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Research Paper

First record of *Borrelia burgdorferi* B31 strain in *Dermacentor nitens* ticks in the northern region of Parana (Brazil)

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Abstract

The aim of this study was to investigate the presence of DNA of *Borrelia burgdorferi* sensu lato (s.l.) in ticks that feed on horses used for animal traction in rural Jataizinho, Parana, Brazil. Between February and June 2008, a total of 224 ticks was collected of which 75% were identified as *Dermacentor nitens* and 25% as *Amblyomma cajenense*. To amplify *B. burgdorferi* s.l. DNA, the intergenic space region (ISR) between the 5S (*rrf*) 23S (*rrl*) rRNA genes was used as targets for nested-PCR. Two ticks of the *D. nitens* species were positive for *B. burgdorferi* s.l. Both species showed a fragment of 184 bp, but the sequencing revealed 99.9% homology with the *B. burgdorferi* sensu stricto (s.s.) strain B31. These results showed, for the first time, the presence of spirochete DNA infecting ticks that parasitize horses used for animal traction, in the rural municipality mentioned. In conclusion, this study opens up promising prospects for determining the infection rate of *B. burgdorferi* s.s. genospecies or other species in the equine population, as well as the impact of the infection rate on Lyme disease in the state of Parana.

Key words: ticks, *Dermacentor nitens*, *Borrelia burgdorferi* sensu lato, equine, rural area.

Introduction

Lyme borreliosis or Lyme disease is a multisystemic disorder caused by tick-transmitted spirochetes of the complex *Borrelia burgdorferi* s.l., which infects several species of wild and domestic animals, as well as humans. Lyme borreliosis is currently considered to be an emerging zoonosis, and it occurs in different continents, including South America. The disease may have different clinical manifestations depending of which *Borrelia* species is involved (Baranton *et al.*, 2001).

The maintenance of *B. burgdorferi* s.l. in a particular region depends on the presence of reservoir hosts (Durden *et al.*, 2001). Wild animals (deers, rodents, marsupials and birds) act as natural reservoirs (Soares *et al.*, 2000). Domestic animals can become either accidental reservoirs of these spirochetes or hosts for vectors, especially in residential areas (Anderson, 1988). Humans can be accidentally infected when traveling or working in environments infested with infected ticks (Yoshinari *et al.*, 1995).

Tick-borne diseases have arisen in South America, particularly in Colombia (Palácios *et al.*, 1999) and Bolivia (Ciceroni *et al.*, 1997). In Brazil, the first case of Lyme disease was found in the state of São Paulo (Yoshinari *et al.*, 1993a). In some Brazilian states, serological surveys have reported the presence of antibodies anti-*Borrelia burgdorferi* s.l., which is a Lyme borreliosis simile in hu-

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mans as well as in different species of wild and domestic animals (Yoshinari *et al.*, 1997; Fonseca *et al.*, 2005). These previous studies suggest the presence of Lyme borreliosis in Brazil, and they show not only the importance of this disease in terms of public health but also the role of ticks as vectors in transmission of disease agents to the human to the human population. However, the causal agent of Lyme Borreliosis is unknown according to Dantas-Torres (2008).

The purpose of this study was to investigate the presence of DNA of *Borrelia burgdorferi* s.l. in ticks that feed on equines used as animal traction in the municipality of Jataizinho, Parana, Brazil.

Material and Methods

Sample collection and taxonomic identification

Between February and June 2008, 224 ticks were collected from equines used for animal traction, at 22 rural properties in the municipality of Jataizinho. These properties were described by INCRA (National Institute of Colonization and Agrarian Reform) as small properties with limited extension and minifundia. Jataizinho is located in the northern region of the state of Parana at the following coordinates: 56°14'15" S and 50°58'48" W. This area is bordered by the Tibagi River with an area of 201,847 km^2, 346 m above sea level and a wet subtropical climate. According to the official administrative data, the rural population was estimated to be 1,850 inhabitants.

