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Original Article

Rickettsial infection in equids, opossums and ticks in the municipality of Monte Mor, state of São Paulo, Brazil

Infecção por *Rickettsia* spp. em equídeos, gambás e carrapatos do município de Monte Mor, estado de São Paulo, Brasil

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Abstract

The aim of this study was to investigate rickettsial infection in equids, opossums and ticks in the municipality of Monte Mor, a place where a Brazilian spotted fever case occurred in 2005. In addition, characteristics possibly associated with seropositivity in horses were analyzed. Serum samples from horses, mules and opossums (*Didelphis albiventris*) were subjected to indirect immunofluorescence assay (IFA) against *Rickettsia rickettsii*. The ticks collected from the animals were identified and *Amblyomma sculptum* ticks from the equids were tested using PCR for *Rickettsia* spp. Anti-*R. rickettsii* antibodies were detected in 22.6% (14/62) of the horses, none of the mules and 21.7% (5/23) of the opossums. Among the variables analyzed, only age > 12 years showed a statistically significant association with seropositivity among horses. All of the 166 *A. sculptum* ticks tested using PCR were negative. The results showed that rickettsiae of the spotted fever group was circulating in the municipality of Monte Mor when the samples were collected and indicate a need for surveillance of Brazilian spotted fever in this region.

Keywords: Rickettsiales, spotted fever, equine, *Didelphis*, São Paulo.

Resumo

Este trabalho objetivou pesquisar a infecção por *Rickettsia* spp. em equídeos, gambás e carrapatos, do município de Monte Mor, local que teve um caso de febre maculosa brasileira, em 2005. Além disso, características possivelmente associadas com a soropositividade nos equinos foram analisadas. Soros de equinos, muares e gambás *Didelphis albiventris* foram submetidos à reação de imunofluorescência indireta (RIFI) contra *Rickettsia rickettsii*. Os carrapatos coletados dos animais foram identificados e os carrapatos *Amblyomma sculptum* dos equídeos foram testados pela PCR para *Rickettsia* spp. Anticorpos anti-*R. rickettsii* foram detectados em 22,6% (14/62) equinos, zero muares e 21,7% (5/23) gambás. Entre as variáveis analisadas, apenas a idade maior que 12 anos mostrou associação estatisticamente significante com a soropositividade em equinos. De 166 carrapatos *A. sculptum* testados pela PCR, todos foram negativos. Os resultados mostram que riquétsias do grupo da febre maculosa estavam circulando no município de Monte Mor, quando as amostras foram coletadas, e apontam para a necessidade de vigilância para a febre maculosa brasileira nessa região.

Palavras-chave: Rickettsiales, febre maculosa, equinos, *Didelphis*, São Paulo.

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Introduction

The genus *Rickettsia* consists of multiple species of obligate intracellular bacteria. Some of them are pathogenic to humans and animals, while others are not pathogenic or have unknown pathogenicity (Weinert et al., 2009). These bacteria have been divided into groups according to their serological or molecular profile (Raoult & Roux, 1997; Weinert et al., 2009). The spotted fever group (SFG) contains one of the most pathogenic rickettsiae in the world, *Rickettsia rickettsii*, which causes Brazilian spotted fever (BSF) in Brazil (Parola et al., 2013). In this country, the vectors for *R. rickettsii* are the ticks *Amblyomma sculptum* and *Amblyomma aureolatum* (Szabó et al., 2013).

Specifically, in the region of Brazil where the municipality of Monte Mor is located, the vector is the tick *A. sculptum* (Szabó et al., 2013). Horses and opossums are good sentinels for *R. rickettsii* in areas where the vector is *A. sculptum*, since these animals are hosts for this tick and, when they come into contact with *R. rickettsii*, they produce antibodies at high level in large quantities that continue to be present for at least six months in opossums or at least two years in horses (Horta et al., 2009; Ueno et al., 2016). Opossums are also amplifier hosts of *R. rickettsii* for *A. sculptum* ticks, because they present rickettsemia and can be source of infection to ticks (Horta et al., 2009). Horses, in opposite, cannot transmit the bacterium to the tick vector (Ueno et al., 2016). There are no studies that assessed the susceptibility of mules to *R. rickettsii* and their role as amplifying hosts.

