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Spotted fever group *Rickettsia* and *Borrelia* sp. cooccurrence in *Amblyomma sculptum* in the Midwest region of Brazil

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Abstract
More than 70 tick species are found in Brazil, distributed over five genera and including main vectors of infectious disease agents affecting both animals and humans. The genus *Amblyomma* is the most relevant for public health in Brazil, wherein *Amblyomma aureolatum*, *Amblyomma ovale* and *Amblyomma sculptum* have been incriminated as vectors of *Rickettsia* and *Borrelia* pathogens. The objective of this study was to investigate the presence of *Rickettsia* spp. and *Borrelia* spp. in ticks in the Brazilian mid-western savannah. DNA extraction, PCR for *Borrelia* spp. (*flgE* gene) and *Rickettsia* spp. (*ompA* and *gltA* genes) and subsequent sequencing were performed. A total of 1875 ticks were collected and identified as *A. sculptum* except for two *Amblyomma coelebs* ticks. Molecular evidence for *Borrelia* spp. and *Rickettsia parkeri* was found in *A. sculptum*. This is the first molecular evidence for *R. parkeri* in *A. sculptum* ticks in the Midwest region and *Borrelia* spp. circulating in a tick of the *Amblyomma* genus in Brazil.

Keywords Tick-borne disease · Pathogen · Savannah · Ixodidae

Introduction

Along with mosquitoes, ticks are considered an important source of pathogens for humans, which places them at the forefront of disease transmission and public health (Colwell et al. 2011). Due to advances in diagnostic methods as well as the effects of climate change

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caused by societal progress, the number of reports of arthropod-borne diseases in humans has increased (Beugnet and Chalvet-Monfray 2013).

With more than 900 described species worldwide, ticks are obligatory blood-sucking ectoparasites that can transmit various types of disease-causing agents, such as viruses, protozoa, and bacteria during the feeding process (Jongejan and Uilenberg 2004). A tick-transmitted disease of great importance in Americas are the rickettsioses caused by obligatory intracellular gram-negative bacteria belonging to the genus *Rickettsia*. First described in the USA in 1899, the disease has been found in Canada, Mexico, Panama, Costa Rica, Argentina, Colombia, and Brazil (Dantas-Torres 2007). The main species of medical importance in Brazil is *Rickettsia rickettsii*, a bacterium that causes Rocky Mountain fever (or Brazilian macular fever). This disease is associated with clinical complications due to its nonspecific symptoms (myalgia, headache, fever) as well as more serious symptoms (neurological disorders, renal failure, respiratory difficulty, jaundice), with maculopapular rash as a pathognomonic sign (Angerami et al. 2009). Currently the main vectors of *R. rickettsii* are *Amblyomma cajennense* (and *A. sculptum*) and *A. aureolatum* (Labruna 2009; Ogrezewalska et al. 2012). Other *Rickettsia* bacteria belonging to the spotted fever group may be reported in other tick species as well (Almeida et al. 2013; Matias et al. 2015).

Lyme disease, another disease related to Ixodidae ticks, is mainly transmitted by ticks belonging to the genus *Ixodes* and is considered to be most common in the Northern Hemisphere (Jaenson 1991; Mead 2015; Durand et al. 2017). Lyme disease is a multisystem inflammatory disease caused by spirochete bacteria belonging to the *Borrelia burgdorferi* sensu lato complex and manifests as classic symptoms such as the presence of erythema migrans in the skin initially and joint, neurological, and cardiac complications at later stages (Sanchez 2015).

The *B. burgdorferi* s.l. complex consists of 18 genospecies recognized in North America, Europe, and Asia, with *Borrelia afzelli*, *Borrelia garinii*, and *B. burgdorferi* being the most pathogenic in humans (Mead 2015). Studies conducted in Brazil using protein-based serology reported some inconsistencies in the diagnostic results for *B. burgdorferi*. These facts, together with the absence of bacterial isolation from tissues or body fluids, have shown that until now, Lyme disease has never been confirmed in Brazil (Oliveira et al. 2018).

