

Serological survey of *Rickettsia* in equids from Vale do Paraíba, São Paulo, Brazil, and their tick identification and molecular investigation of *Rickettsia*

Levantamento sorológico de *Rickettsias* em equinos no Vale do Paraíba, São Paulo, Brasil. Identificação e pesquisa molecular de *Rickettsias* em carrapatos

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ABSTRACT

Brazilian spotted fever is a serious and lethal illness for humans and is caused by the *Rickettsia rickettsii* bacteria. In the state of São Paulo/SP (Brazil), the etiologic agent of this disease is transmitted by the *Amblyomma sculptum* tick. It was already shown that horses infected with this bacteria produce a strong immune response and could be important sentinels for the detection of the disease in a proper region. The present investigation performed a serological survey in horses from five farms of Vale do Paraíba, São Paulo state, Brazil, searching for antibodies against, *Rickettsia rickettsii*, *Rickettsia parkeri*, *Rickettsia amblyommatis*, *Rickettsia rhipicephali*, and *Rickettsia bellii*. In each farm, ticks were also collected that were taxonomically identified and examined by real-time PCR for *Rickettsia* spp DNA. Blood samples were collected from 206 horses, and 334 ticks were picked up from these animals from January to December 2017. Eighty ticks were *A. sculptum* and 254 *Dermacentor nitens*. Of the blood samples, 7.3% seroconverted to *Rickettsia* spp. Of these, 0.97% had a positive serological response to *R. bellii*. None of the 80 *A. sculptum* ticks were positive through real-time PCR for *Rickettsia* spp. Although there was no detection of ticks infected by *Rickettsia* spp in five farms of Paraíba Valley, the horses presented serological positive reactions against this agent. Thus, further large studies should be conducted in the area targeting hosts and vectors to generate data for control measures of the transmission of Brazilian spotted fever.

Keywords: Ticks. *Amblyomma*. *Dermacentor*. qPCR. Host.

RESUMO

A febre maculosa brasileira é uma doença grave e letal para seres humanos causada pela bactéria *Rickettsia rickettsii*. No estado de São Paulo, SP, Brasil, o agente etiológico desta enfermidade é transmitido pelo carrapato *Amblyomma sculptum*. Conforme descrito na literatura científica, os cavalos infectados com esta bactéria produzem uma forte resposta imune e podem ser importantes sentinelas para a detecção da doença. A presente investigação realizou um levantamento sorológico em cavalos de cinco fazendas do Vale do Paraíba, São Paulo, Brasil, à procura de anticorpos contra *Rickettsia rickettsii*, *Rickettsia parkeri*, *Rickettsia amblyommatis*, *Rickettsia rhipicephali* e *Rickettsia bellii*. Em cada fazenda, também foram coletados carrapatos identificados taxonomicamente e examinados por PCR em tempo real para o DNA de *Rickettsia* spp. Foram coletadas amostras de sangue de 206 cavalos e coletados 334 carrapatos desses animais entre os meses de janeiro e dezembro de 2017. Oitenta carrapatos foram identificados como *A. sculptum* e 254 *Dermacentor nitens*. Das amostras de sangue, 7,3% soroconverteram para *Rickettsia* spp., sendo que, 0,97% apresentaram soropositividade homóloga para *R. bellii*. Nenhum dos 80 carrapatos de *A. sculptum* foi positivo com o emprego de PCR em tempo real para *Rickettsia* spp. Embora não tenham sido detectados carrapatos infectados por *Rickettsia* spp em cinco fazendas do Vale do Paraíba, os animais apresentaram reações sorológicas positivas para este agente. Assim, outros estudos abrangentes deverão ser realizados na área investigando hospedeiros e vetores, gerando dados para medidas de controle da transmissão da febre maculosa brasileira.

Palavras-chave: Carrapatos. *Amblyomma*. *Dermacentor*. qPCR. Hospedeiro.

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Introduction

Brazilian spotted fever (BSF) is a tick-borne zoonosis caused by the bacterium *Rickettsia rickettsii*, first described in the United States, where the disease was called Rocky Mountain spotted fever (Ricketts, 1909). In Brazil, the disease was first described in 1929 in São Paulo (Piza, 1932) and its transmission was confirmed by tick species of *Amblyomma sculptum* (former *Amblyomma cajennense*) (Lemos-Monteiro et al., 1932).

