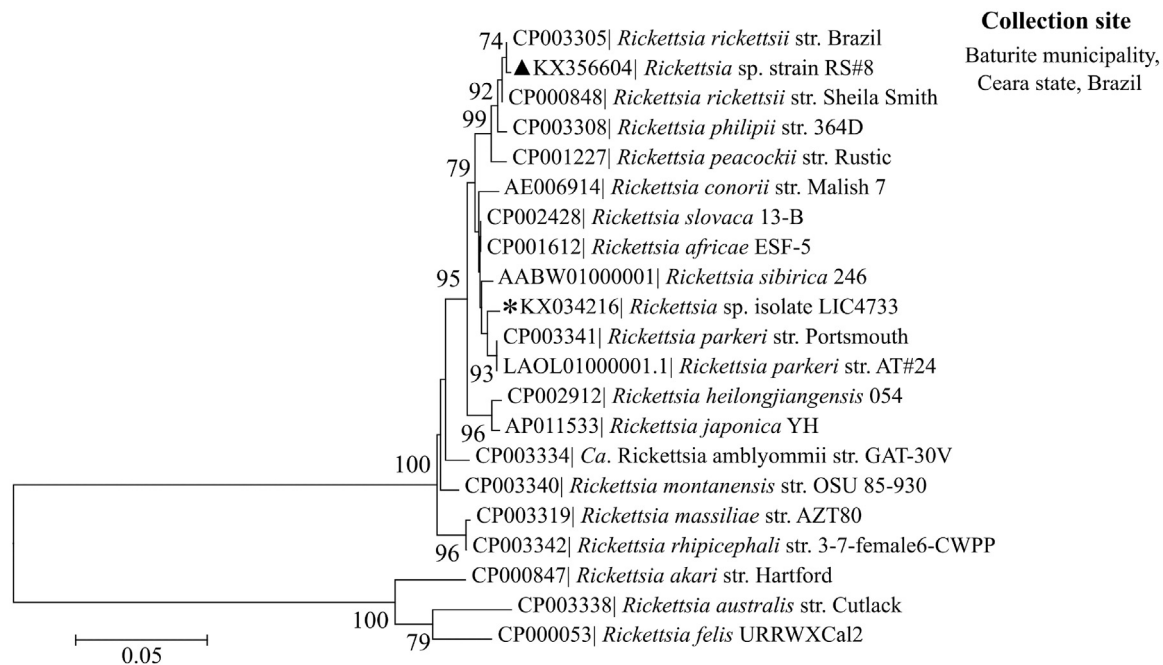
Collection site

Baturite municipality,
Ceara state, Brazil

Fig. 2. Phylogenetic tree of rickettsiae *sca4* gene constructed by the neighbor-joining method, using 2-Kimura-parameters evolutionary model with MEGA 5.2 software. Bootstrap values below 70% are not shown. The black triangle is isolate RS#8 and the star is *Rickettsia parkeri* strain Atlantic rainforest from Rio Grande do Sul State, southern Brazil.

passages in Vero cell culture.

A DNA sample of the infected Vero cells was subjected to a PCR that targeted the rickettsial genes *htrA* and *sca4*. After sequencing, a 394-nucleotide sequence of *htrA* gene, and a 720-nucleotide sequence of *sca4* gene, showed similarity values of 99% with GenBank sequences for several *R. rickettsii* isolates. For *sca4* gene, the sequence had an identity matching of 99% (703/704) with *R. rickettsii* isolates and 98% with *R. parkeri* str. Portsmouth (689/704) (CP003341), and *R. parkeri* strain Atlantic rainforest LIC4733 (601/614) (KX034216). Phylogenetic inferences obtained with a concatenated sequence (1114 bp) of *htrA* and *sca4* clustered the isolated RS#8 into the *R. rickettsii* group (Fig. 1). In addition, a phylogenetic tree was obtained with the *sca4* sequences (619 bp) of the isolate RS#8 and *R. parkeri* strain Atlantic rainforest LIC4733 from Rio Grande do Sul State, southern Brazil (Fig. 2).

4. Discussion

The current study reports the identification and isolation of a *Rickettsial* strain from a naturally infected *R. sanguineus* s.l. collected in the region of Maciço de Baturité, Ceará State, a new endemic area for SF in Brazil (Oliveira, 2006). In this region, the clinical-epidemiological characteristics of the cases indicate the occurrence of a mild SF, with *R. parkeri* strain Atlantic rainforest being the most likely pathogen (Moerbeck et al., 2016; Oliveira 2016).