However, clinical manifestation similar to Lyme disease has been reported in Brazil, including erythrema migrans (Mantovani et al. 2007). According to the authors, there is a possibility that a new tick-borne disease related to a spirochete belonging to the *Borrelia* genus may exist, known as Lyme disease-like syndrome or Lyme Imitator Syndrome.

As *A. cajennense* complex (*A. cajennense* and *A. sculptum*) was speculated in the literature as possible participant in the transmission of Brazilian borreliosis (Dantas-Torres 2008; Yoshinari et al. 2010) and this tick is known as the main transmitters of rickettsial diseases, the objective of the present study was to investigate *Rickettsia* spp. and *Borrelia* spp. by molecular evidence in *A. sculptum* from the Midwest region of Brazil.

Materials and methods

Study site

Tick collection was carried out between December 2017 and August 2018 in the Terenos Municipality in the state of Mato Grosso do Sul, Brazil. The municipality is situated in the

west-central region of the state (22 km from the capital Campo Grande), with a population of ca. 17.146, a size of ca. 2.844 km^2, and an average elevation of 408 m above sea level (https://www.ciudad-brasil.com.br/municipio-terenos.html). The ticks were collected in a forest fragment belonging to the Cerrado biome located in the rural area of the municipality (−20.405580, −55.014690). The entire area covered in the study is surrounded by cattle-breeding pastures, and the presence of wild animals has been reported (e.g., *Myrmecophaga tridactyla* and *Tapirus terrestres*).

Tick collection

Free-living ticks were collected using CO_2 traps, as described by Oliveira et al. (2000), and transported alive to the Embrapa Cattle Tick biology laboratory where they were identified according to Barros-Battesti et al. (2006).

DNA extraction

DNA was extracted individually from adult ticks using the acid guanidinium thiocyanate–phenol–chloroform extraction protocol (Sangioni et al. 2005), and the samples were quantified by spectrophotometry (NanoDrop ND-1000 Uniscience) and subsequently subjected to polymerase chain reaction (PCR). The CS-78 and CS-323 oligonucleotides were used to amplify a 401-bp fragment of the citrate synthase gene (*gltA*) in species of the genus *Rickettsia* (Labruna et al. 2004). The PCR assay was standardized to a final volume of 25 µl, and the DNA concentration used was between 50 and 150 ng/µl, with A_{260} nm/A_{280} nm ≥ 1.8. Positive samples were subjected to another round of PCR using oligonucleotides Rr190.70p and Rr190.602n, which amplify a 530-bp fragment of the *ompA* gene (protein 190 kDa) only for *Rickettsia* species belonging to the Rocky Mountain spotted fever group (Regnery et al. 1991). All samples were also tested for the presence of *B. burgdorferi* using the oligonucleotides *flgE*-F and *flgE*-R, which target the *Borrelia* flagellar hook, amplifying a 262-bp fragment with adaptations (Sal et al. 2008). The PCR-amplified *ompA* and *flgE* products were visualized on 1.5% agarose gels stained with ethidium bromide (EtBr) and purified using a PureLink Quick Gel Extraction Kit (Invitrogen). The products were subsequently cloned and sequenced using the Sanger method (Sanger et al. 1977) with an ABI 3130 Genetic Analyzer (Applied Biosystems). The consensus sequence was obtained using BioEdit software (Hall 1999) and compared with data available in GenBank. A BLASTn search was performed for sequence identity (Altschul et al. 1990), and phylogenetic analyses were conducted using MEGA v.7.0 software (Kumar et al. 2016).

Sequence alignment and phylogenetic tree construction

The *ompA* (GenBank: MK231013) and *flgE* (GenBank: MK231014) sequences were aligned with those in GenBank using BLASTn, and a database was constructed that contained all similar sequences obtained from the analysis. The MEGA v.6.0 program (Tamura et al. 2013) was applied to align the sequences.

