

Serological evidence of *Rickettsia* in horses from a semi-arid Brazilian region

Evidência sorológica de *Rickettsia* em cavalos da região semiárida do Brasil

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How to cite: Farias IF, de Souza EAR, Serpa MAC, Palha FS, de Oliveira GMB, Labruna MB, et al. Serological evidence of *Rickettsia* in horses from a semi-arid Brazilian region. *Braz J Vet Parasitol* 2021; 30(2): e026220. <https://doi.org/10.1590/S1984-29612021011>

Abstract

Brazilian spotted fever (BSF) is a common tick-borne disease caused by *Rickettsia rickettsii*. Horses are the primary hosts of the main vector, *Amblyomma sculptum*, and are considered efficient sentinels for circulation of *Rickettsia*. Therefore, the aim of this study was to detect antibodies reactive to *R. rickettsii* antigens in horses from a non-endemic area in the north-central region of Bahia state, Brazil. Blood samples and ticks were collected from 70 horses from the municipalities of Senhor do Bonfim, Antônio Gonçalves, Pindobaçu, and Campo Formoso. The sera obtained were tested by an indirect immunofluorescence assay to detect anti-*Rickettsia* antibodies. Overall, 5.7% (4/70) of the horses reacted to *R. rickettsii* antigens. Ticks were collected from 18.6% (13/70) of the horses and were identified as *A. sculptum* and *Dermacentor nitens*. Despite being a non-endemic area, seropositive animals were identified in our study, suggesting circulation of rickettsial agents in the region. This is the first serological survey of this agent in horses from the north-central region of Bahia, and further studies are needed to understand the epidemiology of BSF in these locations.

Keywords: *Amblyomma sculptum*, Brazilian spotted fever, rickettsiosis, horse.

Resumo

A Febre Maculosa Brasileira (FMB) é uma importante zoonose causada por *Rickettsia rickettsii*, sendo seu vetor o *Amblyomma sculptum*. Cavalos são os principais hospedeiros desse carrapato e considerados sentinelas eficientes para detectar a circulação de *Rickettsia*. O objetivo deste estudo foi detectar anticorpos reativos a antígenos de *R. rickettsii* em cavalos de uma área não-endêmica na região Centro-Norte da Bahia, Brasil. Foram coletadas amostras de sangue e carrapatos de 70 cavalos nos municípios de Senhor do Bonfim, Antônio Gonçalves, Pindobaçu e Campo Formoso. Os soros obtidos foram testados pela reação de imunofluorescência indireta para detectar anticorpos anti-*Rickettsia*. No geral, 5,7% (4/70) dos cavalos reagiram sorologicamente para antígenos de *R. rickettsii*. Os carrapatos foram coletados em 18,6% (13/70) dos cavalos e foram identificados como *A. sculptum* e *Dermacentor nitens*. Embora seja uma área não endêmica, foram observados animais soropositivos, sugerindo a circulação de riquetsias na região estudada. Este é o primeiro estudo de levantamento sorológico desse agente em cavalos do Centro-Norte da Bahia. São necessários mais estudos para compreender a epidemiologia da FMB nesses locais.

Palavras-chave: *Amblyomma sculptum*, febre maculosa brasileira, riquetsioses, cavalo.

Received November 11, 2020. Accepted January 25, 2021.

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Brazilian spotted fever (BSF) is a common tick-borne disease caused by the bacterium *Rickettsia rickettsii* and is considered the most important rickettsial disease in Brazil due to > 50% fatality rates (Luz et al., 2019). This disease is endemic in areas of the Atlantic Forest biome in southeastern and southern Brazil (Moraes-Filho et al., 2009). The Brazilian territory has adopted a compulsory notification for BSF since 2001 (Ordinance N°. 1,943 of 18/10/2001) due to its growing prevalence (Brasil, 2001). The Notifiable Diseases Information System (SINAN) confirmed 2,127 cases of BSF between 2007 and 2019. The southeastern region, especially the state of São Paulo, has the maximum number of cases (n=1,555), followed by the southern region with 520 confirmed cases. In addition to BSF, cases of a mild spotted fever caused by the bacterium *Rickettsia parkeri* have also been reported in humans in different areas of the Atlantic Forest biome. This condition has been reported in the northeastern region in addition to the southeastern and southern regions of the country (da Paixão Sevá et al., 2019).

