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Brief communication

Molecular evidence of *Borrelia burgdorferi sensu lato* in patients in Brazilian central-western region

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ABSTRACT

We aimed to detect DNA of *Borrelia burgdorferi* in whole blood and serum samples of patients with clinical symptoms and epidemiology compatible with Brazilian Lyme-like disease. Four patients with positive epidemiological histories were recruited for the study. Blood samples were collected, screened by serologic testing by ELISA and Western blotting and molecular identification of *B. burgdorferi* by amplifying a fragment of the conserved gene that synthesizes the hook flagellar *flgE*. The results showed positive serology and for the first time, the presence of *B. burgdorferi sensu lato* in humans in the Midwest region of Brazil. The resulting sequences were similar to GenBank corresponding sequences of *B. burgdorferi flgE* gene. By neighbor-joining the phylogenetic analysis, the *flgE* sequence of the Brazilian strain clustered in a monophyletic group with the sequence of *B. burgdorferi sensu lato* under 100% bootstrap support. This study opens up promising perspectives and reinforces the need for additional studies to determine the epidemiological characteristics of the disease, as well as the impact of the prevalence of Brazilian borreliosis in Mato Grosso do Sul State, Brazil.

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Evidência molecular de *Borrelia burgdorferi sensu lato* em pacientes no centro-oeste brasileiro

Palavras-chave:
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Brasil

RESUMO

Este estudo promoveu a detecção de DNA de *Borrelia burgdorferi sensu lato* em amostras de sangue e soro de pacientes com manifestações clínicas e epidemiologia compatíveis com a doença de Lyme-símile brasileira ou síndrome de Baggio-Yoshinari. Para tanto, foi feita triagem sorológica pelos métodos de Elisa e *Western blotting* e a identificação molecular de *B. burgdorferi* por meio da amplificação de um fragmento do gene conservado que sintetiza o gancho flagelar (*flgE*). Os resultados demonstraram sorologia positiva e, pela primeira vez, a presença de DNA de *Borrelia burgdorferi sensu lato* em humanos na Região Centro-Oeste do Brasil. A análise genética das sequências dos isolados mostrou similaridade às sequências disponíveis no GenBank. Pela análise filogenética inferida pela sequência parcial do gene *flgE*, a cepa brasileira agrupou-se com a sequência de *B. burgdorferi sensu lato*. Este estudo abre perspectivas promissoras e reforça a necessidade de estudos adicionais a fim de determinar as características epidemiológicas da doença, bem como o impacto da prevalência da borreliose brasileira no Estado de Mato Grosso do Sul, Brasil.

Introduction

Lyme disease (LD) is an emerging multisystemic zoonosis, caused by spirochetes of the *Borrelia burgdorferi sensu lato* group and transmitted by ticks of the *Ixodes ricinus* complex.[1]

This is a disease with wide geographical distribution, and its clinical manifestations vary according to the species of *B. burgdorferi sensu lato* complex found in a certain geographical location.[2,3] The etiological and antigenic diversity explains the organotropism and the appearance of different clinical and laboratory pictures in different regions, presenting an increasing challenge to the management of this emerging zoonosis.[1]

In Brazil, the leading cases were described in the state of São Paulo in 1992,[4] and since then other cases have been described with the use of serological and molecular techniques in several Brazilian states: Mato Grosso do Sul,[5–7] Amazonas,[8] Tocantins,[9] and Paraná.[10]

Differences observed in epidemiological, clinical and laboratory characteristics in relation to LD described in the northern hemisphere allowed characterizing the Brazilian Lyme Disease-like Syndrome (BLDS) or Baggio-Yoshinari Syndrome[11,12] which, despite the classic migratory erythema and the usual systemic complications found in LD, frequently occurs with a high frequency of recurrences and the production of autoantibodies over its lengthy clinical evolution.[13]

Studies using molecular methods from human samples with BLDS-compatible symptomatology have reinforced the existence of cases of borreliosis in Brazil[10,14] Thus, this study aimed to investigate the presence of *B. burgdorferi* DNA in samples from patients with a clinical and serological diagnosis of borreliosis in Mato Grosso do Sul, Brazil.

Patients and methods

Four female patients with a mean age of 33.3 (± 11.9) years old and presenting clinical manifestations and BLDS-compatible epidemiology treated at the Rheumatology Department, Hospital Universitario Maria Aparecida Pedrossian, Universidade Federal de Mato Grosso do Sul (HUMAP-UFMS), were selected. These patients had a history of tick bites and of visits to high-risk areas in southeastern and midwestern regions of Brazil; they met the Brazilian criteria for the diagnosis of borreliosis, as adopted by the Laboratório de Investigação em Reumatologia (Rheumatologic Research Laboratory), Hospital das Clínicas, Faculdade de Medicina, Universidade de São Paulo (LIM-17), a reference center in Brazil.[12,15]