The municipality of Monte Mor belongs to the microregion of Campinas, in the state of São Paulo (Figure 1), in southeastern Brazil, which encompasses 16 municipalities. To date, only one case of BSF (which occurred in 2005) has ever been confirmed in this municipality (Brasil, 2019). However, over the whole microregion of Campinas, 379 BSF cases were confirmed from 2001 to 2017 (Brasil, 2019). This region has the highest number of notifications of the disease in Brazil.

Monte Mor is characterized by urban pockets that have been formed along the SP-101 highway. Among these urban areas and around them there are rural properties and regions of environmental preservation that have been anthropized and occupied irregularly (Cutolo et al., 2014). The city is crossed by several watercourses, the main

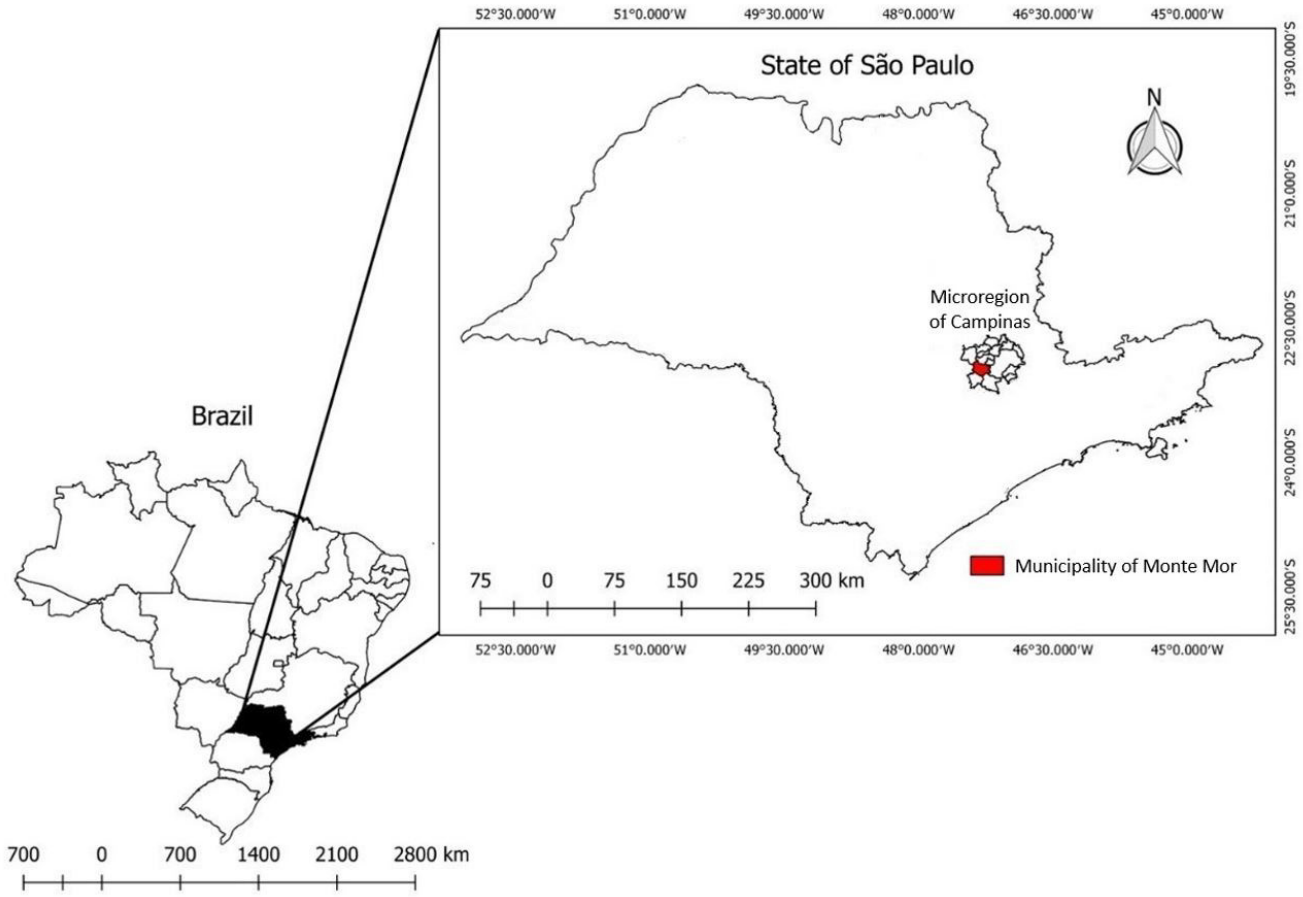

Figure 1. Location of the municipality of Monte Mor, in the state of São Paulo, Brazil.

one being the Capivari River, which belongs to the Tietê River basin. The municipality has few forest fragments, consisting mainly of riparian forest around the rivers and secondary forests scattered throughout the rural area. Part of the rivers and riparian forests are in the urban area of the city (Instituto Florestal, 2009).