Areas in the northeast are considered non-endemic for BSF, possibly due to the lack of suspicion or diagnosis (Oliveira, 2016). However, infection caused by *R. parkeri* in the Atlantic Forest has been confirmed among humans in the state of Bahia in the municipality of Ilhéus (da Paixão Sevá et al., 2019), Chapada Diamantina National Park (Silva et al., 2011), and in the state of Ceará in the municipalities of Aratuba, Baturité, Guaramiranga, and Pacoti (Oliveira, 2016). In the endemic area of Ilhéus, seroreactivity of dogs and horses for spotted fever group rickettsiae have also been reported (Oliveira et al., 2019).

The vectors of BSF are ticks of the species *Amblyomma aureolatum*, identified only in the São Paulo metropolitan region, and *Amblyomma sculptum*, which is responsible for transmitting the highest number of cases. *A. sculptum* is the most common human-biting tick in Brazil and the main vector of *R. rickettsii*, and is distributed mainly in the Cerrado and Pantanal biomes and in degraded areas of the Atlantic Forest biome (Moraes-Filho et al., 2009). In contrast, there are limited records of the presence of *A. sculptum* in the Caatinga biome, for example, in the north-central region of Bahia state, which represents a narrow invagination of the tropical climate (Martins et al., 2016). However, there is no information regarding tick-borne rickettsiae in this remote region.

Horses and capybaras are among the most common hosts for all parasitic stages of *A. sculptum*, especially in human-modified landscapes (Luz et al., 2019). Horses do not play a direct role in the epidemiology of BSF, since they do not develop rickettsemia or serve as a source of *R. rickettsii* for ticks (Ueno et al., 2016). On the other hand, horses elicit an effective humoral response on exposure to *R. rickettsii*-infected ticks; therefore, horses are considered suitable sentinels for epidemiological surveillance of BSF (Ueno et al., 2016).

BSF can be confused with other febrile and exanthematic diseases in humans (Traeger et al., 2015). Owing to the scarcity of studies, knowledge of the circulation of spotted fever group (SFG) rickettsiae in the northeastern region of Brazil is of great importance, especially where the tick vector has already been identified. Thus, the aim of this study was to assess the prevalence of anti-*Rickettsia* antibodies in horses in a BSF-non-endemic area within the semi-arid Caatinga biome, where the presence of *A. sculptum* was previously reported by Martins et al. (2016).

This study was approved by the Committee on Ethics in the Use of Animals (CEUA) under protocol no. 0004/180418. It was carried out in the microregion of Senhor do Bonfim, located in the north-central area of the state of Bahia, Northeastern Brazil, composed of nine municipalities, four of which were included in this study: Senhor do Bonfim (10° 27' 46" S; 40° 11' 27" W), Antônio Gonçalves (10° 34' 12" S; 40° 16' 20" W), Pindobaçu (10° 44' 46" S; 40° 21' 28" W), and Campo Formoso (10° 30' 41" S; 40° 19' 21" W) (Figure 1). This study region belongs to the Caatinga biome; however, this area has tropical climate in contrast to the general semi-arid climate in the biome (Martins et al., 2016).

Blood was collected from 70 horses selected according to convenience, aged between 9 and 180 months old, from May 2017 to July 2017, based on the availability of rural owners in the region. After physically restraining the animals, blood samples were collected from the jugular vein after disinfection with 70% alcohol, by using a 25 mm × 8 mm sterile needle. Blood samples were stored in sterile glass tubes without anticoagulant at room temperature. After clot retraction (two to six hours), the samples were centrifuged at 5,000 g for 15 minutes to obtain sera, which were stored at -20 °C until laboratory analysis.

Horse sera were tested for the presence of antibodies reactive to SFG rickettsiae by the indirect immunofluorescence assay, with *R. rickettsii* crude antigens as described in previous studies (Horta et al., 2007). Briefly, *R. rickettsii* strain Taiaçu was grown in Vero cells and harvested when nearly 100% of the cells were infected. The infected cells were centrifuged at 12,000 g for 10 minutes and the pellet was washed in 0.1 M phosphate-buffered saline (PBS, pH 7.4), centrifuged again, and resuspended in PBS containing 1% bovine calf serum (Sigma, St Louis, USA), and 0.1% sodium azide (Sigma, St Louis, USA). Ten microliters (≈500 rickettsiae-infected cells) were applied onto each well of 12-well slides. The antigens on the slides were air-dried and then fixed in acetone for