The primary hosts for *A. sculptum* in endemic areas for BSF are capybaras (*Hydrochoerus hydrochaeris*) and horses (*Equus caballus*) (Aragão, 1936; Vieira et al., 2004). Equines are animals that can play a fundamental role in the epidemiological chain of BSF because they have free movement and can disseminate infected ticks, spreading the disease to different regions (Cardoso et al., 2006; Medeiros et al., 2013). Ueno et al. (2016) evaluated horses after either intravenous inoculation of *R. rickettsii* or infestation by *R. rickettsii*-infected ticks, demonstrating that, in both cases, this bacteria was able to infect horses, inducing immune response, but without bacteremia and clinical manifestations of the disease. These experimentally infected horses were not a source of infection for the uninfected ticks that had fed on them during *R. rickettsii* infection. Therefore, Ueno et al. (2016) demonstrated that although horses were not amplifying hosts of *R. rickettsii* for ticks, these animals are efficient sentinels for the epidemiological surveillance of rickettsial diseases, since they develop an effective and lasting humoral immune response after *R. rickettsii* infection.

The Paraíba Valley, in the state of São Paulo, Brazil, is an area where rural tourism is of significant economic importance, with several horse stables and riding centers.

It is an area characterized by abundant vegetation and the presence of wild animals, such as capybaras, which may favor the maintenance of tick species vectors of Brazilian spotted fever and, consequently, *Rickettsia* spp. The region has presented human cases of Brazilian spotted fever in the last 10 years, with emphasis on the cities of São José dos Campos and Jacarei, with disease lethality rates of 50% and 100%, respectively (Centro de Vigilância Epidemiológica Prof. “Alexandre Vranjac, 2019).

Thus, this work performed a serological survey of *Rickettsia* spp antibodies in horses of farms located in the Paraíba Valley, São Paulo state, Brazil, collected ticks from these horses, and investigated the presence of *Rickettsia* spp DNA on them by real time PCR.

Material and Methods

The population of this study was horses from five intensive farms located in the Vale do Paraíba of the state of São Paulo. The farms were named as A (23°17'46.87”S; 46°01'05.98”O), B (23°17'57.55”S; 46°01'11.97”O), C (23°05'53.42”S; 46°31'03.93”O), D (23°16'07.46”S; 46°14'07.40”O) and E (23°15'53.46”S; 45°57'30.03”O), according to Figure 1.

The region has a subtropical climate, with rainy and warm summers, and dry and cool winters. The local vegetation is a remaining of Atlantic Rainforest, with annual temperatures ranging from 18 to 19 °C (Martinelli, 2010).

All field activities were performed with consent of animal owners and all procedures were authorized by Ethics Committee of Universidad Santo Amaro, protocol number 31/2016. Blood samples and ticks were collected from January to December 2017.

Blood samples were aseptically obtained by jugular venipuncture with 30x7 mm needle and 5 ml syringe, being kept at room temperature and then centrifuged to obtain the serum, which was aliquoted and stored at -20° C until the time of processing. From the 206 equines examined, 136 were from farm A, five from farm B, 11 farm C, 43 farm D, and 11 farm E.

Equine sera were tested by immunofluorescence assay (Horta et al., 2004). The used antigens were *R. rickettsii* (Taiacu strain), *R. parkeri* (At24 strain), *R. bellii* (Mogi strain), *R. amblyommatis* (published as *Rickettsia amblyommii*) (Ac37 strain) and *R. rhipicephali* (HJ#5 strain), maintained at the FMVZ-USP Parasitic Disease Laboratory, in cell culture (vero cells) (Labruna et al., 2004, 2007; Pinter & Labruna, 2006; Silveira et al., 2007). Samples that had a positive reaction at the screening dilution (1/64) were retested for determination of the endpoint titer (Horta et al., 2004).

