Original Article

Morphological, molecular and phylogenetic characterization of *Borrelia theileri* in *Rhipicephalus microplus*

Caracterização morfológica, molecular e filogenética de *Borrelia theileri* em *Rhipicephalus microplus*

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Abstract

This study aimed to perform a morphological, molecular and phylogenetic characterization of *Borrelia theileri* obtained from infected *Rhipicephalus microplus* in Brazil. Fifty engorged *R. microplus* females from cattle in the municipality of Seropédica, Rio de Janeiro, were analyzed for spirochetes by hemolymph smear. Macerated eggs and positive ticks, as well as blood from the bovine infested by these ticks, were analyzed the *glpQ*, *flaB* and *hpt* genes by PCR. The PCR products were purified and sequenced for analysis and construction of a phylogenetic tree. Only 2% (1/50) of the ticks generated a positive result by both smear and PCR. The spiral forms (*n* = 50) had (media ± SD) a mean length of 19.17 ± 4.12 μm, diameter of 0.2935 ± 0.0469 and number of turns 8.44 ± 2.59. Sequence alignments of the three evaluated genes exhibited 98% similarity to *B. theileri* isolates, occurring in a clade highly related to *B. theileri* strain KAT. Egg maceration samples were positive for the three evaluated genes, whereas bovine blood was negative by PCR. This is the most detailed characterization of *B. theileri* in the Americas to-date, presenting morphological, molecular and phylogenetic data, including the transovarial transmission of the spirochete in the host tick.

Keywords: Relapsing fever-like, *Borrelia*, Ixodidae, phylogenetic characterization.

Resumo

O estudo teve como objetivo realizar a caracterização morfológica, molecular e filogenética de *Borrelia theileri* obtida de *Rhipicephalus microplus* naturalmente infectado em bovino no estado do Rio de Janeiro, Brasil. Um total de 50 fêmeas de *R. microplus* ingurgitadas foram analisadas para espiroquetas por meio de esfregaço de hemolinfa. Ovos macerados e carrapatos, assim como sangue de bovinos infectados por esses carrapatos, foram analisados os genes *glpQ*, *flaB* e *hpt* por PCR. Os produtos de PCR foram purificados e sequenciados para análise e construção de uma árvore filogenética. Apenas 2% (1/50) dos carrapatos geraram um resultado positivo tanto pelo esfregaço como pela PCR. As formas espirais (n = 50) apresentaram (média ± DP) comprimento médio de 19,17 ± 4,12, diâmetro de 0,2935 ± 0,0469 e número de voltas de 8,44 ± 2,59. Os alinhamentos das sequências dos três genes avaliados exibiram 98% de similaridade aos isolados de *B. theileri*, ocorrendo em um clado altamente relacionado à linhagem de *B. theileri* KAT. As amostras de maceração de ovos foram positivas para os três genes avaliados, enquanto o sangue bovino foi negativo pela PCR. Esta é a mais completa caracterização de *B. theileri* nas Américas, apresentando dados morfológicos, moleculares e filogenéticos, incluindo a transmissão transovarial da espiroqueta no carrapato hospedeiro.

Palavras-chave: Febre recorrente, *Borrelia*, Ixodidae, caracterização filogenética.

Introduction

Species in the phylum Spirochaetes (order: Spirochaetales) are thin, spiral-shaped or wave-like, highly motile bacteria that are visualized by darkfield microscopy.

Borrelia theileri is a tick-borne spirochete known to infect cattle and other mammals like horse, sheep, and deer. In cattle, the infection can be characterized by fever, lethargy and anemia. This agent is particularly important because *B. theileri* infection induces cross-reactive antibodies that can interfere with the interpretation of seroepidemiological studies of other bovine spirochetes (ROGERS et al., 1999).

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The subclinical aspects of *B. theileri* infection were described by Rogers et al. (1999) and Guedes et al. (2008). This species of spirochete is one of the least-described pathogenic tick-borne Borreliae. Historically, this spirochete has been identified in Africa, Australia, and North and South America (MCCOY et al., 2014). In southeast Brazil, Yparraguirre et al. (2007) detected a *Rhipicephalus microplus* tick infected with *Borrelia* sp. strain BR, which was highly correlated with *B. theileri* and *B. lonestari*. Therefore, this study aimed to characterize the morphological, molecular and phylogenetic features of a *B. theileri* isolate obtained from naturally infected *R. microplus* in Brazil.