The aims of this study were to investigate antibodies against *R. rickettsii* in horses, mules and opossums in the municipality of Monte Mor, to identify characteristics associated with seropositivity among horses and to investigate rickettsial DNA in *A. sculptum* ticks found in these animals.

Material and Methods

Sample collection

From January to July 2011, as part of an equid health program in the municipality of Monte Mor, a convenience sample of horses and mules from the urban and periurban region of the municipality was subjected to blood collection via jugular vein puncture and tick collection (Cutolo et al., 2014).

From December 2009 to November 2011, opossums (*D. albiventris*) were caught manually or using nets in urban or periurban residential areas of the municipality of Monte Mor, after the residents had notified the municipality's Zoonosis Control Department regarding the presence of these animals. From June 2011 to May 2012, other opossums were caught by means of Tomahawk traps in the rural area of Monte Mor as part of a study on opossum parasites (Teodoro et al., 2019). The sampling was also by convenience. The opossums thus caught were then subjected to blood collection by means of puncture of the ventral caudal vein and collection of ticks.

Indirect immunofluorescence assay

The equid and opossum blood samples were subsequently centrifuged at 3000 *g* for ten minutes to separate the serum, which was stored in microtubes at –20 °C until processing. In order to test for the presence of IgG antibodies against rickettsiae of the SFG, the serum samples were subjected to indirect immunofluorescence assay (IFA), as previously described (Horta et al., 2004), using the Taiaçu strain of *R. rickettsii* as the crude antigen. The secondary antibodies used were anti-horse IgG conjugate (Sigma, St Louis, MO, USA) and anti-opossum IgG conjugate (produced by the Zoonosis Control Center of São Paulo, Brazil). The serum samples were first tested at the dilution 1:64, and those that showed a positive result were retested at twofold serial dilutions, to determine the endpoint titers. On each slide, positive and negative control serum samples from horses (derived from the work of Ueno et al., 2016) or opossums (derived from the work of Horta et al., 2009) were processed simultaneously with the samples.

Statistical analysis

At the time of sample collection, some information about the horses was obtained from the current owner. This included the animals' sex and age, the neighborhood in which they lived, the activity for which they were used and the length of ownership. These data were input to bivariate analysis by using chi-squared test, G test or Fisher's exact test and calculating prevalence ratio and respective binomial confidence interval of 95% (Martinez et al., 2017) to identify variables associated with seropositivity. Differences were considered significant when $p < 0.05$. The analyses were performed on R environment (R Core Team, 2019) with the package "epiR" (Stevenson, 2020).

Tick identification

The ticks collected from the equids and opossums were stored in Falcon plastic tubes containing 70% ethanol, until processing. The adult ticks of *Rhipicephalus microplus*, all the stages of *Dermacentor nitens* and larvae of *Amblyomma* spp. were identified in accordance with Barros-Battesti et al. (2006). The adults of *A. sculptum* were identified according to Nava et al. (2014). Nymphs of the genera *Amblyomma* were identified according to Martins et al. (2010). The mean abundance and mean intensity were calculated according to Bush et al. (1997).

Molecular analysis

The ticks from equids that were identified as *A. sculptum*, a recognized vector for BSF, were subjected to DNA extraction using guanidine isothiocyanate-phenol solution, as previously described (Sangioni et al., 2005). Then, the samples were tested for the presence of *Rickettsia* spp. DNA by means of PCR targeting the *gltA* gene, using the

primers CS78 and CS323 (Labruna et al., 2004). Each reaction was prepared to a final volume of 25 µL, containing 1x buffer, 2 mM of $MgCl_2$, 0.25 mM of each dNTP, 0.6 µM of each primer, 1.5 U of DNA polymerase, 10.85 µL of ultrapure water and 2.5 µL of DNA. The reactions were carried out under the following conditions: initial denaturation at 95 °C for 3 min, then 40 cycles of 95 °C for 15 s, 48 °C for 30 s and 72 °C for 30 s, with final extension at 72 °C for 7 min. The other ticks recovered from equids that are not recognized as vectors for BSF were not tested. The ticks recovered from opossums were deposited in the tick collection "Coleção Nacional de Carrapatos Danilo Gonçalves Saraiva" (CNC) of the FMVZ-USP, so they were not tested for rickettsial DNA.