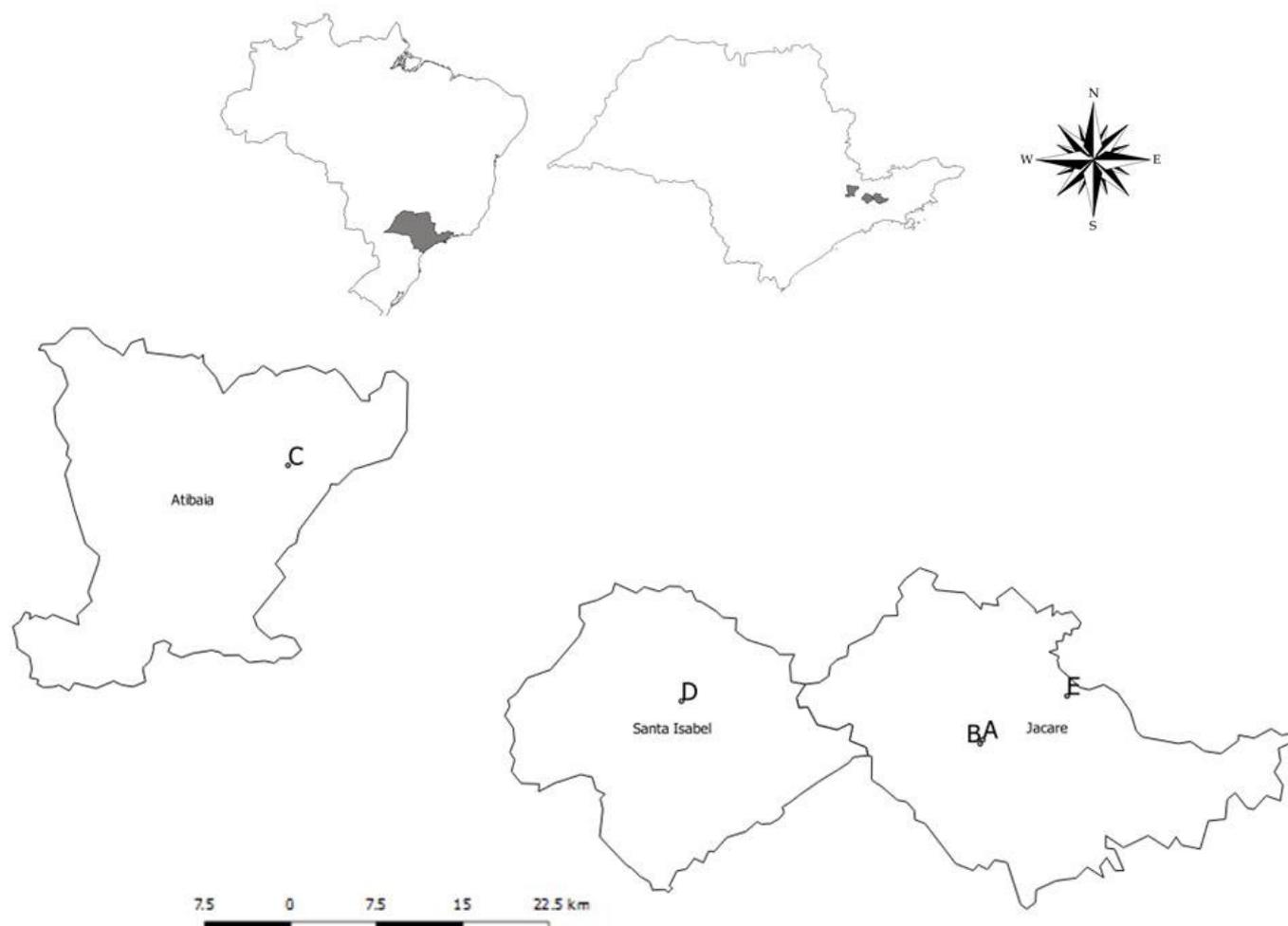


Figure 1 – Map with the locations of collection points.

The collected ticks were kept in bottles with 70° alcohol, identified according to Barros-Battesti et al. (2006) and processed individually in the laboratory for DNA extraction, according to Sangioni et al. (2005). For detection of *Rickettsia* spp. through real-time TaqMan PCR, each extracted DNA sample was tested according to Guedes et al. (2005) and Labruna et al. (2004).