Phylogenetic analysis performed with a concatenated sequence of *sca4* and *htrA* genes showed the RS#8 isolate to be most closely related to *R. rickettsii* (Fig. 1). In addition, an analysis performed using only *sca4* gene showed that the isolate was phylogenetically distant from *R. parkeri* strain Atlantic rainforest, the other pathogenic species previously identified at the collection site of the tick analyzed here (Moerbeck et al., 2016) (Fig. 2).

In Brazil, *R. sanguineus* s.l. ticks seem to be involved in some BSF cases. This tick species have been indicated as the probable vector of a *R. rickettsii* strain which caused the death of five employees of a dog shelter in an urban area in southeastern Brazil (Rozental et al., 2015). Although the reports of *R. sanguineus* s.l. parasitizing humans are not frequent in Brazil (Dantas-Torres et al., 2006; Serra-Freire et al., 2011), it should be noted that, under both laboratory and natural conditions, it has been shown that *R. sanguineus* s.l. can act as a vector for *R. rickettsii*

(Piranda et al., 2011; Eremeeva et al., 2011).

In southeast Brazil, where most BSF cases have been recorded, the disease occurs mainly within an already established ecological scenario, with high levels of *A. sculptum* environmental infestations maintained either by its principal host, the capybara (*Hydrochoerus hydrochaeris*) and horses (*Equus caballus*), or involving *A. aureolatum* and domestic dogs living close to forested areas (Szabó et al., 2013b; Souza et al., 2016; Pinter et al., 2016). Some of the ecological characteristics of *A. sculptum* in southeastern Brazil indicate that it is favored by anthropic factors and associated with the Cerrado biome (Veronez et al., 2010; Szabó et al., 2013b). However, this tick is not often found in the Atlantic rainforest biome, although it may appear soon in degraded areas of this former closed forest vegetation type (Szabó et al., 2009).

The fragmentation of natural environments due to their conversion to anthropized habitats can result in loss of biodiversity (Didham et al., 1996; Viana and Pinheiro 1998), which can lead to an ecological imbalance in parasite-host interactions, with the possible establishment of novel infectious and/or parasitic diseases (Schmidt and Ostfeld 2001; Queirogas et al., 2012). Such areas under heightened anthropic pressure are especially prone to SF cases (Corrêa and Passos, 2001; Pinter et al., 2016), notably when they meet such predisposing factors as ticks, *Rickettsia* pathogens and humans in combination.

The epidemic cycle associated with *R. rickettsii* is not yet established in the area of the Maciço de Baturité, since the vectors of *R. rickettsii* in Brazil, are currently found at very low frequency in the region (Moerbeck et al., 2016). However, it is possible that the anthropization of the environment where *R. parkeri* strain Atlantic rainforest and mild SF currently occurs, may cause a change in the clinical profile of the disease. This could result in the appearance of serious cases or deaths, due to a change in the population of potential BSF vectors in the area, and a likely increase in the circulation of *R. rickettsii*.

5. Conclusion

Although serious cases have yet to be reported in the region of the Maciço de Baturité, the detection of *R. rickettsii* in the studied area, infecting *R. sanguineus*, a species with a recognized vectorial capacity to *Rickettsia*, alert to possible changes in the profile of the disease and, consequently, to the grave impact to the public health on the region.

Thus, in conclusion, we suggest the increase of epidemiological and environmental surveillance in the area, in order to prevent BSF cases.

Acknowledgments

References

Barros-Battesti, D.M., Arzua, M., Bechara, G.H., 2006. Carrapatos de importância médico veterinário da região Neotropical. Um guia ilustrado para identificação de espécies. Modelos Biológicos. Vox; ICTTD-3; Butantan, São Paulo 223 p.

Corrêa, S.H.R., Passos, E.C., 2001. Wild animals and public health. In: Fowler, M.E., Cubas, Z.S. (Eds.), Biology, Medicine and Surgery of South American Wild Animals. Iowa University Press, Ames, pp. 493–499.

Dantas-Torres, F., Figueredo, L.A., Brandão-Filho, S.P., 2006. *Rhipicephalus sanguineus* (Acari: ixodidae), o carrapato vermelho do cão, parasitando humanos no Brasil. Rev. Soc. Bras. Med. Trop. 39, 64–67.

Didham, R.K., Ghazoul, J., Stork, N.E., Davis, A.J., 1996. Insects in fragmented forest: a functional approach. Trends Ecol. Evol. 11, 255–260.

Eremeeva, M.E., Zambrani, M.L., Anaya, L., Beati, L., Karpathy, S.E., Santos-Silva, M.M., et al., 2011. *Rickettsia rickettsii* in *Rhipicephalus* ticks, Mexicali, Mexico. J. Med. Entomol. 48, 418–421.

Giménez, D.F., 1964. Staining rickettsiae in yolk-sac cultures. Stain Technol. 39, 135–140.