Materials and Methods

Ticks and hemolymph smears and examination

Rhipicephalus microplus ticks of the Porto Alegre strain free of *Babesia* spp. and *Anaplasma* spp. kindly provided by Dr. Itabajara da Silva Vaz Junior of the Universidade Federal do Rio Grande do Sul in December, 2014 (RECK et al., 2009). The ticks were maintained for two generations (January and March, 2015) on infested apparently healthy bovines (*Bos taurus*), *Babesia* spp. and *Anaplasma* spp. free. The animals were acquired from the dairy cattle sector of the Universidade Federal Rural do Rio de Janeiro (UFRRJ) in the municipality of Seropédica, Rio de Janeiro.

A hemolymph screen was performed on 50 engorged females collected after completing their second generation on the infested animal in UFRRJ. The specimens were placed in Petri dishes and fixed in plaster with the ventral region facing upwards. During the non-parasitic phase, the ticks were kept in an incubator at 27 ± 1 °C and a relative humidity above 80%.

On the 8th day of oviposition, hemolymph smears were generated by sectioning the distal region of the first legs of each tick. Hemolymph was dispersed on a 12-cell screen-printed slide for individual evaluation. The hemolymph smears were fixed in methanol and stained with 10% Giemsa and examined under an optical microscope using a 100x oil-immersion objective.

Morphometric analysis

The morphometric analysis of the spiral forms was performed by optical microscopy using the oil-immersion objective, with a coupled photo documentation system (D 'Cell software - Olympus®). The evaluated parameters were a diameter, total length, and number of spirals for each form.

Molecular analysis

Females that were positive for spiral forms in the smear, their eggs, and the infested bovine blood collected 15 days after tick feeding were submitted for molecular analysis. DNA extraction was performed using a commercial Dneasy® tissue kit (Qiagen®) following the protocol recommended by the manufacturer.

Amplification of DNA fragments of three *Borrelia* genes was attempted by PCR. For the Glycerophosphodiester Phosphodiesterase gene (*glpQ*), reactions were performed using primers GlpQ F+1 (5'-GGGGTTCTGTTACTGCTAGTGCCATTAC3') and GlpQ F-1

(5'-CAATTTTAGATATGTCTTTACCTTGTTGTTTATGCC-3') and for the hypoxanthine-guanine phosphoribosyltransferase gene (*hpt*) were performed using primers hptdegF (5'-GCAGAYATTACAAGAGARATGG-3') and hptdegR (5'-CYTCRTCACCCCATTGAGTTCC-3'). For the flagellin B gene (*flaB*) reactions were performed using primers FlaLL (5'-ACATATTCAGATGCAGACAGAGGT-3') and FlaRL (5'-GCAATCATAGCCATTGCAGATTGT-3') (MCCOY et al., 2014).

The PCR mixtures contained 1.0 U of GoTaq® DNA Polymerase (Promega®, Brazil), 1x Green GoTaq® Reaction Buffer, 1.5 mM MgCl₂, 0.2 mM dNTPs, 1.25 pmoles of each primer and 2.5 µl of DNA template. Samples were initially heated to 95 °C for 5 min to denature the template and activate the polymerase, followed by 40 cycles of denaturation at 95 °C for 60 seconds, annealing at 55 °C (*flaB*), 53 °C (*hpt*) or 57 °C (*glpQ*) for 60 seconds, and extension at 72 °C for 60 seconds, followed by a final extension at 72 °C for five minutes.

PCR products were analyzed by gel electrophoresis (1.5% agarose). For each reaction, one positive (DNA of *Borrelia anserina* strain AL) and two negative controls (water) were included in the reaction.

