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AMPLIFICATION OF THE *flgE* GENE PROVIDES EVIDENCE FOR THE EXISTENCE OF A BRAZILIAN BORRELIOSIS

Elenice MANTOVANI, Roberta G. MARANGONI, Giancarla GAUDITANO, Virgínia L.N. BONOLDI & Natalino H. YOSHINARI

SUMMARY

Introduction: The symptoms of Brazilian borreliosis resemble the clinical manifestations of Lyme disease (LD). However, there are differences between the two in terms of epidemiological and laboratory findings. Primers usually employed to diagnose LD have failed to detect *Borrelia* strains in Brazil. **Objective:** We aimed to identify the Brazilian *Borrelia* using a conserved gene that synthesizes the flagellar hook (*flgE*) of *Borrelia burgdorferi sensu lato*. **Method:** Three patients presenting with erythema migrans and positive epidemiological histories were recruited for the study. Blood samples were collected, and the DNA was extracted by commercial kits. **Results:** The gene *flgE* was amplified from DNA of all selected patients. Upon sequencing, these positive samples revealed 99% homology to *B. burgdorferi flgE*. **Conclusion:** These results support the existence of borreliosis in Brazil. However, it is unclear whether this borreliosis is caused by a genetically modified *B. burgdorferi sensu stricto* or by a new species of *Borrelia* spp.

KEYWORDS: Lyme disease; Lyme disease-like; Spirochaetales.

INTRODUCTION

The Brazilian borreliosis resembles the classic Lyme disease (LD) of North America and Eurasia with characteristics of clinical features including erythema migrans (EM) skin lesions[6,16] and osteoarticular, neurological and cardiac symptoms as the bacteria disseminates[27,35,36].

However, the epidemiology and laboratory assays indicate that the causative agent inducing borreliosis in Brazil is different from commonly occurring borreliosis in North America. The ticks commonly associated with borreliosis to humans, *Ixodes ricinus* complex, have not been identified in regions of Brazil that possess a high incidence of borreliosis[7,10]. *Borrelia burgdorferi*, the LD etiological agent, was not present in BSK II medium cultures of patient blood samples[36], ticks[1,10] or reservoir animals in Brazil[1]. Moreover, specific serologic tests (ELISA and Western blotting), which identify antibodies to *B. burgdorferi sensu lato* of North American or European origins, have shown low sensitivity when applied to Brazilian patients' samples[22,26].

Previous attempts to amplify genes specific to *B. burgdorferi,* including outer surface protein A (OspA) and genes for flagella filament (major flagellin FlaB, and minor flagellin FlaA), have failed in Brazilian samples. Ribosomal protein primers (16S rRNA) have also failed[5,9]. Other conserved genes that are necessary for *Borrelia* infection and survival in a variety of vertebrate and invertebrate hosts have also been investigated. The first targeted gene belonged to the cp32 plasmid group[31], which was found to be preserved in the majority of *B. burgdorferi* strains. Due to strong homology with human DNA, gene products from cp32 could not be sequenced. Finally, genes encoded on lp 25 and lp 28-1,[13,23] which are important for *Borrelia* virulence and infectivity, were investigated. The results were also negative.

The motility and morphology of *B. burgdorferi* is controlled by periplasmic flagella located between cell cylinder and outer membrane sheath. This structure is composed of a basal body, a hook and a filament (proteins FlaA and FlaB). Recently, SAL *et al.* have studied the importance of the flagella hook in *B. burgdorferi* biology by targeting the gene that encodes the hook structural protein FlgE[25]. Since previous Molecular Biology studies had failed to identify genes codifying flagella proteins, the aim of the present study was to test whether PCR targeting *flgE* gene would be helpful to identify the etiological agent of Brazilian borreliosis.

MATERIALS AND METHODS

Patients: We selected three patients presenting with EM between November 2008 and October 2009. One patient was female and two were male, and the mean age was 47.3 (± 28.2) years. They had a history of a tick bite and had visited high-risk areas in different regions of Brazil. All patients presented with flu-like symptoms, including fever, headache, myalgia, arthralgia, chills and fatigue (Table 1). All subjects fulfilled the Brazilian criteria for diagnosing borreliosis, as adopted by

Faculdade de Medicina da Universidade de São Paulo, Sao Paulo, SP, Brazil.
Correspondence to: Natalino Hajime Yoshinari, MD, PhD, Av. Dr. Arnaldo 455, 3° andar (sala 3184), Cerqueira Cesar, 01246-903 Sao Paulo, SP, Brasil. Phone. +55.11.30617496, Fax. 5511 30617490. E-mail: yoshinari@lim17.fm.usp.br

the Laboratório de Investigação em Reumatologia, Hospital das Clínicas, Faculdade de Medicina, University of São Paulo (LIM-17), the reference center in Brazil[16,36].