Kelly, P.J., Raoult, D., Mason, P.R., 1991. Isolation of spotted fever group rickettsias from triturated ticks using a modification of the centrifugation-shell vial technique. Trans. R. Soc. Trop. Med. Hyg. 85, 397–398.

Kimura, M., 1980. A simple method for estimating evolutionary rates of base substitutions through comparative studies of nucleotide sequences. J. Mol. Evol. 16, 111–120.

Krawczak, F.S., Muñoz-Leal, S., Guztazaky, A.C., Oliveira, S.V., Santos, F.C.P., Angerami, R.N., et al., 2016a. *Rickettsia* sp. strain Atlantic rainforest infection in a patient from a spotted fever-endemic area in southern Brazil. Am. J. Trop. Med. Hyg. 95, 551–553.

Krawczak, F.S., Binder, L.C., Oliveira, C.S., Costa, F.B., Moraes-Filho, J., Martins, T.F., et al., 2016b. Ecology of a tick-borne spotted fever in southern Brazil. Exp. Appl. Acarol. 70, 219–229.

Labruna, M.B., Whitworth, T., Horta, M.C., Bouyer, D.H., Mcbride, J.W., Pinter, A., et al., 2004. *Rickettsia* species infecting *Amblyomma cooperi* ticks from an area in the State of São Paulo, Brazil, where Brazilian spotted fever is endemic. J. Clin. Microbiol. 42, 90–98.

Labruna, M.B., 2009. Ecology of *Rickettsia* in South America. Ann. N. Y. Acad. Sci. 1166, 156–166.

Melo, A.L.T., Witter, R., Martins, T.F., Pacheco, T.A., Alves, A.S., Chitarra, C.S., et al., 2016. A survey of tick-borne pathogens in dogs and their ticks in the Pantanal biome, Brazil. Med. Vet. Entomol. 30, 112–116.

Moerbeck, L., Vizzoni, V.F., Machado-Ferreira, E., Cavalcante, R.C., Oliveira, S.V., Soares, C.A., et al., 2016. *Rickettsia* (Rickettsiales: Rickettsiaceae) vector biodiversity in high altitude Atlantic forest fragments within a semiarid climate: a new endemic area of spotted-fever in Brazil. J. Med. Entomol. 53, 1458–1466.

Nava, S., Beati, L., Labruna, M.B., Cáceres, A.G., Mangold, A.J., Guglielmone, A.A., 2014. Reassessment of the taxonomic status of *Amblyomma cajennense* (Fabricius, 1787) with the description of three new species, *Amblyomma tonelliae* n. sp., *Amblyomma interandinum* n. sp. and *Amblyomma patinoi* n. sp., and reinstatement of *Amblyomma mixtum* Koch, 1844, and *Amblyomma sculptum* Berlese, 1888 (Ixodida: ixodidae). Ticks Tick Borne Dis. 5, 253–276.

Oliveira, S.V., Caldas, E.P., Colombo, S., Gazeta, G.S., Labruna, M.B., Santos, F.C.P., et al.,

2016a. A fatal case of Brazilian spotted fever in a non-endemic area in Brazil: the importance of having health professionals who understand the disease and its areas of transmission. Rev. Soc. Bras. Med. Trop. 49, 653–655.

Oliveira, S.V., Guimarães, J.N., Reckziegel, G.C., Neves, B.M.C., Araújo-Vilges, K.M., Fonseca, L.X., et al., 2016b. An update on the epidemiological situation of spotted fever in Brazil. J. Venom. Anim. Toxins Incl. Trop. Dis. 22, 22.

Oliveira, S.V., 2016. Tick-borne spotted fever in the northeast of Brazil: the series of cases a new endemic area. Rev. Med. UFC 56, 8–9.

Otto, T.D., Vasconcellos, E.A., Gomes, L.H.F., Moreira, A.S., Degrave, W.M., Mendonça-Lima, L.M., et al., 2008. ChromaPipe: a pipeline for analysis, quality control and management for a DNA sequencing facility. Genet. Mol. Res. 7, 861–871.

Paddock, C.D., Allerdicel, M.E.J., Karpathyl, S.E., Nicholson, W.L., Levinl, M.L., Smith, T.C., et al., 2017. Unique strain of *Rickettsia parkeri* associated with the hard tick *Dermacentor parumapertus* Neumann in the western United States. Appl. Environ. Microbiol. 83, e03463–16.

Piranda, E.M., Faccini, J.L.H., Pinter, A., Pacheco, R.C., Cançado, P.H.D., Labruna, M.B., 2011. Experimental infection of *Rhipicephalus sanguineus* ticks with the bacterium *Rickettsia rickettsii*, using experimentally infected dogs. Vector Borne Zoonotic. Dis. 11, 29–36.