At least, five ticks were removed from each equine and packed in sterile plastic bags and date and place of capture were recorded. At the laboratory, ticks were kept at -20 °C for further taxonomic identification and molecular analysis in order to detect the presence of *B. burgdorferi* s.l. Identification of taxa (genus and species) was performed using dichotomous keys based on Brazilian Ixodidae fauna (Aragão *et al.*, 1961; Barros-Battesti *et al.*, 2006). Prior to DNA extraction, ticks were, firstly, rinsed with a solution of sodium hypochlorite followed by another washing with a solution of ethanol 70° and distilled water for five minutes (Couceiro *et al.*, 2003). Ticks were preserved in sterile tubes at -20 °C for further molecular analysis.

DNA extraction, nested PCR (Polymerase Chain Reaction)

Genomic DNA from the ticks was extracted by alkaline hydrolysis according to previously described methodologies (Guy and Stanek, 1991; De Michelis *et al.*, 2000).

A culture of *B. garinii* (strain PBi) containing about 2×10^7 cells/mL was used as positive control. DNA of this genospecies was extracted by the Puregene™ Gentra Cell & Tissue Kit (Qiagen, Valencia, CA, USA) following the manufacturers protocol. DNA lysates were stored at 4 °C for immediate use or at -20 °C for further use.

To avoid contaminations, all procedures were performed in different rooms and in sterile chambers. Amplification of *B. burgdorferi* s.l. DNA was performed by nested PCR targeting the 5S *(rrf)* 23S (*rrl*) intergenic spacer region. For each nested PCR reaction, two sets of oligonucleotide sequences were used as previously described (Rijpkema *et al.*, 1995; Kurtenbach *et al.*, 1998; De Michelis *et al.*, 2000; Couceiro *et al.*, 2003) and DNA amplifications were performed in a thermocycler (MyCycler ™, Bio-Rad, Hercules, CA, USA). The positive control corresponds to the 380 bp and 230 bp fragments obtained in the first and second amplification, respectively. To prevent false results, negative controls were incorporated into the PCR procedures. The amplicons obtained were electrophoresed with 1.5% agarose gels (Bioline, London, UK), stained with 0.5 mg/ml of ethidium bromide (Bio-Rad, Hercules, CA, USA) and visualized under UV illumination with Dolphin-1D Gel Image Analysis Software (Wealtec Corp. NV, USA). The samples that produced amplicons at approximately 230 bp to 380 bp were kept at -20 °C for further use.

PCR products were purified and directly sequenced for both strands by the Sequencing Service Macrogen, Inc. (Seoul, Korea). The primers used for DNA amplification were used for sequencing. The results obtained were compared with the *Borrelia* genospecies sequences, registered in GenBank database, using the BLAST sequence analysis tool (www.ncbi.nlm.nih.gov/blast/Blast).

Results

A total of 224 adult ticks was removed from equines, most of which (n = 168) were engorged female. Based on morphological observations, ticks were taxonomically identified as *Dermacentor nitens* (75.0%) and *Amblyomma cajenense (*25.0%), both genera belonging to the Ixodidae family.

The nested PCR showed *Borrelia* DNA in two ticks with an amplicon of 230 bp approximately. However, considering the intensity, of the band (amplicon) in the gel only one PCR product was sequenced. The amplicon (Figure 1), corresponding to a specimen identified as *Dermacentor nitens* (an engorged female) and designated as Jataí 01 according to its geographic origin. DNA sequencing of the PCR (on both strands) showed a sequence with 184 pb (Table 1) and a 99.9% similarity with the *B. burgdorferi* s.s. strain B31.