Table 1
Epidemiological and clinical characteristics of the patients with Brazilian borreliosis

Features	Number (%) of patients
Positive epidemiologic history	3 (100)
Tick bite history	3 (100)
Flu-like symptoms	3 (100)
Erythema migrans	3 (100)
Arthritis	1 (33.3)
Arthralgia	3 (100)

Two patients were in the acute stage (diagnosed within three months of disease onset) and one was in the latent stage (diagnosed more than three months after disease onset) of borreliosis. The patient who was in the latent disease stage had developed arthritis. Two (66.6%) subjects had serological test results positive for *B. burgdorferi* G39/40 of North American origin, as determined by ELISA or WB according to Brazilian standardization[18]. The patient with negative serological test results was taking antibiotics at the time of blood sample collection (Table 2).

Thirty healthy individuals without a history of tick bite or recent travel to high-risk areas were included in the control group. Nineteen of the controls were female (63.3%) and 11 were male (36.7%). The mean age was 38.1 (± 22) years.

This study was approved by the Ethics Committee in Research, CAPPesq of the Clinical Board of the School of Medicine - University of São Paulo, as stated in Protocol of Research number 0895/05. All patients and controls provided informed written consent.

METHODS

DNA was extracted using the QIAamp® DNA Blood Midi Kit (Qiagen GmbH) from 2 mL of peripheral blood, following the manufacturer´s instructions.

Based on the *flgE* gene, one primer pair was designed using the Primer3 online software[24]. These primers were as follows: *flgE* 470 FW (5'-CGCCTATTCTAACTTGACCCGAAT - 3') and *flgE* 470 Rev (5'-TTAGTGTTCTTGAGCTTAGAGTTG - 3').

Amplification was performed in a 50 µL final volume reaction containing 5 pmoles of each primer, 10 mM Tris-HCl, 1.5 mM $MgCl_2$, 1.25 mM dNTP and 1.5 U TaqDNA polymerase in DNAse/RNAse free H_2O with 5 µL of the DNA sample. DMSO (1.5 µL) was added immediately before cycling (3% final concentration).

PCR cycle conditions consisted of an initial denaturation for 3 min at 95 ºC, and 40 repetitive cycles of 45s at 95 ºC, 45s at 64 ºC, 45s at 72 ºC, followed by a final extension for 7 min at 72 ºC. Good laboratory practice to avoid contamination was always followed, and in every reaction, we included one negative control to check for possible contamination. *B. garinii* was used as a positive control in all reactions. PCR products were eletrophoresed through a 1.5% agarose gel, stained with SYBR Gold (Invitrogen) and examined by UV transillumination.

Positive samples were purified with the QIAEX ® II Gel Extraction Kit (Qiagen GmbH), and sequencing of genomic regions was performed with the BigDye Terminator Cycle Sequencing kit, version 3.1 (Applied Biosystems) and the ABI Prism 3730 DNA analyzer (Applied Biosystems). All sequences were examined for potential overlaps using the SeqManII program (DNAStar, Inc.). Prior to assembly, sequence ends were trimmed and primer sequences were removed manually. The sequences obtained were submitted for BLAST analysis to identify the sequences most closely related to the sample sequences[2].

RESULTS

PCR targeting of the *flgE* gene, which amplifies a fragment of 470bp, yielded products in all patients evaluated (Fig. 1). The samples of the control group were not amplified by the primers used in this study.

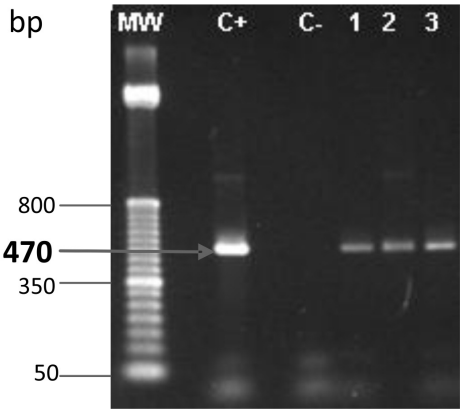

Fig. 1 - PCR *flgE*: MW = molecular weight (50bp); C+ = *B. garinii*; C- = control group; 1, 2 and 3 = Brazilian borreliosis patients.