Results

The immunofluorescence assay detected antibodies in 15 (7.3%) samples for at least one of the antigens analyzed: a) nine (4.4%) against *R. rickettsii*, with titers ranging from 64 to 2,048; b) six (2.9%) against *R. parkeri*, titers ranging from 64 to 1,024; c) eight (3.9%) against *R. belli*, titers ranging from 64 to 1,024; d) 11 (5.4%) against *R. amblyommatis*, titers ranging from 64 to 1,024; e) eight (3.9%) against *R. rhipicephali*, titers ranging from 64 to 1,024.

Some samples obtained from all collection sites presented titers for a given antigen at least four times higher when

compared to the values found for the other four etiologic agents analyzed, which is defined as most probable homologous antigen (PHA) (Ueno et al., 2016). For the other animals, it was not possible to determine which was the most probable infecting agent, since they presented similar values for the five antigens analyzed. Of the seroconverted samples for at least one species of *Rickettsia*, 13.4% (2/15) had probable *R. belli* homologous antigen (Table 1).

Ticks (n=334) were collected only from horses of farms A (n=253) and D (n=81).

All of the ticks collected in farm A were taxonomically identified as *Dermacentor nitens*, with eight nymphs, two larvae, 158 females and 85 males.

The ticks collected in farm D were identified as 80 *Amblyomma sculptum*: 17 males, 57 females and six nymphs, and one *D. nitens* identified as a female.

All total of 80 tick specimens of the species *A. sculptum* were tested by real-time PCR for *Rickettsia* spp and none of them contained rickettsial DNA.

Table 1 – Titers of positive samples of horses from Vale do Paraíba, SP, obtained by immunofluorescence assay against five different *Rickettsia* spp antigens. Blood collections performed from January to December 2017

| Sample | Farm | <i>R. rickettsii</i> | <i>R. parkeri</i> | <i>R. bellii</i> | <i>R. amblyommatis</i> | <i>R. rhipicephali</i> | PHA* |
|--------|------|----------------------|-------------------|------------------|------------------------|------------------------|-----------------------|
| 3 | A | 2048 | 1024 | 64 | 1024 | 1024 | <i>Rickettsia</i> spp |
| 5 | A | 128 | Negative | Negative | 128 | 128 | <i>Rickettsia</i> spp |
| 20 | A | 512 | 64 | Negative | 512 | 256 | <i>Rickettsia</i> spp |
| 34 | A | Negative | Negative | 512 | 256 | 128 | <i>Rickettsia</i> spp |
| 65 | A | 128 | Negative | Negative | 64 | Negative | <i>Rickettsia</i> spp |
| 78 | A | Negative | Negative | Negative | 64 | Negative | <i>Rickettsia</i> spp |
| 81 | A | Negative | Negative | 1024 | Negative | Negative | <i>R. bellii</i> |
| 83 | A | 512 | 64 | 512 | 128 | 64 | <i>Rickettsia</i> spp |
| 87 | A | Negative | Negative | 64 | Negative | Negative | <i>Rickettsia</i> spp |
| 97 | A | Negative | Negative | Negative | 64 | Negative | <i>Rickettsia</i> spp |
| 98 | A | 128 | 256 | 1024 | 256 | 1024 | <i>Rickettsia</i> spp |
| 116 | A | 128 | 64 | 256 | 256 | 128 | <i>Rickettsia</i> spp |
| 119 | A | Negative | Negative | 128 | Negative | Negative | <i>R. bellii</i> |
| 120 | A | 64 | 256 | Negative | 64 | 512 | <i>Rickettsia</i> spp |
| 148 | C | 64 | Negative | Negative | Negative | Negative | <i>Rickettsia</i> spp |

*PHA: probable homologous antigen.

Discussion

The Paraíba Valley, in the state of São Paulo, Brazil is an area where rural tourism is of significant economic importance, with several horse stables and riding centers. It is an area characterized by abundant vegetation and the presence of wild animals, such as capybaras, which may favor the maintenance of tick species vectors of Brazilian spotted fever and, consequently, *Rickettsia* spp. The region presented human cases of BSF in recent years, with lethality in some municipalities of 100% (Centro de Vigilância Epidemiológica Prof. “Alexandre Vranjac, 2019).

Equines are primary hosts of *A. sculptum* in the state of São Paulo, and can seroconvert after infestation by ticks containing *Rickettsia* bacteria, demonstrating that, although they do not present clinical alterations, they are able to develop a humoral immune response, which enables them to be used as an excellent sentinel for BSF surveillance (Labruna et al., 2002; Ueno et al., 2016).