Sequencing and phylogenetic analysis

PCR products of the expected size were purified using a PuriLink® PCR purification kit (Life Technology®) and sequenced using a capillary-type Sanger platform in an ABI 3730 DNA Analyzer (Applied Biosystems, Life Technologies®). The sequences generated were compared to published data using the NCBI Nucleotide BLAST program. Multiple sequence alignments were performed with sequences obtained from this study and sequences from GenBank using MUSCLE, in the SeaView v.4 program (GOUY et al., 2010). The best-fit evolutionary model was determined using jModelTest version 2.0 (DARRIBA et al., 2012). The *Borrelia* phylogenetic tree was constructed by the maximum likelihood method with MEGA 7 (KUMAR et al., 2016). The support for the tree nodes was measured using a heuristic search with 1000 bootstrap replicates.

Results

Of the 50 hemolymph smears of engorged female *R. microplus* ticks analyzed, only one was positive, thus indicating a 2% infection rate. Microscopic analysis of Giemsa-stained smears illustrated a typical morphology for *Borrelia* spp. (Figure 1). Free forms were observed in the hemolymph (Figure 1A-E) and forms in contact with the surface of the hemocytes (Figure 1C, E and F). In total, 50 spirochetes were identified across all fields of the hemolymph smears. The spirochete detected is herein referred to as *B. theileri* strain C5. The average number of spirals, length, and diameter of 43 spirochetes measured in thick hemolymph smears is shown in Table 1. Longer spirochetes (greater than 27 µm) were suspected to be in the process of dividing or attached to another spirochete in tandem.

The engorged female *R. microplus* ticks hemolymph positive, and their egg sample was positive for *Borrelia* by PCR. From the *flaB*, *glpQ* and *hpt* PCRs, gene fragments of 593 bp, 548 bp and 389 bp were generated, respectively. No PCR products were amplified from the DNA in blood samples of the infested cattle. The sequences
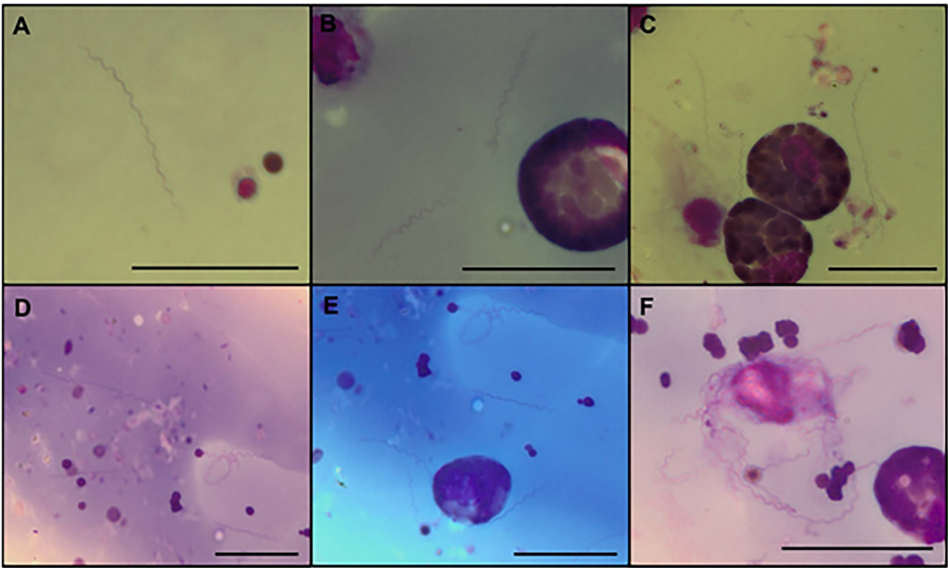
Figure 1. Typical forms of *Borrelia theileri* in hemolymph smears of *Rhipicephalus microplus* (A-F). Giemsa. Scale bar: 20 μm.

Table 1. Morphometric data (μm) for spirochetes forms of *Borrelia theileri* strain C5 and published data from other *Borreliae* species.