Discussion

Lyme borreliosis presents several clinical pictures, particularly, in humans (Yoshinari *et al.*, 1993b; Galo, 2006). In Brazil, this disease was first reported in serologic studies in humans by Yoshinari *et al.* (1993a) and in animals by Fonseca and collaborators (Fonseca *et al.*, 1994). So far, diverse serological cases in the human population have been reported in some states, including Rio de Janeiro, São Paulo, Mato Grosso do Sul and in the Amazonia region

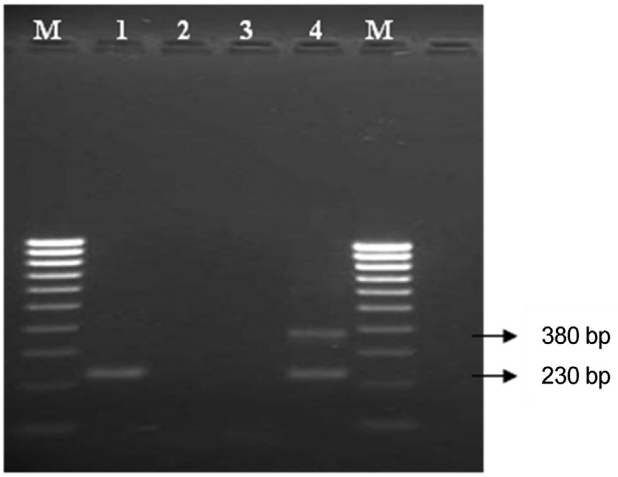

Figure 1 - DNA amplification from *Dermacentor nitens* by nested-PCR targeting the *B. burgdorferi* s.l. 5S (*rrf*) - 23S (*rrl*) intergenic spacer region. Lane 1: Tick sample Jataí 01- (*D. nitens*); lanes 2 and 3: negative controls (extraction and nested-PCR amplification); lane 4: positive control with PBi strain of *B. garinii*); M - DNA marker (100 bp).

Table 1 - Sequence identification of the *B. burgdorferi* s.s., B31 strain obtained from Jataí 01 tick (*D. nitens*), collected in the municipality of Jataizinho - Parana - Brazil.

CCCCTTGANNNNACTTACTTTGATTTTATTTTTATGTTTTTTA
TATTGGTGTTTTTGAATGTGTTGTTTAAATAACATAAAAAAT
AAAATATATATTGACATGTATTAAACAAAGATATATATTATT
TTATGTTGTATAAATAAATTGGCAAAATAGAGATGGAAGAT
AAAAATATGGTCAAAG

(Azulay *et al.*, 1991; Talhari *et al.*, 1992; Costa *et al.*, 2001). All of these cases have shown significant seroprevalence rates in humans and domestic animals, suggesting that the etiologic agent of Lyme borreliosis is present in the environment (Costa *et al.*, 2001).

According to some studies, the presence of *B. burgdorferi* s.l. has been recorded in different tick species, particularly in North America, Europe, Asia and South America (Guglielmone *et al.*, 1987; Palácios *et al.*, 1999; Clark *et al.*, 2001; Gonzalo *et al.*, 2001; Baptista *et al.*, 2004; Gaumond *et al.*, 2006; Hiraoka *et al.*, 2007). In Brazil, despite the wide geographic distribution of vertebrate hosts that are susceptible to tick infestation, there are few studies concerning the transmission cycle of *B. burgdorferi* in ticks from the Ixodidae family (Martins *et al.*, 1996). Thus, vector species for *Borrelia* spp in Brazil are not well known, although there is strong possibility that the species responsible for the sylvatic cycle may belong to the *Ixodes* genus, while the *Amblyomma* genus could be involved in transmission of the spirochetes to domestic animals and humans (Dantas-Torres, 2008).