One of the patients was in an acute phase (diagnosed within three months of onset of disease) and the other three were in a late phase (diagnosed more than 3 months after the onset of the disease) of borreliosis. The patient who had early Lyme presented flu-like symptoms, including fever, headache, myalgia, and arthralgia. The other three patients, who had late Lyme disease, developed arthritis and also cognitive disorders, manifesting with nonspecific symptoms, including memory loss, sleep disturbances and mood changes, a depressive mood with social indifference and loss of appetite, and recurrence of flu-like symptoms and chronic fatigue. For all patients studied, serological tests for *B. burgdorferi* G39/40, of North American origin, were positive, using ELISA and WB[16] methodologies according to the standardization and interpretation recommended by LIM-17, a national reference laboratory.[12,15]

Forty individuals in good general condition, with no history of tick bite or of a recent trip to high-risk areas, provided blood samples and were included in the control group. Thirty of these were female (75.0%) and 10 were male (25.0%). The mean age was 34.6 (± 19.2) years old.

DNA was extracted using QIAamp DNA Kit (QIAGEN®) from samples of 100 μL of peripheral blood and of serum, according to the manufacturer's instructions. Purity (260 nm/280 nm) and total DNA concentration (ng/μL) were determined by optical density spectrophotometry (BioDrop® Touch Duo Spectrophotometer by BioDrop England).

The primers used were designed by Rezende et al. (Instituto Nacional da Propriedade Industrial [National Institute of Industrial Property] – INPI – Process BR 10 2016 021522 6) for amplification of a 262-bp fragment from the coding region of the conserved gene that synthesizes the flagellar hook (flgE) of *B. burgdorferi* described by Sal et al.[17] The following primers were used: flgE 262 FW (5′-TCCTCCGGGATTCATACAAG-3′) and flgE 262 Rev (5′-TGGGTGCAAATGTAGGTGAA-3′).

Amplification was performed in 25 μL of reaction final volume containing 17.55 μL of DNAse/RNAse-free ultrapure H_2O, 2.50 μL of 10× PCR Buffer, 0.75 μL of $MgCl_2$ (1.5 mM), 0.50 μL Of dNTP mix (10 mM), 0.25 μL of each primer (10 pmoles), 0.2 μL of Platinum™ TaqDNA Polymerase and 3 μL of DNA extracted from the sample.

PCR cycle conditions consisted of an initial denaturation at 95 °C for 3 min, followed by 45 repetitive cycles consisting of denaturation at 95 °C for 20 s, annealing at 62 °C for 25 s, extension at 72 °C for 25 s, followed by a final extension at 72 °C for 5 min. Good laboratory practices were followed to avoid contamination and, in each reaction, a negative control (water) was included to rule out the possibility of contamination. *Borrelia anserina* was used as a positive control in all reactions. PCR products were analyzed on 1.5% agarose gel electrophoresis, stained with SYBR Gold (Invitrogen) and analyzed by UV transillumination.

Positive samples were purified with QIAEX® II Gel Extraction Kit (Qiagen GmbH) and sequenced in ABI Prism 3130 automatic DNA sequencer (Applied Biosystems®) in both directions using BigDye Ready Reaction mix (ABI Corporation®). Alignment was performed on Clustal W2Â® software using the sequences obtained in this study and the sequence available on GenBank (Accession Number: CP009656.1). Phylogenetic analysis was inferred for the flgE gene using the MEGA 7.0® program and a dendrogram was constructed by the Neighbor-Joining method.[18] Confidence values for the individual branches of the resulting tree were determined by bootstrap analysis with 100 replicates.

The study was approved by the Research Ethics Committee (REC) of Universidade Federal de Mato Grosso do Sul (UFMS) as indicated in the research protocol number 1065681 of May 15, 2015 – CAAE 42325815.1.0000.0021. All controls and patients signed a consent form.

Results

The presence of anti-*Borrelia* antibodies and amplification of *B. burgdorferi sensu lato* DNA for the flgE gene were detected in all samples analyzed (Table 1).

For PCR reaction, eight samples (four whole blood and four sera) and 100% (4/4) of the whole blood samples were submitted and 25% (1/4) of the human serum samples were positive (Fig. 1).

Table 1 – Results of serology for *Borrelia burgdorferi* by ELISA and Western blotting and of PCR for the flgE gene in patients samples.

Patient	ELISA		Western blotting		PCR
	IgM	IgG	IgM	IgG	*flgE*
1	Negative	Positive	Negative	Positive	Positive
2	Positive	Positive	Positive	Positive	Positive
3	Negative	Positive	Positive	Positive	Positive
4	Negative	Positive	Negative	Positive	Positive

The sequences obtained in this study showed homology with sequences of *B. burgdorferi sensu lato* deposited in GenBank, allowing identification at the species level. According to the GenBank sequences, the sequenced and assessed extent of the flgE gene was 228 bp, which encode 76 amino acids.

