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Original article

Borrelia burgdorferi sensu lato in *Ixodes longiscutatus* ticks from Brazilian Pampa

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ABSTRACT

Borrelia burgdorferi sensu lato (s.l.) complex includes the agents of Lyme disease/borreliosis in North America, Europe, and Asia, such *Borrelia burgdorferi* sensu stricto, *Borrelia afzelii*, *Borrelia garinii*, *Borrelia bavariensis*, *Borrelia spielmanii*, *Borrelia bissettiae*, and *Borrelia mayonii*. In 2013 *B. burgdorferi* s.l. was reported for the first time in the Neotropical region, from *Ixodes aragaoi* ticks in Uruguayan Pampa. In addition, from 2011 to 2016, 17 suspected human cases of borreliosis-like syndrome were reported in Rio Grande do Sul (RS) state, Brazil, which contains only part of country in the Pampa biome. The goal of this work is to report the results of a state surveillance program conducted in order to investigate the presence of *B. burgdorferi* s.l. in its classic vector, *Ixodes* spp. ticks, from the Brazilian Pampa. For this, we searched for *Ixodes* spp. ticks in 307 rodents from 11 municipalities of RS state. We then tested the ticks for the presence of *B. burgdorferi* s.l. DNA using PCR analysis. Of 35 *Ixodes* spp. ticks tested, one larva and one nymph of *Ixodes longiscutatus* ticks tested positive for *Borrelia* sp. DNA. The phylogenetic analysis of the *flaB* fragment grouped our samples (referred as *Borrelia* sp. haplotype Pampa) into *B. burgdorferi* s.l. group in a particular branch with other South American haplotypes, and this group was close to *Borrelia carolinensis*, *B. bissettiae*, and *Borrelia californiensis*. This is the first evidence of *B. burgdorferi* s.l. circulation in ticks of the genus *Ixodes* in Brazil. These results highlight the need for the implementation of public health policies for the diagnosis and prevention of potential cases of human borreliosis in Brazil. Further studies are needed to fill the gaps in our knowledge of the distribution, pathogenicity, reservoirs, and vectors of these emerging South American *B. burgdorferi* s.l. haplotypes.

1. Introduction

Since the first isolation of the Gram-negative spirochete *Borrelia burgdorferi* from *Ixodes* spp. ticks and its association in the etiology of Lyme Disease (LD) (Burgdorfer et al., 1982), several other members of the Spirochaetaceae family have been identified throughout the world as emergent vector-borne bacteria. Among these bacteria, some *Borrelia* species and a set of still unnamed haplotypes of *Borrelia* species that are genetically related to *Borrelia burgdorferi* have been grouped into the *Borrelia burgdorferi* sensu lato (s.l.) complex (Postic et al., 2007). In addition to *Borrelia burgdorferi* sensu stricto (s.s.), the agent of LD in North America, the complex also comprises the other Lyme borreliosis agents in Europe and Asia, *Borrelia afzelii* and *Borrelia garinii*, respectively (Steere et al., 2016); as well as other emerging pathogens, including *Borrelia bissettiae*, *Borrelia spielmanii*, and *Borrelia bavariensis* (Collares-Pereira et al., 2004; Rudenko et al., 2011, 2008). Due to the emergence of new species and variants, it is likely that the number of members of *B. burgdorferi* s.l. complex will increase during next few years.

In general, *B. burgdorferi* s.l. is maintained in nature through complex interactions among ticks, particularly *Ixodes* spp., and a variety of vertebrate hosts. As a rule, *B. burgdorferi* s.l. has been detected in ticks only from the Northern Hemisphere. In 2013, *B. burgdorferi* s.l. was reported for the first time in the Neotropical region (Barbieri et al., 2013). However, borreliosis cannot be dismissed as a neglected public health issue in this region. Indeed, during 2009–2016, Brazil registered 4078 suspected cases of borreliosis-like disease, also known as Baggio-Yoshinari syndrome. Of those cases, 679 people were positive for

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Borrelia spp. by Western blotting and enzyme immunoassay tests (Oliveira et al., 2017). Moreover, despite the fact that since 1992 there are reports of LD-like syndrome in Brazil, to date *Borrelia* spp. has never been detected in *Ixodes* spp. ticks in that country, and there is no conclusive evidence regarding the potential tick species associated with the transmission of Baggio-Yoshinari syndrome (Basile et al., 2017).