Bayesian phylogenetic analysis was performed using the MrBayes v.3.2.6 program (Ronquist and Huelsenbeck 2003). For the data set used in this study, approximately 10^7 generations were found to be sufficient for generating topologies; plots were prepared using the FigTree v.1.4.2 program (Tree Bio 2016). All analyses for *ompA* and *flgE* were initiated

with random starting trees and run for 10^6 generations, with sampling every 1000 generations. To determine the stationarity of the Markov chain, the log-likelihood scores of sample points were plotted against the generation time. The first 25% of samples was discarded as burn-in for each data set, and the remaining samples were retained for generating consensus trees. Each sample included a tree topology that incorporates branch length and substitution model parameter values. These topologies were used to generate a 50% majority rule consensus tree, with the percentage of sample recovering any particular clade representing the posterior probability of a clade (1 = 100%). No manual editing of the trees was performed. *Rickettsia australis* (GenBank: AF149108) and *Treponema pallidum* (GenBank: CP021113) were used as outgroups in the phylogenetic analyses.

Results

A total of 1875 ticks (1873 *A. sculptum* and 2 *A. coelebs*) were collected from the environment during the study period, as shown in Table 1. Of these, 144 ticks (including the *A. coelebs* specimens) were subjected to PCR to detect *Rickettsia* and *Borrelia* spp. genetic material. An adult *A. sculptum* tick presented molecular evidence of *Rickettsia* spp. based on *ompA* as the target region for PCR. Another individual of the same species presented molecular evidence of *Borrelia* sp. DNA, which was confirmed by PCR targeting the *flgE* gene. No molecular evidence for any of the aforementioned bacteria was found for the *A. coelebs* specimens.

Both pathogens were sequenced for identity confirmation, and BLASTn analysis of the amplified fragment revealed 100% identity with the Atlantic Forest strain of *Rickettsia parkeri* (*ompA* gene, 530 bp; GenBank: MF536975), 98.5% with *B. burgdorferi* B31 (GenBank AE000783.1) and 99.6% with *B. burgdorferi* LS2 (GenBank: KY073268.1) (*flgE* gene, 262 bp). A phylogenetic tree was generated using the *ompA* sequences obtained in this study and GenBank MK231013 sequences (Fig. 1). The same procedure was performed for the *B. burgdorferi* sequence (GenBank: MK231014), with another phylogenetic tree generated (Fig. 2).

Discussion

Among the tick species collected in the present study, *A. sculptum* was the most prevalent as well as the most abundant in the environment. *Amblyomma sculptum* exhibits a wide distribution in the environment, a wide range of hosts, and a high affinity for humans (Parola and Raoult 2001). Thus, this species is commonly related to cases of parasitism in humans in South America (Guglielmone et al. 2006) and *A. sculptum* is known to be the main transmitter of *R. rickettsi* (Labruna 2009).

Table 1 Number of adult female (F) and male (M) ticks collected on various dates in the Terenos municipality, MS, Brazil

Collection	11/16/2017		02/23/2018		05/03/2018		08/28/2018	
Species	F	M	F	M	F	M	F	M
Amblyomma sculptum	200	194	441	337	198	265	150	90
Amblyomma coelebs	0	0	1	1	0	0	0	0