Species	Country	Host	Length Mean ± SD* (μm) (Minimum-Maximum)	Diameter Mean ± SD (μm) (Minimum-Maximum)	Spiral numbers Mean ± SD (Minimum-Maximum)	References
Borrelia theileri strain C5	Brazil	*Rhipicephalus microplus*	19.17 ± 4.12 (10.34-26.84)	0.2935 ± 0.0469 (0.20-0.31)	8.44 ± 2.59 (5-11)	This study
Borrelia theileri	Brazil	*Rhipicephalus microplus*	ND** (10-19)	ND	ND	Martins et al. (1996)
Borrelia theileri	Mexico	*Rhipicephalus microplus*	17.66 ± 0.46	ND	ND	Smith et al. (1978)
Borrelia theileri	Mexico	Bovine	11.65 ± 0.58	ND	ND	Smith et al. (1978)
Borrelia theileri	Botswana	Bovine	13.2 (9-18.5)	ND	Numerous	Sharma et al. (2000)
Borrelia theileri	Australia	Bovine	12.1 (6-19.5)	ND	ND (3-7)	Callow (1967)
Borrelia theileri	Australia	*Rhipicephalus microplus*	ND (8.3-18.3)	ND	ND (4-8)	Callow (1967)
Borrelia theileri	South Africa	Bovine	13.2 (9.6-18.9)	ND	ND (3-7)	Callow (1967)
Borrelia theileri	South Africa	Equine	5.7 (3.5-9)	ND	ND (1-3)	Callow (1967)
Borrelia lonestari	United States	Culture	15.48 (11-25)	ND	ND	Varela et al. (2004)
Borrelia turcica	Turkey	*Hyalomma aegyptium*	ND (10-25)	ND (0.2-0.28)	ND	Güner et al. (2003)

*SD = Standard Deviation; **ND = Not determined.

generated from the PCR-amplified genes were analyzed to determine the similarity to other species in the GenBank Nucleotide database. The partial sequence of the *flaB* gene showed 100% coverage and 99% similarity to that of *B. theileri* strain KAT (access number KF569936). It also showed 99% similarity and 99% coverage with sequences of *Borrelia* sp. strain BR (access number EF141022). For the *glpQ* gene, there was 98% similarity to *B. theileri* strain KAT (query coverage 99%) and 100% similarity to *Borrelia* sp. BR (query coverage 57%). The PCR products of the *hpt* gene aligned with 99% of the query sequence (query coverage 91%) of *B. theileri* strain KAT and 90% of the query sequence (query coverage of

99%) of *B. miyamotoi* strain CT13-2396. The novel sequences were deposited in the GenBank with the following accession numbers MG601737 (*fla*B), MG601738 (*glpQ*) and MG601739 (*hpt*).