Rio Grande do Sul (RS), the southernmost state of Brazil, is the only part of the country that contains the Pampa biome; which also occurs in Uruguay and in part of Argentina. From 2011–2016, 17 suspected human cases of Lyme-like borreliosis were recorded in 11 municipalities of RS, including one patient with positive serology to *B. burgdorferi* (data kindly provided by Dr. Stefan Vilges de Oliveira, Secretaria de Vigilância em Saúde, Brazilian Health Ministry). Taking into account the first epidemiological evidences of human borreliosis cases in RS state (Oliveira et al., 2017), it is plausible to hypothesize that *Borrelia* spp. may circulate in ticks from southern Brazil. This hypothesis is reinforced by the recent reports of *B. burgdorferi* s.l. in *Ixodes aragaoi* ticks from the nearby areas of RS, as Uruguay (Barbieri et al., 2013). Thus, the aim of this work is to report the results of a state surveillance program conducted in order to determine whether *B. burgdorferi* s.l. occurs in its classic vector, *Ixodes* spp. ticks, in the Brazilian Pampa.

2. Materials and methods

2.1. Study area and sample collection

The study area consisted in 11 municipalities of RS state: Barra do Quaraí (30°11′32.67″S/57°.31′22.17″W), Chuí (33°43′57.37″S/53°22′12.96″W), Herval (32°01′42.19″S/53°23′37.62″W), Porto Alegre (30°03′05.70″S/51°10′50.65″W), Santa Maria (29°39′38″S/53°49′8″W), Santa Vitória do Palmar (32°30′44.50″S/52°32′47.39″W), Santana da Boa Vista (30°51′59.94″S/53°12′00.87″W), Santana do Livramento (30°48′31.35″S/55°37′19.97″W), São Francisco de Assis (29°32′40.33″S/55°07′37.61″W), São Francisco de Paula (29°30′15.01″S/50°13′05.54″W) and Uruguaiana (29°45′38.78″S/57°05′12.82″W).

Ticks were obtained from wild rodents (Rodentia), since they are considered to be the main reservoirs of Neotropical *Ixodes* species (Barros-Battesti et al., 2006). Between 2014 and 2016 small rodents were sampled using live traps (Sherman and Tomahawk) and traps of interception and fall (pitfall traps with drift fences). Briefly, the captured animals were sedated with ketamine hydrochloride and xylazine hydrochloride for collection of ectoparasites, and to collect biometric data and photographic recordings for taxonomical identification. All procedures described in this study were approved by our Institutional Animal Care Committee (number 14/13 CEUA-IPVDF) and by Brazilian Biodiversity Guidelines (numbers 39496-2 and 43919-3, SISBIO). More information on the captured rodents and a full record of their ectoparasites are shown elsewhere (Michel, 2016). Ticks were also obtained by direct collection from vegetation. For this, the flagging/dragging technique was used (Sonenshine, 1993). Briefly, white flannel cotton cloths (90 × 70 cm) were dragged along the soil and shrubby vegetation in forested areas.

2.2. Tick identification

Ticks were collected and stored individually in 96% ethanol and transferred to the laboratory for taxonomic determination. The identification of ticks was carried out by morphology (Barros-Battesti et al., 2006; Durden and Keirans, 1996; Marques et al., 2004; Nava et al., 2017; Onofrio et al., 2009, 2014; Venzal et al., 2005, 2008).