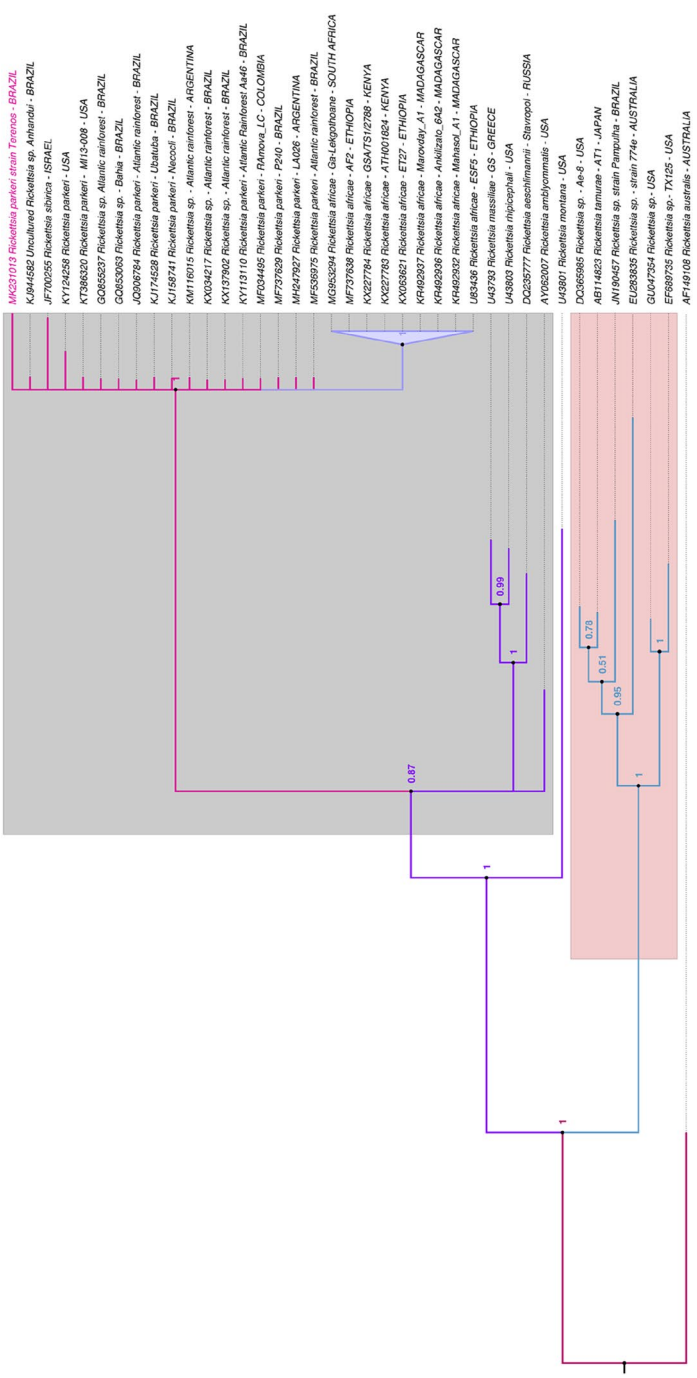

Fig. 1 Phylogenetic tree of *Rickettsia parkeri* (GenBank: MK231014). Evolutionary history was based on the Bayesian inference tree with probability scores for the *ompA* gene. The scale bar indicates 0.02 changes per nucleotide position. The sample sequences obtained in this study are shown in pink. (Color figure online)