Queirogas, V.L., Del Claro, K., Nascimento, A.R., Szabó, M.P., 2012. Capybaras and ticks in the urban areas of Uberlândia, Minas Gerais, Brazil: ecological aspects for the epidemiology of tick-borne diseases. Exp. Appl. Acarol. 57, 75–82.

Rozental, T., Ferreira, M.S., Gomes, R., Costa, C.M., Barbosa, P.R.A., Bezerra, I.O., et al., 2015. A cluster of *Rickettsia rickettsii* infection at an animal shelter in an urban area of Brazil. Epidemiol. Infect. 143, 2446–2450.

Santos, F.L.A., Medeiros, E.M., Souza, M.J.N., 2012. Contexto Hodroclimático do Enclave Úmido do Maciço de Baturité-Ceará: Potencialidades e Limitações ao Uso da Terra. Ver Geon 2, 1056–1065.

Schmidt, K.A., Ostfeld, R.S., 2001. Biodiversity and the dilution effect in disease ecology. Ecology 83, 609–619.

Sekeyova, Z., Roux, V., Raoult, D., 2001. Phylogenu of *Rickettsia* spp. inferred by comparing sequences of 'geneD', which enconders an intracytoplasmic protein. Int. J. Syst. Evol. Microbiol. 51, 1353–1360.

Serra-Freire, N.M., Sena, L.M.M., Borsoi, A.B.P., 2011. Parasitismo Humano por Carrapatos na Mata Atlântica, Rio de Janeiro, Brasil. EntomoBrasilis 4, 67–72.

Silva, N., Eremeeva, M.E., Rozental, T., Ribeiro, G.S., Paddock, C.D., Ramos, E.A.G., et al., 2011. Eschar-associated spotted fever Rickettsiosis, Bahia, Brazil. Emerg. Infect. Dis. 17, 27–28.

Souza, C.E., Camargo, L.B., Pinter, A., Donalisio, M.R., 2016. High seroprevalence for *Rickettsia rickettsii* in equines suggests risk of human infection in silent areas for the brazilian spotted fever. PLoS One 11, e0153303.

Spolidorio, M.G., Labruna, M.B., Mantovani, E., Brandao, P.E., Richtzenhain, L.J., Yoshinari, N.H., 2010. Novel spotted fever group rickettsiosis, Brazil. Emerg. Infect. Dis. 16, 521–523.

Szabó, M.P.J., Labruna, M.B., Garcia, M.V., Pinter, A., Castagnolli, K.C., Pacheco, R.C., et al., 2009. Ecological aspects of free-living ticks (Acari: Ixodidae) on animal trails in an Atlantic Rainforest of southeastern Brazil. Ann. Trop. Med. Parasitol. 103, 57–72.

Szabó, M.P.J., Nieri-Bastos, F.A., Spolidorio, M.G., Martins, T.F., Barbieri, A.M., Labruna, M.B., 2013a. *In vitro* isolation from *Amblyomma ovale* (Acari: Ixodidae) and ecological aspects of the Atlantic rainforest *Rickettsia*, the causative agent of a novel spotted fever rickettsiosis in Brazil. Parasitology 140, 719–728.

Szabó, M.P.J., Pinter, A., Labruna, M.B., 2013b. Ecology, biology and distribution of spotted-fever tick vectors in Brazil. Front. Cell. Infect. Microbiol. 3, 1–9.

Tamura, K., Peterson, D., Peterson, N., Stecher, G., Nei, M., Kumar, S., 2011. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Mol. Biol. Evol. 28, 2731–2739.

Veronez, V.A., Freitas, B.Z., Olegário, M.M.M., Carvalho, W.M., Pascoli, G.V.T., Thorga, K., et al., 2010. Ticks (Acari: Ixodidae) within various phytophysiognomies of a cerrado reserve in Uberlândia, Minas Gerais, Brazil. Exp. Appl. Acarol. 50, 169–179.

Viana, V.M., Pinheiro, L.A.F.V., 1998. Conservação da biodiversidade em fragmentos florestais. Série Técnica IPEF 12, 25–42.

Vizzoni, V.F., Silva, A.B., Cardoso, K.M., Santos, F.B., Stenzel, B., Amorim, M., et al., 2016. Genetic identification of *Rickettsia* sp. strain Atlantic rainforest in an endemic area of a mild spotted fever in Rio Grande do Sul State, Southern Brazil. Acta Trop. 162, 142–145.

Webb, L., Carl, M., Malloy, D.C., Dasch, G.A., Azad, A.F., 1990. Detection of murine typhus infection in fleas by using the polymerase chain reaction. J. Clin. Microbiol. 28, 530–534.