2.3. Molecular detection of Borrelia spp. and phylogenetic analysis

After taxonomic identification of ticks, genomic DNA was extracted from individual specimens using the protocol according to Aljanabi and Martinez (1997). The investigation of *Borrelia* spp. DNA was performed using two Polymerase Chain Reaction (PCR) assays, as reported by Barbieri et al. (2013). Briefly, nested-PCR was performed targeting the Flagellin B gene (*flaB*) of *Borrelia* spp. Positive samples were further used to amplify a fragment of the *rrfA-rrlB* intergenic spacer region (*IGS*). PCR products of these reactions that matched the expected size were purified, sequenced, and then compared with sequences available in GenBank using BLAST algorithm. A phylogenetic tree of the *flaB* gene was constructed using MEGA 7 software (Kumar et al., 2016); the Maximum Likelihood algorithm and Kimura-2-parameter with gamma distribution served as the DNA substitution model, and 1000 bootstraps were used for the phylogeny test.

3. Results

Of the 307 rodents investigated, 17 were parasitized by *Ixodes* spp. ticks. A total of 35 *Ixodes* spp. ticks were collected from rodents and vegetation, as follows: *I. aragaoi* (one nymph, one larva), *Ixodes longiscutatus* (one female, five nymphs, one larva), *Ixodes fuscipes* (one nymph), *Ixodes loricatus* (three nymphs), *Ixodes auritulus* (two nymphs), and *Ixodes* sp. (20 larvae).

The molecular analysis of these ticks showed that one larva and one nymph of *I. longiscutatus* (Fig. 1) tested positive for the presence of *Borrelia* spp. DNA using *flaB* nested-PCR and *IGS* PCR. These ticks were collected from *Oxymycterus nasutus* and *Oligoryzomys nigripes* (Rodentia: Cricetidae) from Chuí and Santa Vitória do Palmar municipalities, respectively. Fig. 2 summarizes the distribution of *Ixodes* spp. ticks and *Borrelia* sp. in the investigated areas.

The amplified fragment sequences from both positive samples were identical. The BLAST analysis of the partial sequence of the *flaB* gene revealed 100% of identity to the sequence of uncultured *Borrelia* sp. clone C (GenBank JX082313), a member of the *B. burgdorferi* s.l. group, detected in *I. aragaoi* (previously classified as *Ixodes parariciunus*) ticks from Uruguay. The analysis of the *IGS* fragment showed 99% identity (best match) to *Borrelia* sp. SCW–30 h (GenBank AF221673), a member of the *B. burgdorferi* s.l. group, genospecies *B. bissettiae*, detected in an *Ixodes minor* nymph tick from South Carolina, USA. The sequences of *flaB* (KY657353) and *IGS* (KY657352) fragments were deposited in GenBank as *Borrelia* sp. haplotype Pampa. The phylogenetic analysis of the *flaB* fragment (Fig. 3) showed that *Borrelia* sp. haplotype Pampa was grouped together with the *Borrelia* species of the *B. burgdorferi* s.l. group, which includes LD spirochetes. Indeed, it seems that *Borrelia* sp. haplotype Pampa clusters in a particular interior branch with *Borrelia* sp. clones A, B and C from Uruguay, close to *Borrelia carolinensis*, *B. bissettiae* and *Borrelia californiensis*.

4. Discussion and conclusion

Since the first report of *B. burgdorferi* s.l. in ticks of the genus *Ixodes* in South America in 2013 (Barbieri et al., 2013), molecular surveys conducted in other countries have provided evidence of the transboundary circulation of *Borrelia* spp. across the continent. Several haplotypes of *B. burgdorferi* s.l. have recently been registered in *I. parariciunus*, *Ixodes cf. neuquenensis* and *Ixodes sigelos* ticks from Argentina (Nava et al., 2014; Saracho Bottero et al., 2017; Sebastian et al., 2016). Also, DNA of *Borrelia chilensis* was detected in samples of *Ixodes stilesi* ticks from Chile (Ivanova et al., 2014; Verdugo et al., 2017).