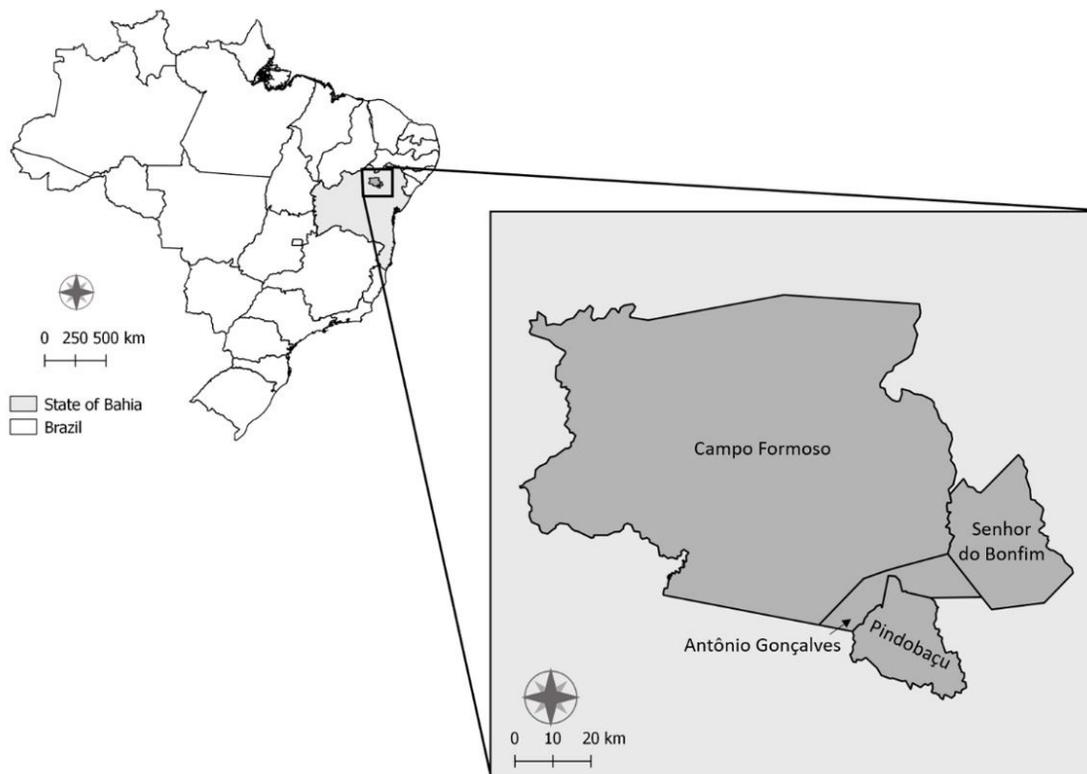


Figure 1. Municipalities of Antônio Gonçalves, Campo Formoso, Pindobaçu, and Senhor do Bonfim located in the north-central area of the state of Bahia, Brazil.

10 minutes. Slides were kept at -20°C until used. All procedures of antigen preparation were performed within a class II biosafety cabinet.

Horse sera were diluted in twofold increments with PBS starting from the 1:64 dilution. Ten microliters of diluted sera were added to each well of the antigen slides. The slides were incubated at 37°C for 30 minutes in a humidity box. The slides were rinsed once, then washed twice for 10 minutes per wash in PBS. The slides were incubated with fluorescein isothiocyanate-labeled goat anti-horse IgG (Sigma, St Louis, USA), and then mounted with buffered glycerin under coverslips. The slides were read using an ultraviolet microscope (Olympus, Tokyo, Japan) at 400x magnification. Serum was considered to contain antibodies against the rickettsiae if it displayed a reaction at the 1:64 dilution; however, sera reactive at this dilution were titrated to determine the endpoint titer.

Sampled horses were also examined for the presence of ticks, which were collected and stored in microtubes containing holes in the lid to maintain viability. Taxonomic identification of the adult ticks was performed according to Nava et al. (2014). At the time of sample collection, horse owners answered a questionnaire with information about the horses, such as sex, functionality (sports activities, walking, and/or work), the environment in which they lived, and the type of breeding. Breeding was classified as extensive, semi-extensive, and intensive systems. In the extensive breeding system, horses were reared in native fields with access to forest areas and streams, where they were potentially in contact with wild animals (such as marsupials, rodents, carnivores). In semi-intensive breeding, the animals spent one part of the day in stalls and the other in native fields. In the intensive system, the animals were always in the stall, with no access to native fields.

A total of 70 horses were evaluated (21 females and 49 males), from the municipalities of Antônio Gonçalves (8), Campo Formoso (49), Pindobaçu (7), and Senhor do Bonfim (6). Anti-*Rickettsia* reactive antibodies were detected in 5.7% (4/70) of the sampled horses, with endpoint titers varying from 128 to 2,048 (Table 1). Of these, two horses belonged to the Pindobaçu and two to the Campo Formoso municipalities. These four seropositive horses were three males and one female, aged between 48 and 144 months old, and were bred extensively for varied purposes (sports, reproduction, and walking), and did not have history of travelling to other municipalities. Although ticks were not seen on any of the seropositive horses, owners reported that all horses had already presented ticks at some moment in their lives.