The phylogenetic relationship analysis based on the *flaB*, *glpQ* and *hpt* gene sequences obtained in this study formed a clade highly related to *B. theileri* strain KAT, which was well-supported as being monophyletic (Figures 2, 3 and 4). In the phylogenetic tree of the *flaB* gene, the clade included *Borrelia* sp. strain BR, followed by the sequence obtained from this study and *B. theileri* strain KAT (Figure 2).
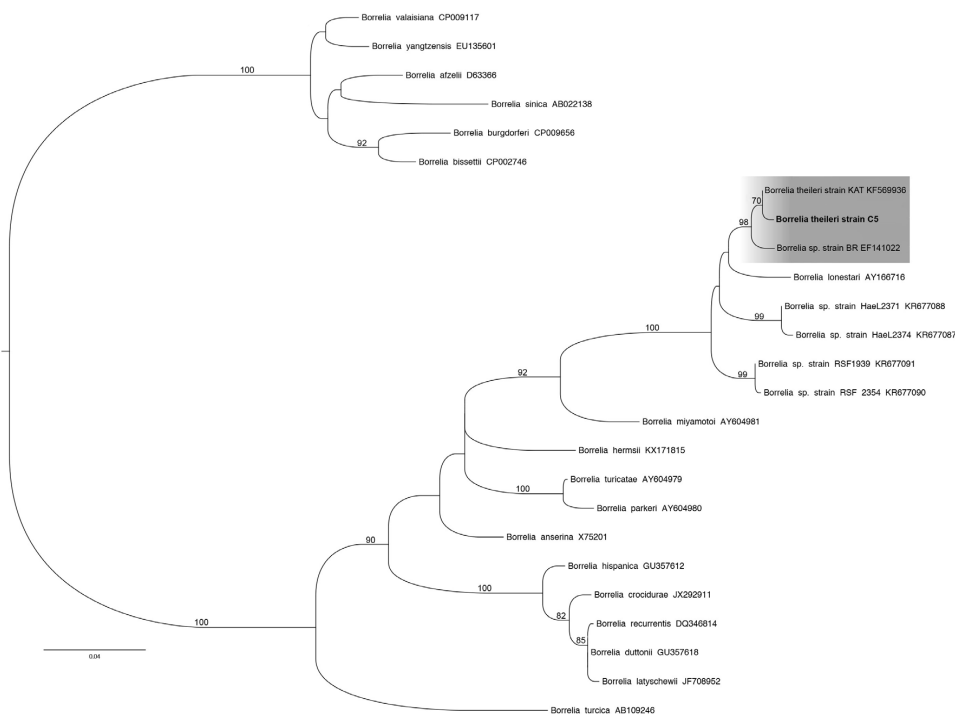
Figure 2. Phylogenetic relationships between *Borrelia* based on maximum likelihood analysis generated from comparisons of a 497 bp fragment of the *flaB* sequence. The scale bars indicate an evolutionary distance of 0.04 substitutions per position in the sequence. The numerical value ≥ 0.7 at the node indicates the bootstrap replicates supported by the inner branch. The branch labels include GenBank accession numbers. The Tamura 3-parameter model with gamma-distributed heterogeneity (T92 + G) was selected as the best-fit evolutionary model.
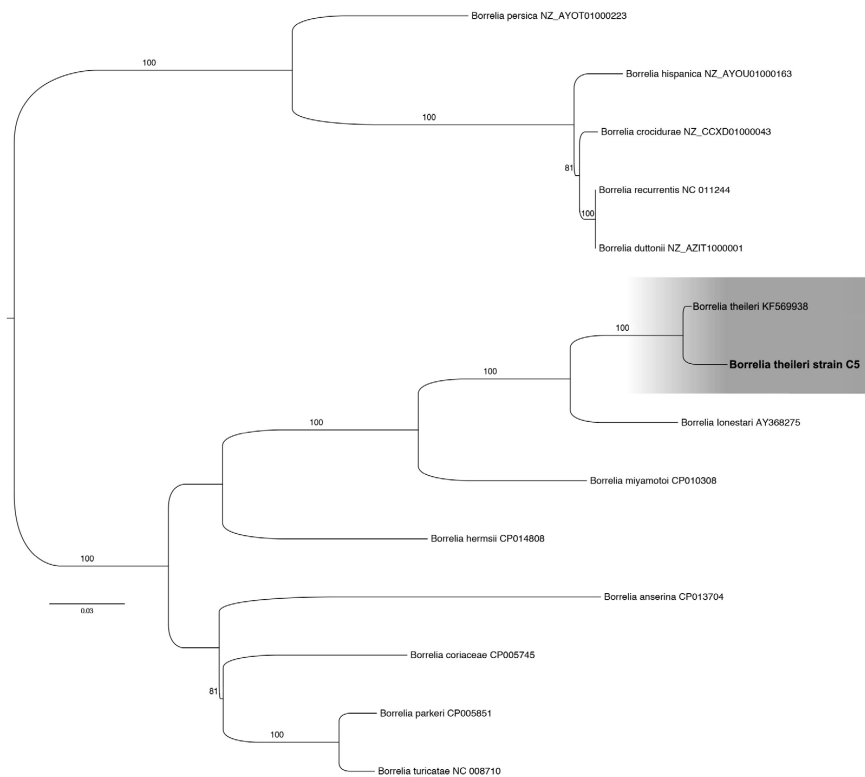