Institutional permissions

This study was approved by the Ethics Committee for Animal Use of the State University of Campinas (CEUA/UNICAMP) (protocol number 2546-1). Authorization to handle wild opossums was obtained from the Brazilian Institute for the Environment and Renewable Natural Resources (IBAMA) (protocol number 31724 -1).

Results

Serum samples were obtained from 62 horses and three mules in ten neighborhoods of the municipality of Monte Mor. In addition, serum samples from 23 *D. albiventris* opossums were obtained (17 opossums were caught in urban and periurban areas after notification from residents and six were caught by means of traps in rural areas).

Among the 62 samples from horses that were tested using IFA, 14 (22.6%) were positive for *R. rickettsii* (Table 1). The titers ranged from 64 to 2048. No mule samples were positive in IFA. Among the opossum samples, 5/23 (21.7%) were positive for *R. rickettsii* (Table 1), with titers ranging from 64 to 256.

The bivariate analysis on the characteristics of the horses (sex, age, neighborhood, use and length of ownership) showed that only age > 12 years was associated with seropositivity for *R. rickettsii* (prevalence ratio = 3.13; $p = 0.010$), as shown in Table 2. Horses aged > 12 years presented 2.13 more chances to be seropositive than horses ≤ 12 years old.

Among the 62 horses examined for the presence of ticks, 46 were infested. The mean abundance of ticks was 6.9 and the mean intensity was 9.2. Out of a total of 425 ticks that were collected, 59.8% (254/425) were identified as *D. nitens*, 38.6% (164/425) as *A. sculptum* and 1.6% (7/425) as *R. microplus* (Table 3).

Among the three mules that were examined, only one was parasitized. The mean abundance of ticks was 0.7 and the mean intensity was 2.0. Two adult *A. sculptum* ticks were removed from this animal.

Twenty-three opossums were examined and six were found to be parasitized by ticks. In these animals, the mean abundance of ticks was 2.0 and the mean intensity was 7.8. A total of 47 ticks were recovered, among which 44.7% (21/47) were *A. sculptum* nymphs, 38.3% (18/47) were *A. dubitatum* nymphs and 17.0% (8/47) were larvae of the genus *Amblyomma* (Table 3).

All the 166 *A. sculptum* ticks (98 males, 67 females and 1 nymph) that were found on the horses and mules were subjected to PCR for the *gltA* gene of *Rickettsia* spp. and were shown to be negative.

Table 1. Antibodies against *Rickettsia rickettsii* in horses, mules and opossums (*Didelphis albiventris*) in the municipality of Monte Mor, SP, which were tested by means of the indirect immunofluorescence assay (IFA ≥ 64).

Animal	Titers no. of positive samples according to the titer/total no. of positive samples						Total (no. of positive samples/no. of samples tested)
	64	128	256	512	1024	2048	
Horses	2/14 (14.3%)	2/14 (14.3%)	4/14 (28.6%)	3/14 (21.4%)	2/14 (14.3%)	1/14 (7.1%)	14/62 (22.6%)
Mules	0	0	0	0	0	0	0/3
Opossums (*D. albiventris*)	1/5 (20%)	1/5 (20%)	3/5 (60%)				5/23 (21.7%)

Table 2. Bivariate analysis performed to investigate associations between the characteristics of the horses and the presence of antibodies against *R. rickettsii*.

Variables	Categories	Total no. of horses	No. of seropositive horses (%)	Prevalence ratio (95% CI)[a]	*p*-value[b]
Age	Up to 12 years	47	7 (14.9)	Reference	
	> 12 years	15	7 (46.7)	3.13 (1.31-7.49)	0.010
Length of possession by the current owner	Up to 5 years	48	11 (22.9)	Reference	
	> 5 years	9	2 (22.2)	1.03 (0.27-3.89	0.349
Sex	Female	30	6 (20)	Reference	
	Male	32	8 (25)	1.25 (0.49-3.18)	0.639
Neighborhood	Panorama	11	1 (9.1)	Reference	0.760
	Moreira	5	1 (20)	2.20 (0.17-28.53)	
	Nova Alvorada	10	2 (20)	2.20 (0.23-20.72)	
	Paviotti	20	6 (30)	3.30 (0.45-24.02)	
	São Rafael	9	3 (33.3)	3.67 (0.46-29.49)	
	Others*	7	1 (14.3)	1.57 (0.12-21.26)	
Use	Foal not yet used	7	0 (0)	Reference	
	Cart[+]	18	5 (27.8)	4.63 (0.29-74.27)	0.164
	Leisure[#]	37	9 (24.3)	4.00 (0.26-61.93)	