Here, we show for the first time evidence of the presence of *B. burgdorferi* s.l. in its classic vector, *Ixodes* spp. ticks, in Brazil. The DNA of *Borrelia* sp. haplotype Pampa was found only in one species of tick, *I. longiscutatus*. In general, *B. burgdorferi* s.l. has been reported in ticks belonging to the *Ixodes ricinus* complex (e.g., *Ixodes scapularis* and *I. aragaoi*). However, considering the reports from Chile and Argentina, it seems that tick species unrelated to the *I. ricinus* complex (such as *I. stilesi* and *I. sigelos*) might be associated with *B. burgdorferi* s.l. in South America. Indeed, *I. longiscutatus* is not genetically related to the *I. ricinus*

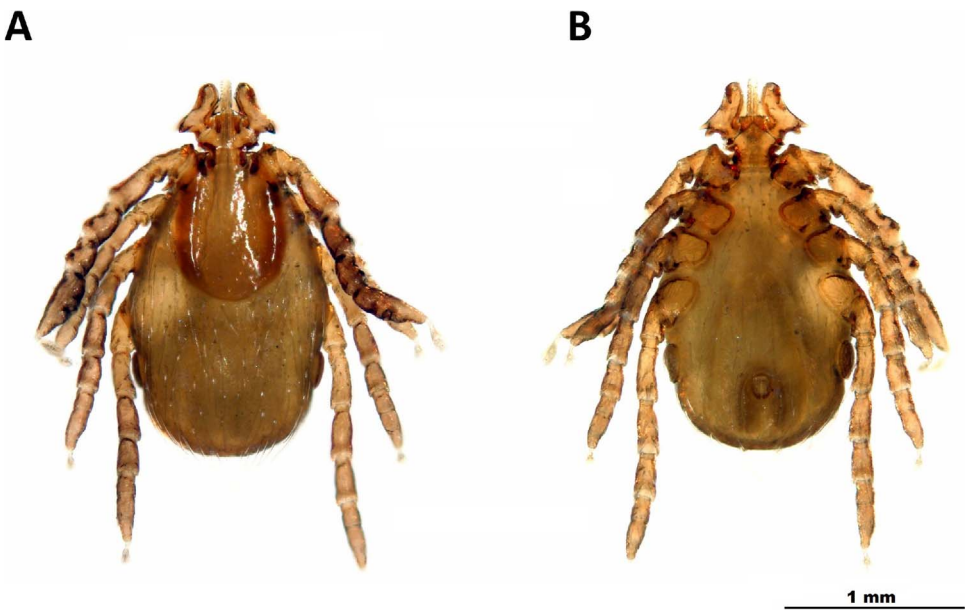

Fig. 1. *Ixodes longiscutatus* nymph. Panel A, dorsal view. Panel B, ventral view.

complex and is considered a monophyletic tick species (Guglielmone et al., 2005, 2006). It was previously assumed that this tick species was restricted to Argentina and Uruguay (Venzal et al., 2008), since it was just recently reported for Brazil (Michel, 2016). To date, little is known about *I. longiscutatus* hosts and distribution in Brazil.

Borrelia sp. haplotype Pampa seems to be closely related to other *Borrelia* spp. haplotypes previously identified in *I. aragaoi* from Uruguayan Pampa. Indeed, in our work, positive samples for *Borrelia* sp. were found only in the southern part of RS state that borders Uruguay. These haplotypes may represent a novel clade of *Borrelia* spp., that has an unknown pathogenicity and naturally circulates in both *Ixodes* species (*I. longiscutatus* and *I. aragaoi*) and wild rodents in South America. It is important to note that southern RS and Uruguay share the Pampa biome and that there are no naturally defined borders (rivers or mountains) between Brazil (RS) and Uruguay. In actuality, in the South American Pampa, *I. longiscutatus* and *I. aragaoi* ticks occur sympatrically and both of these species can share the same rodent hosts (Venzal et al., 2005, 2008; Onofrio et al., 2014).