Figure 3. Phylogenetic relationships between *Borrelia* based on maximum likelihood analysis generated from comparisons of a 548 bp fragment of *glpQ* sequences. The scale bars indicate an evolutionary distance of 0.03 substitutions per position in the sequence. The numerical value ≥ 0.7 at the node indicates the bootstrap replicates supported by the inner branch. The branch labels include GenBank accession numbers. The General Time Reversible model with gamma-distributed heterogeneity (GTR + G) was selected as the best-fit evolutionary model.
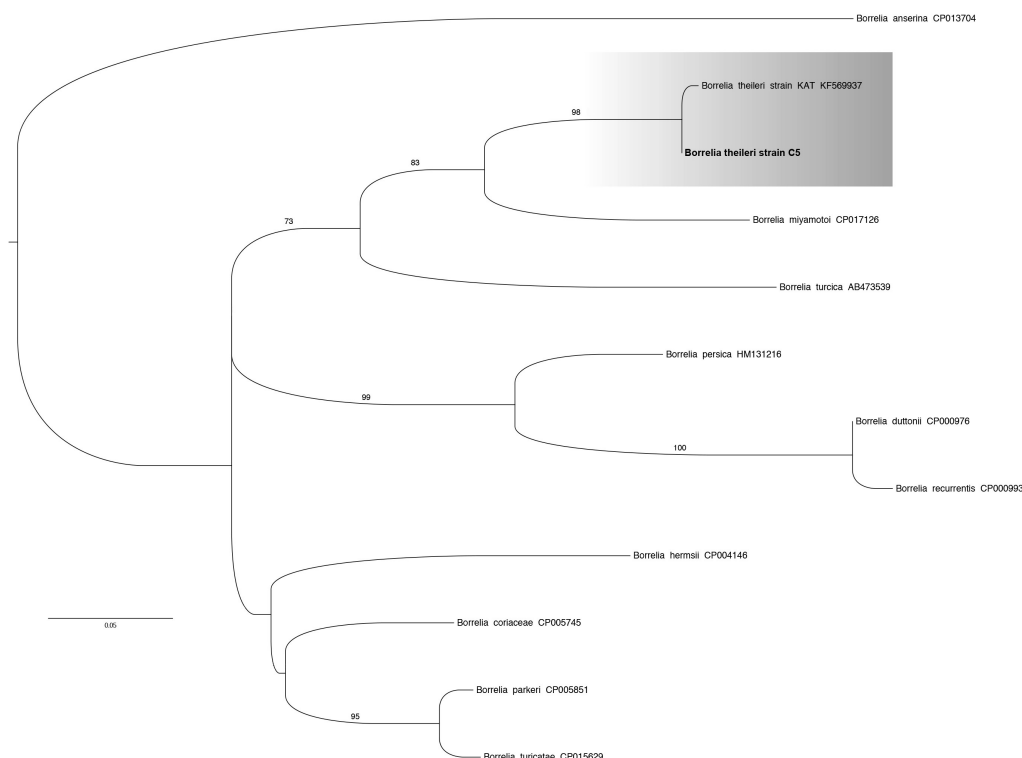
Figure 4. Phylogenetic relationships between *Borrelia* based on maximum likelihood analysis generated from comparisons of a 382 bp fragment of *hpt* sequences. The scale bars indicate an evolutionary distance of 0.05 substitutions per position in the sequence. The numerical value ≥ 0.7 at the node indicates the bootstrap replicates supported by the inner branch. The branch labels include GenBank accession numbers. The General Time Reversible model with gamma-distributed heterogeneity (GTR + G) was selected as the best-fit evolutionary model.

Discussion

This study presents the most complete description of *B. theileri* in *R. microplus* in the Americas to-date and includes molecular data and some morphological data regarding *B. theileri* in *R. microplus* ticks, in addition to the detection of transovarial transmission (TOT).

The description of this spirochete in *R. microplus* ticks in South America was made only by incidental findings in hemolymph smears (MARTINS et al., 1996; SOARES et al., 2000). In addition to these findings, Yparraguirre et al. (2007) reported a molecular isolate of *Borrelia* sp. BR in the Southeastern region of Brazil. However, the scarcity of molecular data for *B. theileri* meant that there was insufficient evidence for accurate identification of the species. However, the alignment of its sequences with *B. theileri* strain KAT and two other sequences of *B. theileri* from GenBank possibly identify this spirochete as *B. theileri* species (MCCOY et al., 2014).