*The neighborhoods Engenho, Fahrid Calil, Centro, Clube de Campo and Colina were put together in the "others" group because of the very small number of horses in each of them; [+]The cart category corresponds to the horses that pulled carts used mainly to collect recyclable material; [#]The leisure category corresponds to the horses used to riding, to draw leisure carts or as pets; [a]Confidence interval of 95% for prevalence ratio; [b]Probability that null hypothesis is true.

Table 3. Ticks that were identified from horses, mules and opossums (*Didelphis albiventris*) in the municipality of Monte Mor, SP.

Host	Tick stage	Ticks identified				
		Amblyomma sculptum	***Amblyomma dubitatum***	***Amblyomma* spp.**	***Rhipicephalus microplus***	***Dermacentor nitens***
Horses	Adults	163			7	185
	Nymphs	1				65
	Larvae					4
	Total	164/425 (38.6%)			7/425 (1.6%)	254/425 (59.8%)
Mules	Adults	2				
	Total	2/2 (100%)				
Opossums (*D. albiventris*)	Nymphs	21	18			
	Larvae			8		
	Total	21/47 (44.7%)	18/47 (38.3%)	8/47 (17.0%)		

Discussion

The IFA test showed that 22.6% of the horses and 21.7% of the opossums were positive, which indicates that SFG rickettsiae was circulating in the municipality of Monte Mor at the time when samples were obtained. More recent research is needed to check whether SFG rickettsiae are still circulating in the city nowadays.

In this study, a strain of *R. rickettsii* was used as the antigen in IFA. However, since cross-reactions occur between different *Rickettsia* species of the SFG (Horta et al., 2004), it is not possible to determine the species of *Rickettsia* responsible for infections. In Brazil, at least seven SFG rickettsiae have been reported, namely *R. rickettsii*, *R. parkeri*, *R. amblyommatis*, *R. rhipicephali*, "*Candidatus* R. andeanae" (Parola et al., 2013), "*Candidatus* R. colombianensi" (Luz et al., 2018) and "*Candidatus* R. paranaensis" (Peckle et al., 2019).

Because the municipality of Monte Mor is located in a BSF-endemic region, it is possible that *R. rickettsii* was the bacterium that caused some of the infections. In other studies that were conducted previously in municipalities neighboring Monte Mor, the frequencies of horses seropositivity for *R. rickettsii* ranged from 40.8% to 90% (Horta et al., 2004, 2007; Souza et al., 2016), while among opossums the frequency was 78.6% (Horta et al., 2007). In these studies, the probable infectious rickettsiae (as determined from differences of antibody titers against different *Rickettsia* antigens) were *R. rickettsii*, *R. parkeri* or *R. bellii*.

Caution should be taken when analyzing the data from the present survey. As the sampling of the animals was done by convenience, it is not possible to extrapolate the results to the entire population of horses, mules and opossums of the municipality of Monte Mor. For the same reason, comparisons among the percentages of seropositive animals found in this study with frequencies found in other surveys should be done carefully.

Horses can be used as indicators for circulation of *R. rickettsii* in regions where the vector is *A. sculptum*, because when they come into contact with the bacterium, they produce long-lasting antibodies (Ueno et al., 2016). Moreover, these are domestic animals that can easily be handled for blood collection. Even in regions where there have never been cases of BSF or where cases have not occurred for a long time, such as Monte Mor, which has only had one case (reported in 2005), serological tests on horses have great value for active surveillance of BSF (Souza et al., 2016). On the other hand, horses are not good sentinels in places where the vector is probably not *A. sculptum* (Cunha et al., 2014; Oliveira et al., 2019).