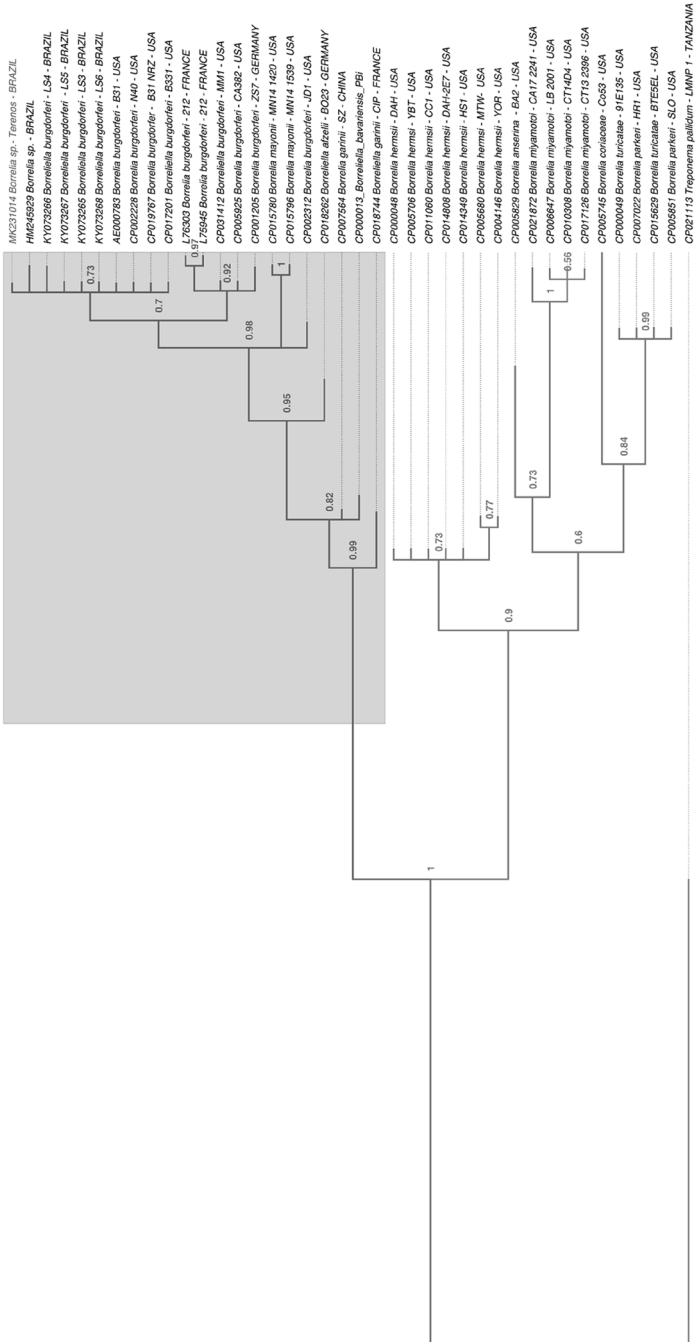

Fig. 2 Phylogenetic tree of *Borrelia* spp. (GenBank: 231013). Evolutionary history was based on the Bayesian inference tree with probability scores for the *flgE* gene. The scale bar indicates 0.03 changes per nucleotide position. The sample sequences obtained in this study are shown in blue. (Color figure online)

As well as *R. rickettsii*, *R. parkeri* belongs to the Rocky Mountain spotted fever bacterial group and is recognized as the etiological agent of rickettsial disease in Brazil (Spolidorio et al. 2010). Compared with other rickettsial diseases, spotted fever associated with *R. parkeri* presents milder symptoms, primarily fever, eschar, lymphadenopathy, rash and lethal cases have not been reported (Silveira et al. 2007).

In Brazil, *A. ovale* is considered one of the main vectors of *R. parkeri* (Sabatini et al. 2010), beside *A. tigrinum* and *A. triste* (Faccini-Martínez et al. 2018). According to the genotypes pre-established in the literature, various phylogenetic groups exist (Nieri-Bastos et al. 2018) as do various ticks related to *R. parkeri* (Table 2). It is important to state that, according to the literature and Table 1, the Atlantic Forest strain is related to *A. ovale* and may be the main etiological agent of the disease (Spolidorio et al. 2010; Nieri-Bastos et al. 2018).

In this work, molecular evidence of a 100% identity *R. parkeri* Atlantic Forest strain was obtained from a free-living *A. sculptum* adult tick. As shown in Table 2, most reports of *R. parkeri* related to ticks are described in the South (41.9%) and Southeast (29%) regions, corroborating with other available data in literature (Spolidorio et al. 2010; Krawczak et al. 2016).

The first time that *R. parkeri* was found in *A. sculptum* was in Minas Gerais state (Szabó et al. 2019). According to the authors, the relationship between *R. parkeri* and *A. sculptum* remains unclear. Simultaneous parasitism with *A. nodosum* was recorded, however, with no DNA amplification in this tick. In our study a free-living female tick was found with *R. parkeri* but, until an experiment with infected and non-infected *A. sculptum* is performed, we can only suggest that transstadial transmission may occur.

In addition to molecular evidence of the *Rickettsia* described above, another *A. sculptum* adult tick showed DNA amplification for a borrelial gene (*flgE*). Whereas Lyme borreliosis has a wide distribution in the Northern Hemisphere, which is linked to the presence of its main vector *Ixodes* spp. (Gray 1998), a similar disease is present in Brazil and the corresponding vector has remained unclear (Dantas-Torres 2008), which has inspired new studies, as shown in Table 3.

According to Table 3, we can highlight the importance of serological diagnoses for both humans and animals in epidemiological investigations of the disease in Brazil. Indeed, serological investigations can identify potential reservoirs and suggest areas of infection risk. Most diagnoses for humans are also achieved through serological tests and clinical evaluations as reported in Table 3 and in agreement with the studies by Yoshinari et al. (2010) and Mantovani et al. (2012). However, it is important to state that serological cross-reactions may occur (Magnarelli et al. 1987) and these findings do not state that classical Lyme disease exists in Brazil (de Oliveira et al. 2018).