In this research, the infected ticks were identified as *Dermacentor nitens*. This genus includes about 33 species with a worldwide distribution, except in Australia. In Eurasia, some species of *D. marginatus* and *D. reticulates* as well as of *D. variabilis* and *D. andersoni* from North America, infect livestock and other domestic animals. In Africa, these ticks do not play a significant role in infecting livestock (Jongejam and Uilenberg, 2008). In several Brazilian states, ticks identified as *D. nitens* have been found feeding on horses (Figueiredo *et al.*, 1999; Martins *et al.*, 2009) and humans (Guglielmone *et al.*, 2006). These findings show the importance of these ticks as vectors of pathogenic agents of Lyme disease and other diseases (Fernandez-Soto *et al.*, 2006).

In this research, the equine population was chosen due to prior knowledge that horses are the preferred hosts for ticks belonging to the *Ixodes* and *Amblyomma* genera in Brazil. Both genera are known vectors of *Borrelia* spirochetes. In fact, several studies carried out in animals in the states of Rio de Janeiro and Pará have reported the presence of antibodies against *B. burgdorferi* s.l., as well as the isolation of this spirochete from *Boophilus microplus* ticks (Salles *et al.*, 2002; Galo, 2006; Madureira, 2007).

In this study, only two infected specimens with *B. burgdorferi* s.l. were obtained. However, this result may be underestimated due to potential inhibitors present in the blood of engorged ticks. These inhibitors included hemoglobin, which limits PCR amplification and affects the detection of specific DNA fragments (Basta and Hulínská 1999; Sparagano *et al.*, 1999). PCR inhibitors usually affect the PCR reaction by interacting with DNA or interfering with DNA polymerase. In fact, inhibitors can survive DNA extraction/purification procedures by binding directly to single or double-stranded DNA (Bessetti, 2007). Moreover, in this study, extraction and purification of DNA from ticks were not performed with a standard kit. Instead, samples were processed with mechanical action followed by boiling the samples in an alkaline hydrolysis solution which may have enhanced their inhibitory effect.

Unlike the study by Ataliba (2006) in the state of São Paulo whose objective was to investigate *Borrelia* spp., in ticks parasitizing horses by nested PCR amplification for the flagelin B (*flab*) gene, the same technique was used in this study, but a different amplification target was employed. By using the intergenic spacer region of 5S (*rrf*) -23S (*rrl*) rRNA genes, the detection of DNA from *B. burgdorferi* s.s., in two ticks identified as *D. nitens* (Neumann, 1889) was accomplished for the first time.

So far, the studies regarding the identification of etiologic agents for Lyme disease in Brazil have shown negative results. However, serological diagnoses with positive results have been reported in several publications, which suggest prior exposure to the agent in both domestic animals and humans. In this study, our sequencing results showed 99.9% similarity with the *B. burgdorferi* s.s. strain B31, which is the first report concerning the presence of this important agent for Lyme borreliosis in the state of Parana and in Brazil.

Thus, the presence of these tick species in the equine population shows that further studies on ticks are needed to better understanding the prevalence of different tick vectors and the infection rate in domestic animals. Regarding the latter aspect, it is also important to emphasize that these equines parasitized by ticks have close contact with the human population, which justifies the development of new studies to determine its role in the transmission cycle of *B. burgdorferi* s.s. and/or other *Borrelia* genospecies in the peridomestic environment.

In conclusion, DNA of *B. burgdorferi* s.s. was detected for the first time in ticks parasitizing an equine population from the municipality of Jataizinho, Paraná state. This finding is promising for the understanding of this species and/or other genospecies of *B. burgdorferi* s.l. distribution in the state of Parana and other Brazilian states, pointing to the importance of the host community in the ecology of Lyme borreliosis. These methods could also be used in the future to clarify the prevalence of genospecies of *Borrelia burgdorferi* complex and the vectorial capacity of ticks and thus prevent the damages caused by this zoonosis in human and animal populations.

Ethics Committee

The "Zoonosis in rural residents from the municipality of Jataízinho, Parana, Brazil" project was approved on February 13, 2007, by the Ethics Committee in Animal Experimentation at the State University of Londrina (UEL) in Brazil. The study was registered under no. 58/06.

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