Although, there are no confirmed cases of human parasitism by *I. longiscutatus* to date, it is noteworthy that, in some areas, the majority of ticks that serve as maintenance vectors of *B. burgdorferi* s.l. usually do not bite humans. Nevertheless, these species appear to be more important in the enzootic cycle of borreliosis than the "bridge" vectors, i.e., those that feed on vertebrate reservoirs and humans (Oliver, 1996).

Despite hundreds of human cases of Baggio-Yoshinari borreliosis-like syndrome in Brazil during the past few years, the country is not considered a traditional risk area for human borreliosis. It could be explained because, to date, *B. burgdorferi* s.l. was never isolated from human patients or even detected in its classic vector, *Ixodes* spp. ticks, in Brazil. However, the lack of reports of *B. burgdorferi* s.l. in ticks in Brazil may be due to the absence of a sound surveillance program or surveys focused on *Borrelia* spp. detection.

Although it is not possible, at this point, to determine whether an association between *Borrelia* sp. haplotype Pampa and human

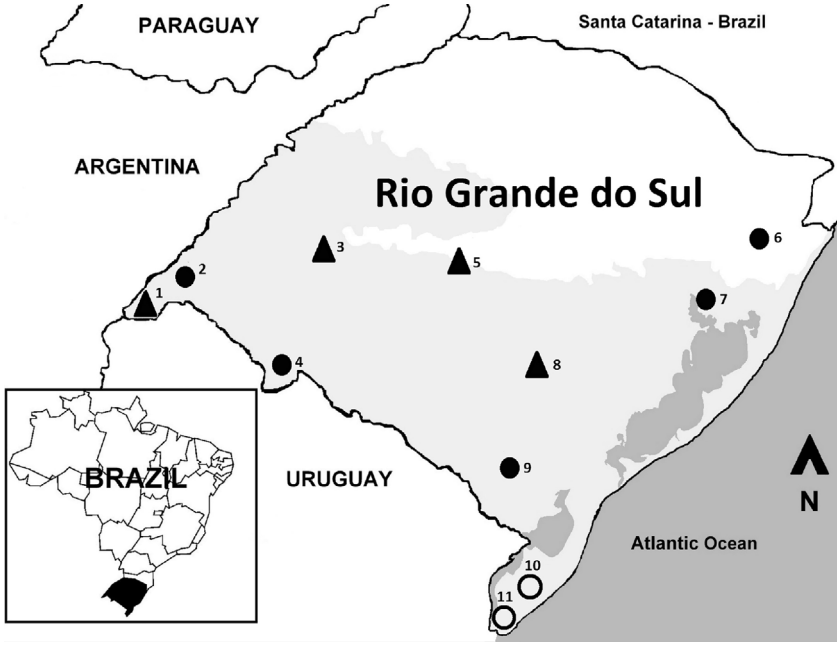

Fig. 2. Setting for investigation of tick infection with *Borrelia burgdorferi* sensu lato in Rio Grande do Sul state, Brazil, 2014–2016. Rio Grande do Sul state, Brazil, and neighboring countries. Light gray shading indicates the Pampa biome; dark gray shading indicates water bodies. Black triangles indicate sampled places in which *Ixodes* spp. ticks were not found; black circles indicate sampled places in which *Ixodes* spp. ticks negative to *Borrelia* sp. were collected; and open circles indicate sampled places in which *Ixodes longiscutatus* ticks positive to *Borrelia* sp. were collected. Numbers indicate the sampled municipalities, as following: Barra do Quaraí (1), Uruguaiana (2), São Francisco de Assis (3), Santana do Livramento (4), Santa Maria (5), São Francisco de Paula (6), Porto Alegre (7), Santana da Boa Vista (8), Herval (9), Santa Vitória do Palmar (10), and Chuí (11).