The sequences obtained in this study were deposited in GenBank under accession numbers KU712208, KU712209, KU712210, KY073265 and KY073266. The sequences were grouped into a monophyletic group by phylogenetic analysis by the Neighbor-Joining method, with 100% bootstrap support (Fig. 2).

Discussion

Our results provide evidence of *B. burgdorferi* sensu lato in whole blood samples and the unprecedented detection of amplified spirochete DNA for the flgE gene in a human serum sample in our region.

The gene that synthesizes the flagellar hook (flgE) is a unique chromosomal gene, approximately 1 kb, that encodes the flagellin protein (41 kDa). Because it is highly conserved, its diversity is valuable in distinguishing *Borrelia* species.[19,20] Phylogenetic analysis using the flagellin gene sequence confirmed homology among the isolates.

All analyzed whole blood samples showed positivity and only a single serum sample was positive in the PCR reaction. Coburn et al.[21] demonstrated the binding ability of *B. burgdorferi* to host platelets and Goodman et al.[22] state that perhaps this is the reason for the higher concentration of plasma versus serum spirochetes and suggest that plasma is the preferred sample for research.

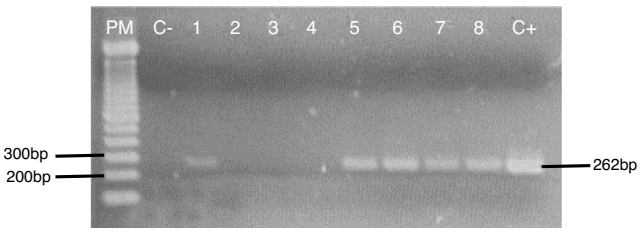

Fig. 1 – PCR of flgE gene analyzed on 1.5% agarose gel stained with ethidium bromide: MW, molecular weight (100 pb); C–, negative control; 1, 2, 3 and 4, serum samples from patients with Brazilian borreliosis; 5, 6, 7 and 8, whole blood samples from patients with Brazilian borreliosis; C+, *Borrelia anserina*.

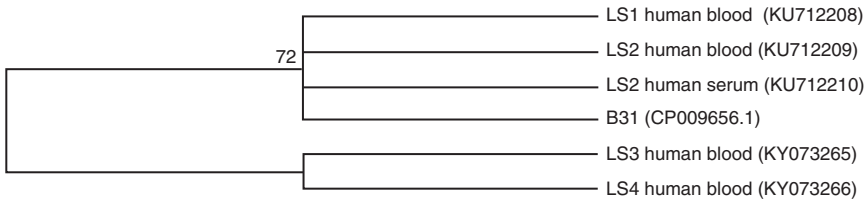

Fig. 2 – Phylogenetic tree based on the comparison of the coding region of the flgE gene of *Borrelia burgdorferi*.

In Brazil, patients diagnosed with characteristics suggestive of borreliosis present a history of exposure to ticks, a suggestive clinical picture with frequent recurrences, a positive serology but with low, little persistent titers, including the visualization of structures suggestive of spirochetes in blood samples of patients with a compatible clinical profile.[12,13]

The presence of anti-*Borrelia* antibodies detected by ELISA and WB techniques has been demonstrated in symptomatic and asymptomatic patients, with positive epidemiology of contact with ticks in several regions of Brazil.[5,6,8–10]

However, the observation of a serological reactivity pattern different from LD with low sensitivity and variable titers led to the definition of national criteria for the interpretation of serological tests. LIM-17, a reference center in Brazil, adopts a WB evaluation criterion that is based not on the presence of specific bands, but on the quantification of bands: a test is considered positive with the presence of 2 bands for IgM, 4 bands for IgG or yet a combined pattern of 1 IgM band and 2 IgG bands.[12,15]

The description of spirochetes in their L-form, known as cell wall-deficient bacteria and which may alter their morphology when survival conditions are not favorable,[12] and the subsequent detection of *B. burgdorferi sensu stricto* by Mantovani et al.,[14] have aided in the understanding of most controversies on BLDS: a protracted permanence of spirochetes in the host due to the evasion mechanisms of host defenses,[23] difficulties to cultivate these organisms in traditional media such as BSK, although such media describe the visualization of structures suggestive of spirochetes in human blood samples of patients with a compatible clinical profile,[12] low immunological response against the bacteria, causing low, little persistent, titers of antibodies, development of autoimmunity[12] and resistance to their elimination from the host, with clinical and serological relapses.[13,23–25]

These results confirm the existence of borreliosis caused by *B. burgdorferi* sensu lato in the state of Mato Grosso do Sul, Brazil, with symptoms similar to classic LD and suggest the need to continue epidemiological, serological and molecular studies in order to better characterize this emerging zoonosis in the region, which is rarely fatal but of great morbidity when not adequately diagnosed and treated due to recurrences and progressive clinical complications.

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Conflicts of interest

The authors declare no conflicts of interest.

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