In a study conducted in the Brazilian Pampas region, the presence of bacterial DNA belonging to *B. burgdorferi* s.l. was found in larvae and nymphs of *Ixodes longiscutatus* using the *flaB* gene in nested PCR (Dall'Agnol et al. 2017). Despite belonging to the genus of ticks classified as main vectors for this disease in the Northern Hemisphere, this ectoparasite is not very relevant to the direct transmission of TBD to humans in Brazil, and it mainly participates in the maintenance and circulation of the pathogen in wild animals (de la Fuente et al. 2008; Dall'Agnol et al. 2017).

In the state of Mato Grosso do Sul, a molecular investigation utilizing the *flgE* gene revealed evidence for *Borrelia* sp. in the tick *Rhipicephalus microplus* (Rezende et al. 2016). However, this tick is closely related to livestock-related losses, and humans are considered sporadic hosts (Guglielmone et al. 2006; Kaur et al. 2019).

Table 2 Tick species described in the literature as carrying *Rickettsia parkeri* or its various strains

Ticks	*Rickettsia parkeri*	Location	References
Amblyomma ovale	Atlantic forest strain	Itoupava, SC	Barbieri et al. (2014)
		Environmental reserve, SP	Luz et al. (2016)
		Rio Grande do Sul	Voizzoni et al. (2016)
		Poconé, MT	Witter et al. (2016)
		Ibiraçu, ES	Acosta et al. (2018)
		Águas Mornas, Blumenau, Urussanga, SC	Medeiros et al. (2011)
		Environmental reserve, SP	Sabatini et al. (2010)
Amblyomma aureolatum	*R. parkeri*-like	Itoupava, SC	Dall'Agnol et al. (2018)
	R. parkeri s.s. clone RS	Santana do Livramento and Triunfo, RS	Dall'Agnol et al. (2018)
	Atlantic forest strain	Blumenau, SC	Medeiros et al. (2011)
Amblyomma tigrinum	*R. parkeri* s.s. clone RS	Santana do Livramento and Triunfo, RS	Dall'Agnol et al. (2018)
	Rickettsia sp. AF strain	Rosário do Sul, RS	Weck et al. (2017)
Amblyomma nodosum	*R. parkeri*-like	Environmental reserve Paraíba	Lugarini et al. (2015)
	NOD strain	Perdizes and Uberlândia, MG; Bebedouro, SP; Anastácio, MS	Szabó et al. (2019)
		Cuiabá, MT	Witter et al. (2016)
		Pantanal and Cerrado, MT	Ramos et al. (2015)
Amblyomma triste	Pantanal strain At46	Pantanal, MT	Melo et al. (2015)
	Strain At24	Paulicéia, SP	Silveira et al. (2007)
	R. parkeri	Agua Clara, MS	Nieri-Bastos et al. (2013)
Amblyomma calcaratum	NOD strain	Ivinhema, MS	Ogrzewalska et al. (2013)
Amblyomma parkeri	ApPR strain	Juiz de Fora, MG	Zeringota et al. (2017)
		Paraná	Pacheco et al. (2012)
Amblyomma longirostre	NOD strain	Paraná	Pacheco et al. (2012)

Table 2 (continued)

Ticks	*Rickettsia parkeri*	Location	References
Amblyomma dubitatum	*R. parkeri* clone RS	Toropi and Quevedos, RS	Weck et al. (2017)
	Cooperi strain	Pedreira, SP	Labruna et al. (2004)
Rhipicephalus sanguineus	Atlantic forest strain	Blumenau, SC	Medeiros et al. (2011)
		Environmental reserve, SP	Sabatini et al. (2010)
	Rickettsia sp. AF strain	Rosário do Sul, RS	Weck et al. (2017)
Amblyomma sculptum	NOD strain	Sponsorship, MG	Szabó et al. (2019)
	Atlantic forest strain	Terenos, MS	Present study