Table 1. Number of seroreactive horses to *Rickettsia* and the number of ticks collected from the horses, according to the municipalities in the state of Bahia, Brazil.

Municipalities	Seroreactive horses (%)	Tested horses	Number of ticks from horses	
			<i>Amblyomma sculptum</i>	<i>Dermacentor nitens</i>
Antônio Gonçalves	0 (0)	8	1	3
Campo Formoso	2 (4.1)	49	102	18
Pindobaçu	2 (28.6)	7	0	0
Senhor do Bonfim	0 (0)	6	0	0
Total	4 (5.5)	72	103	21

The horses sampled in the present study were bred in extensive or semi-extensive systems. Thus, the animals potentially had contact with other animals as well as access to forest areas and streams. *Rickettsia*-infected ticks from wildlife could infest domestic animals that have access to forest areas or share the same environment as wild fauna (i.e., rodents, marsupials, carnivores). This could be related to their serological status, as reported for seropositive horses in other regions (Medeiros et al., 2013).

At the time of the evaluation, tick infestation was observed in 18.6% (13/70) of the horses, 15.4% (2/13) in the municipality of Antônio Gonçalves and 84.6% (11/13) in the municipality of Campo Formoso (Table 1). Of the total adult ticks collected, 83% (103/124) were identified as *A. sculptum* and 17% (21/124) as *Dermacentor nitens*. Although *A. sculptum* is endemic to the Cerrado and Atlantic Forest biomes, its presence in the Caatinga biome may be related to a narrow invagination of the tropical climate in this biome, where it was first reported (Martins et al., 2016).

The present study demonstrated, for the first time, the presence of anti-*Rickettsia* antibodies in horses from the Caatinga biome, in an area in the state of Bahia where spotted fever has never been reported. These findings suggest the exposure of horses to this microorganism, especially because the seropositive animals did not have a travel history to other locations. Moreover, one animal had a high endpoint titer (2,048), suggesting recent exposure to rickettsiae (Ueno et al., 2016; Guimarães et al., 2017). Furthermore, we clearly demonstrated the reactivity of horse sera to *R. rickettsii* antigens. These results must be interpreted as evidence of exposure to rickettsial agents, since it has been shown that *R. rickettsii*-infected horses demonstrate serum reactivity to different *Rickettsia* species (Ueno et al. 2016) by antigen cross-reactions among members of this bacterial group. Further studies employing methods of direct diagnosis of rickettsiae (i.e., isolation, molecular detection) are warranted to confirm the circulation of SFG rickettsiae in the study region.

Considering that this is the first study to evaluate rickettsial exposure in horses from the Caatinga biome, we could not identify the exact *Rickettsia* species that elicited the antibody response to the *R. rickettsii* antigens. However, among the two tick species found on horses, *D. nitens* and *A. sculptum*, only the latter has been reported to be infected by SFG rickettsiae in Brazil. In fact, *A. sculptum* is the main vector of *R. rickettsii* in humans in southeastern Brazil (Gerardi et al., 2019), although this tick has rarely been found to harbor DNA of other SFG agents, such as *Rickettsia amblyommatis* (Barbieri et al., 2019), *Rickettsia bellii* (Machado et al., 2018), and 'Candidatus *Rickettsia andeanae*' (Witter et al., 2016). In addition, another tick species known to occur throughout the Caatinga biome, *A. auricularium*, has been found to be infected by *R. amblyommatis* in other Caatinga areas in the state of Bahia (Lugarini et al., 2015; Maia et al., 2018). Therefore, further studies are needed to identify the exact *Rickettsia* species that possibly infected the horses in the areas sampled in the present study.

Horses are considered suitable sentinels because they develop an efficient and long-lasting humoral response after infection by SFG rickettsiae (Horta et al., 2007; Ueno et al., 2016). The present study suggested the presence of rickettsial species in a BSF-non-endemic area in the north-central region of Bahia. To date, the risk of the disease becoming endemic in this region is low. Nevertheless, the results of this study are fundamental for increasing knowledge about the risks of the disease and the occurrence of rickettsial agents in the semi-arid Caatinga biome.

Acknowledgments

MBL and MCH are recipients of research fellowships from CNPq (grant number: 301641/2019-6 and 308668/2018-9, respectively).

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