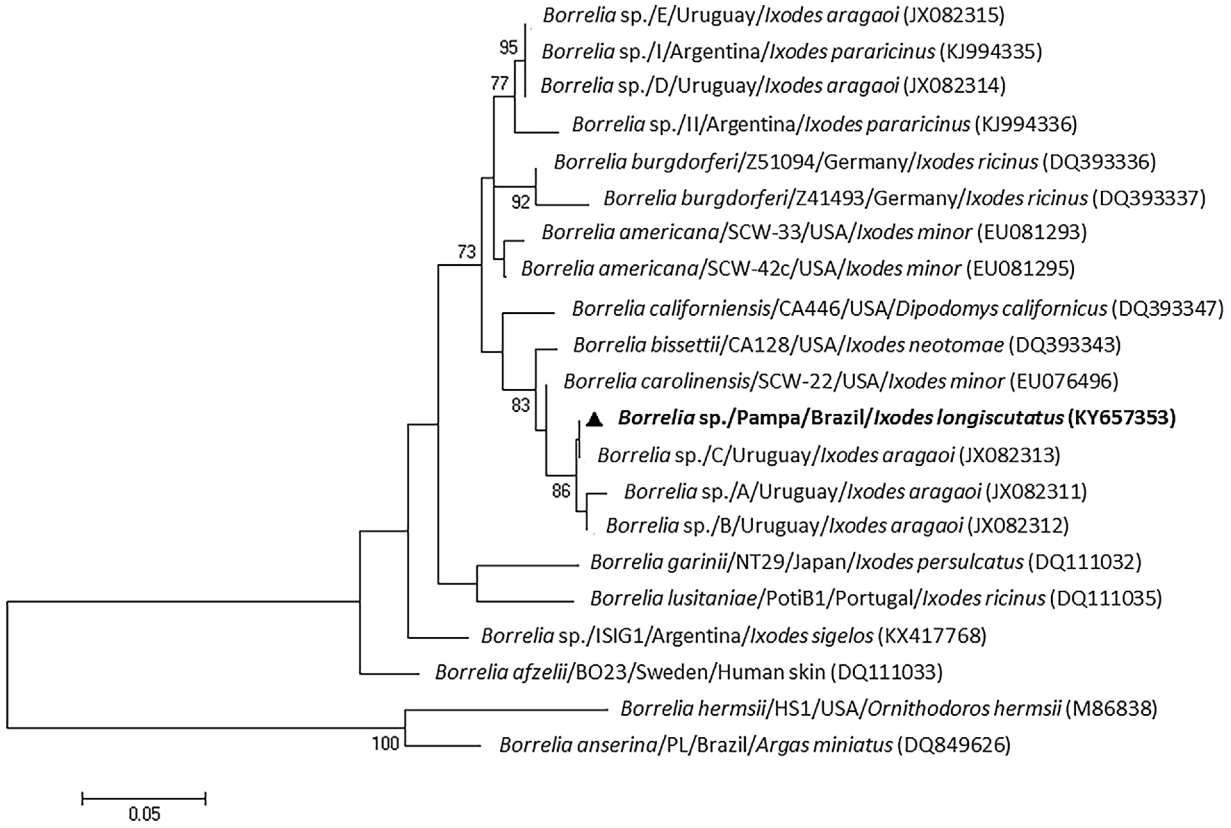

Fig. 3. Maximum likelihood-based phylogenetic tree of *Borrelia* spp. *flaB* gene. The sequence described in this study is showed in bold and indicated by a black triangle. For each sequence presented the following information is provided: species/strain/country/host (GenBank accession number).

borreliosis-like cases exists in Southern Brazil; the first detection of the genetic material from *B. burgdorferi* s.l. in ticks of the genus *Ixodes* in Brazil draws attention to a wider circulation of these bacteria in the Neotropics than was previously assumed. The set of results presented here, in addition to the lack of investigation of suspected human cases of borreliosis, highlights the need for the implementation of public health policies for the diagnosis and prevention of human borreliosis in Brazil. Additionally, further studies are needed to fill the gaps in the distribution, pathogenicity, potential vertebrate reservoirs and vectors of the South American haplotypes of *B. burgdorferi* s.l.

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