Table 3 Records of the detection of borreliosis in Brazil

Location		Pathogen	Vector/host	Method	References
North and Northeast	Castanhal, PA	*B. burgdorferi* s.l	Domestic dog	Serology	Pacheco et al. (2016)
	Belém, PA	*B. burgdorferi* s.l	Human	Clinical, serology	Rodrigues et al. (2007)
	Ananindeua, PA	*B. burgdorferi*	Horse	Serology	Galo et al. (2009)
	Manaus, AM	*B. burgdorferi* s.l	Human	Serology, microscopy	Talhari et al. (2010)
	Porto Nacional and Marianópolis, TO	*Borrelia* sp.	Human	Clinical, serology	Carranza-Tamayo et al. (2012)
Southeast	Rio de Janeiro, RJ	*Borrelia* sp.	Human	Clinical, serology	Azulay et al. (1991)
	Cotia, SP	*B. burgdorferi*	Human	Clinical, serology	Yoshinari et al. (2003)
	Cotia, SP	*Borrelia* sp.	Rodents, tick	Serology	Abel et al. (2000)
	Seropédica, Itaguaí, and Rio de Janeiro, RJ	*Borrelia* sp.	Domestic dog	Serology	Alves et al. (2004)
	São Paulo	*B. burgdorferi*	Human	Serology	Gouveia et al. (2010)
	Several cities, ES	*B. burgdorferi*	Dog, horse, and human	Serology	Spolidorio et al. (2010)
	Santo Cruz Escavaldo and Pingo D'Água, MG	*B. burgdorferi*	Wild and domestic mammals	Serology	Montandon et al. (2014)
	São Paulo	*Borrelia* sp.	Human	PCR	Mantovani et al. (2012)
South	Umuarama, PR	*B. burgdorferi* s.l	Domestic dog	Serology	Gonçalves et al. (2015)
	Jataí, PR	*B. burgdorferi* s.s	*Dermacentor nitens*	PCR	Gonçalves et al. (2013)
	Alvorada do Sul, PR	*B. burgdorferi*	Domestic dog, horse, and human	Serology	Nascimento et al. (2016)
	Chuí and Vitória do Palmar, RS	*B. burgdorferi* s.l	*Ixodes longiscutatus*	PCR	Dall'Agnol et al. (2017)
Midwest	Sinop, MT	*B. burgdorferi*	Horse	Serology	Socoloski et al. (2018)
	Campo Grande, MS	*B. burgdorferi* s.l	Human	Serology, PCR	Lopes et al. (2017)
	Campo Grande, MS	*B. burgdorferi* s.l	Human and *Rhipicephalus microplus*	PCR	Rezende et al. (2016)
	Terenos, MS	*B. burgdorferi*	*Amblyomma sculptum*	PCR	Current study

It is important to note that all reports cited above represent ticks with no direct relevance to human parasitism. Based on epidemiological investigations, the most accepted hypothesis of Brazilian Lyme-like disease transmission to humans in Brazil is via *A. sculptum*, previously known as *A. cajennense* (Dantas-Torres 2008; Beati et al. 2013; Nava et al. 2014). According to Gray (1998), ticks belonging to the genus *Ixodes* possess certain characteristics that cause them to be the main vectors of the disease, such as a heteroxenous biological cycle, parasitization of mainly birds and small and medium-sized mammals in immature stages, and distinct seasonality in the search for hosts with regulation via the diapause mechanism. These ecological and physiological requirements are mostly present in *A. sculptum*, except for specific differences due to different location. Moreover, *A. sculptum* accounted for 99.9% of the specimens collected in our study, and this high availability, along with its anthropophilic characteristics, supports its potential as a vector.

In addition to molecular evidence for the bacterium itself, sequencing of the DNA obtained in this study using *flgE* primers enabled a phylogenetic analysis (Fig. 1). The phylogenetic analyses of the *flgE* fragment grouped our sample into the *B. burgdorferi* clade with other sequences from the USA and Brazil. It is noteworthy that the identity found with Brazilian strains was 99.4%, differing from the sequences found in the USA (98.5%), corroborating with studies by Mantovani et al. (2012).

Conclusions

This study reports for the first time molecular evidence of the Atlantic Forest strain of *R. parkeri* in *A. sculptum* ticks in the Midwest region of Brazil. Moreover, genetic material from *Borrelia* spp. was detected (by PCR) for the first time in a tick of the genus *Amblyomma* in Brazil. Our data emphasize the need for further studies related to *A. sculptum* competence as a vector of the two agents described.

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Compliance with ethical standards

Conflict of interests The authors declare that they have no competing interests.

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