The infection rate identified in our study (2%) was relatively higher than reported in other studies (MARTINS et al., 1996; YPARRAGUIRRE et al., 2007) and (MCCOY et al., 2014) Although the infested animal in this study was negative by PCR, it is possible that it was the source of infection for the positive tick, since *B. theileri* is usually in low parasitemia (CALLOW, 1967) and therefore would have infected only one of the 50 *R. microplus* females.

McCoy et al. (2014) observed that only one of six *Rhipicephalus* spp. from the same animal was positive for *B. theileri* by PCR.

In general, clinical signs of infection in cattle and other animals are mild and variable, but during the latent phase of infection transient increases in rectal temperature up to 39.5 °C and occasional mild depression with anorexia and anemia are observed (MCCOY et al., 2014).

In our study, TOT was observed through PCR amplification of *B. theileri* genes. These results corroborate Smith et al. (1978) who visualized spirochetes on eggs of *R. microplus* females infected with *B. theileri*. In addition, Smith et al. (1978) reported that the bacterium was observed in ovaries, central ganglia and hemolymph, with intense multiplication occurring in hemocytes. In contrast, Martins et al. (1996) were unable to visualize *B. theileri* in macerated eggs from the infected female. Rollend et al. (2013) reported the occurrence of TOT of *B. miyamotoi*, the relapsing fever group spirochete in *Ixodes scapularis*. The study evaluated many *I. scapularis* tick progenies providing strong evidence that *B. burgdorferi sensu stricto* TOT does not occur in *I. scapularis*.

The morphometric analysis of *B. theileri* strain C5 (Table 1) displays variation in the size and number of spirals, similar to that observed in the other studies of *B. theileri* presented in the table. It is important to emphasize; however, that organisms of this genus reproduce by transverse fission, which requires

elongation of the form and can produce variation in form length (VARELA et al., 2004).

Schwarzbach et al. (2015) demonstrated that *B. burgdorferi* pleomorphism is related to the cultivation conditions, which produces forms ranging from typical (elongated and spiraled) to round bodies. The authors also observed that each morphological variant has distinct biochemical markers, which could influence the pathogenesis of Lyme disease. Here, although *B. theileri* showed a wide variation in the length and number of spirals, there was no pleomorphism such as described by Schwarzbach et al. (2015). This lack of pleomorphism may be due to being isolated from a single tick, or this accentuated pleomorphism may not be a characteristic of *B. theileri*.

When first isolating *B. lonestari* in the ISE6 cell line of *I. scapularis*, Varela et al. (2004) demonstrated an intimate relationship between the bacterium and the ISE6 cell membrane by electron microscopy, suggesting a process of interaction between *B. lonestari* and this cell line.

In this study, some spirochetes were in contact with the hemocyte surface of the tick (Figure 1C, E and F), even in great abundance (Figure 1F), suggesting that there may also be an interaction between *B. theileri* and the tick cell. Thus, the methodology used by Varela et al. (2004) may be a candidate approach for a future study of this species.

Phylogenetic analyses of *flaB*, *hpt* and *glpQ* revealed that the sequences generated in this study clearly formed a cluster highly related to other *B. theileri* sequences in GenBank, including *Borrelia* sp. strain BR. This result definitively confirms the circulation of this species of *Borrelia* in South America.

Combining the morphometric and phylogenetic data (but without molecular evaluation), we believe that the spirochete parasitizing the hemolymph of *R. microplus* females in the American continent belongs to the species *B. theileri*. In Brazil, spirochetes are suspected of causing disease in humans. The *R. microplus* tick is among the possible ixodid species involved in the transmission of this bacterium (YOSHINARI et al., 2010). However, to-date, *B. theileri* has only been found in cattle, horses, goats and sheep (CALLOW, 1967) and there is no record of infection in humans. Nonetheless, as presented in the phylogenetic analysis, *B. theileri* is highly related to *B. lonestari* and *B. miyamotoi* two spirochetes of the relapsing fever group involved in human disease (VARELA et al., 2004; ROLLEND et al., 2013). In addition, the pathogenicity of each strain may vary, as we clearly observe a genetic difference between the different isolates, thus justifying the need for studies of the possible involvement of *B. theileri* in the etiology of the disease in Brazil.

Acknowledgements

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