Opossums also produce antibodies that persist for a long time after infection by *R. rickettsii*, and these animals can also be used as indicators for circulation of this microorganism in a region (Horta et al., 2007, 2009). In addition, these animals may have a limited role as amplifier hosts of *R. rickettsii* for *A. sculptum* ticks, since they can infect a small amount of susceptible ticks in situations of rickettsemia (Horta et al., 2009).

In the present study, none of the three mules was seropositive to *R. rickettsii*. Other studies have found percentages of seropositive donkeys lower than percentages observed in horses. Horta et al. (2004) found 0/4 donkey seropositive to *R. rickettsii* in Pedreira, a municipality near Monte Mor, and Otomura et al. (2016) found 2/45 (4.4%) seropositive donkeys in northeastern Paraná. Although there is no research that evaluated the role of the hybrid equid (*Equus caballus* x *Equus asinus*) on the epidemiology of *R. rickettsii*, it is possible that this animal is not a good sentinel, since donkeys are more resistant to the tick vector *A. sculptum* than horses (Castagnolli et al., 2003) and the population of mules in the state of São Paulo is small (IBGE, 2017).

Among the variables analyzed to test possible associations with seropositivity for *R. rickettsii* among horses, only age presented a statistically significant association, with animals aged > 12 years presenting 2.13 more chances to be seropositive. This can be explained by the fact that older animals have had greater opportunity to become infected over the course of their lifespan than younger animals. Silveira et al. (2015) observed that horses that had lived on the same farm for more than 8.5 years were more likely to be seropositive for SFG *Rickettsia* spp.

There was no association between the neighborhoods and the seropositivity of horses. This may have happened due to the small number of samples and the dispersion of horses between the neighborhoods. In addition, the neighborhoods where the horses rested may not be a useful variable in this study because most horses did not stay in these places all the time, but moved around the city (these animals were used to pull carts for transporting people and recyclable material, besides horse riding). Thus, it is not possible to determine where these horses acquired the infection. Another issue to be clarified is whether horses could carry infected ticks from one place to another. In the region of Campinas, where Monte Mor is located, human cases of BSF were associated with the presence of capybaras, watercourses, and the ticks *A. sculptum* and *A. dubitatum*, with tendency to urbanization of cases over time (Nasser, 2014). The only confirmed BSF case in Monte Mor occurred in the urban area of the

city, close to the downtown (Nasser, 2014). Other studies are needed to assess where the risk areas are in the municipality of Monte Mor using georeferencing tools, and which environmental factors are associated with the presence of rickettsiae.

The three species of ticks that were recovered from equids in the present study (*D. nitens*, *A. sculptum* and *R. microplus*) are species commonly found parasitizing equids in several regions of Brazil (Labruna et al., 2001; Dantas-Torres, 2009; Martins et al., 2009; Alves et al., 2014).

In the opossums, *A. sculptum* nymphs, *A. dubitatum* nymphs and *Amblyomma* spp. larvae were found. The ticks *A. sculptum* and *A. dubitatum*, mainly at immature stages, have been found in opossums of the species *D. albiventris* and *D. aurita* in the southeastern and central-western regions of Brazil (Horta et al., 2007; Perez et al., 2008; Saraiva et al., 2012; Silveira et al., 2015; Sponchiado et al., 2015). The main hosts for the tick *A. sculptum* are horses, capybaras and tapirs (Labruna et al., 2001), but it can also parasitize several other animals, including opossums (Estrada-Peña et al., 2004). The main host for all stages of *A. dubitatum* is capybaras, but it is common to find immature stages of this tick on opossums (Horta et al., 2007; Nava et al., 2010).

All the specimens of *A. sculptum* that were tested by means of PCR were negative. This is concordant with data from previous studies, which did not find any positive ticks or found only a very low rate of infected *A. sculptum* ticks, even among ticks collected in BSF-endemic regions (Guedes et al., 2005; Sangioni et al., 2005). This low prevalence of *R. rickettsii* infection in *A. sculptum* ticks can possibly be explained by these ticks' low capacity to acquire the infection (Labruna et al., 2008), decreased reproductive ability in infected ticks and a low rate of transovarian transmission (Soares et al., 2012; Costa et al., 2020). Thus, PCR on ticks may only have minimal usefulness for detecting *Rickettsia* spp. circulation in any given region, compared with serological tests.

Conclusions

We concluded that SFG rickettsiae was present and circulating in the municipality of Monte Mor when the samples were collected. This emphasizes the importance of BSF surveillance in this municipality, even though only one case has been registered so far.

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