Although property A presented horses with serological positive responses to *Rickettsia* spp., no positive ticks were found on this farm. In farm D, where *A. sculptum* ticks were found, none of the 43 horses tested presented any positive result to *Rickettsia* spp., and the ticks were negative by PCR for rickettsia detection. After the results presented in these two studied areas, further studies must be performed to obtain a better knowledge about the probable vector responsible for the transmission of *Rickettsia* bacteria to these animals.

In farm C, no ticks were found, but one animal reacted serologically to *Rickettsia* spp., indicating the possibility of the animal becoming infected in an area outside the farm boundaries. Both farms B and E did not have animals with positive responses to *Rickettsia* and no ticks were found during the blood collection. This is probably related to

the fact that these farms were smaller, with easier animal handling and more intensive tick control.

The animals that responded serologically to *Rickettsia* probably did not become infected on their properties because the vectors of the disease were not found in these places, raising the suspicion that the infection could happen during exhibitions that the creators participated in or in walks in the region, mainly in trails and forest areas, which would facilitate tick contact with these animals.

In this work, a sample of 206 horses were tested for seroreactivity for five species of *Rickettsia*, indicating two seropositive horses homologous to the *R. bellii* antigen and most of the seroreactivity to the species of the spotted fever group were probably cross reactions, indicating that the animals probably were not exposed to agents of the macular fever group. *R. bellii* has been reported as the species of *Rickettsia* most commonly infecting ticks in Brazil (Krawczak et al., 2018; Labruna et al., 2011). This bacteria belongs to a rickettsial basal group that includes a variety of closely related agents infecting leeches, insects, protozoa, or even plants (Murray et al., 2016; Weinert et al., 2009), making it possible that these other organisms could be related to the seropositivity of *R. bellii* in the present study, a condition still not investigated.

Medeiros et al. (2013) demonstrated the occurrence of cross-reaction between different species of *Rickettsia* in horses. Positive serological responses in horses in areas with no tick exposure or negative serology in equines with tick exposure were described in different areas in Brazil (Batista et al., 2010; Pacheco et al., 2011).

Freitas et al. (2010) collected blood samples from 75 carter horses in São José dos Pinhais and found 9.33% positivity in the animals, with titers between 64 and 1,024 in the indirect immunofluorescence test for *Rickettsia* spp.

In the present study, a positivity of 6.25% was found, showing that serological studies with intensively raised animals for *Rickettsia* positivity are lower in this way of breeding to a greater parasitic control in these animals in relation to the vectors of the disease, where tick infestations in these animals are smaller when compared to animals that live extensively.

The present study obtained results similar to those of other investigators who carried out serological studies for the presence of anti-*Rickettsia* spp., where it was found that it is common for the horse to cross-react with more than one type of *Rickettsia* spp. Also found in these studies were animals that reacted to the serological test for the presence of anti-*Rickettsia* spp. in places where the incriminated vectors of the disease were not found, contrasting with places where there were positive arthropods in real-time PCR for *Rickettsia* spp. and no seropositive animals (Batista et al., 2010; Medeiros et al., 2013; Toledo et al., 2009).

Areas that are used for ecotourism or human recreation and have occurrence of tick species known to be disease

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vectors require greater attention given the risk of infection and disease severity. Thus, further large studies should be conducted in the area targeting hosts and vectors to generate data for control measures of the transmission of Brazilian spotted fever.

Conflict of Interest

No conflicts.

Ethics Statement

The scientific research was authorized by the Ethics Committee of the Santo Amaro University (CEUA-UNISA), with approval protocol number 31/2016.

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of results and elaboration of the scientific text. Fernanda Nieri-Bastos: analysis of the results found, elaboration of the scientific text; Arlei Marcili: assistance in conducting molecular tests, analysis of results and elaboration of the scientific text. Marcelo Bahia Labruna: assistance in carrying out serological and molecular tests, analysis of results and elaboration of the scientific text. Jonas Moraes-Filho: conducting serological and molecular tests of the collected samples, analysis of the results found, elaboration of the scientific text, responsible for the scientific project and obtaining financial resources.