Table 2
Demographic, treatment, and laboratory data of the patients with Brazilian borreliosis

Patient	Age (years)	Symptoms onset (days)	Treatment (days)	Serology for *B. burgdorferi*	PCR *flgE*
1	67	11	None	Positive	Positive
2	60	270	None	Positive	Positive
3	15	30	Tetracycline (7)	Negative	Positive

Fig. 2 - Alignment comparison of sequences generated from positive samples with the gene of *Borrelia burgdorferi* flagellar hook protein (*flgE*) (L43849) performed in BLAST. Base differences are outlined in red. All the three positive samples sequenced presented the same base difference when compared with *Borrelia burgdorferi sensu stricto* sequence.

Sequences disclosed an identity of 99% to the gene for *B. burgdorferi flgE* (L43849).

Partial sequences (*flgE* 470) of *B. burgdorferi flgE* generated in this study were 329 bp in length, and they were submitted to GenBank (accession no. HM245929). Upon comparing our sequence to the fragment already deposited in GenBank (L43849), which corresponds to the *B. burgdorferi flgE* gene, we could identify two different base substitutions (Fig. 2).

The sequence of the positive control (*B. garinii)* was compared with that of *B. burgdorferi*. Our positive control was 94% homologous to the gene for *B. burgdorferi* flagellar hook protein (L43849) and only 92% homologous to the sequence of the patients' samples. This eliminates the possibility that contamination had impacted the PCR.

DISCUSSION

Brazilian borreliosis has been investigated since 1989[37]. Understanding its etiology has been a priority for Brazilian researchers. Brazilian patients diagnosed with *Borrelia* infections characteristically had a history of exposure to ticks, showed frequent relapsing symptoms, had low serologic immune response to the etiological agent of LD (*B. burgdorferi sensu lato* antigens) and contained no *Borrelia* species in their blood that were able to be identified *ex vivo*. In the current study, we identified the *flgE* gene in blood samples of patients diagnosed for *Borrelia* infection for the first time. Our study utilized a pair of primers derived from the conservative *Borrelia* gene *flgE*, which had not been previously tested to diagnose LD in the Northern Hemisphere.

The *flgE* gene is identified in several species of Borrelia, including the etiological agents of relapsing fever *(B. hermsii, B. duttonii, B. recurrentis*). In this study, the comparison of our gene sequence in GenBank, showed that Brazilian spirochete had higher homology with microorganisms of *B.burgdorferi sensu lato* complex.

Several cases resembling LD symptoms have been reported in South America, Africa and Australia[3,8,14,19,21,29]. However, *B. burgdorferi* had never been cultured in BSK II medium or identified by molecular procedures in these continents. Recently, TALHARI *et al.* reported the occurrence of Lyme borreliosis in patients from the Brazilian Amazon Forest[32]. They found spirochetes in skin biopsies of EM using specific immunohistochemistry and focus floating microscopy for *B. burgdorferi.* However, the authors failed to isolate and culture these microorganisms in BSK II medium. Also, nested PCRs targeting conserved genes were performed on DNA samples isolated from the biopsies and were negative. Therefore, molecular procedures targeting the *flgE* gene may provide additional tools for understanding zoonosis presenting with a clinical spectrum resembling LD.

The *flgE* gene is formed of 1119 nucleotides and is responsible for the synthesis of the flagella hook structure[15,23]. The Brazilian spirochete

showed consistent differences in two nucleotides bases in comparison with *B. burgdorferi sensu stricto.* This distinctive feature may serve as a fingerprint for Brazilian *Borrelia*.

Importantly, DNA sequencing of positive samples from genus *Rhipicephalus* ticks and bovine peripheral blood demonstrated the same sequence pattern as seen in Brazilian patients. Their nucleotide alignment also showed different arrangement of two bases in comparison to *B. burgdorferi sensu stricto* sequence (unpublished data). Taken together, these findings reinforce the different epidemiology of Brazilian borreliosis from that described in LD.

In the current study, primers for *flgE* were tested on samples from a homogenous group of patients who possessed typical EM and positive epidemiological characteristics. Among the three studied subjects, two of them were at the acute disease stage and one was at the latent stage, complicated with arthritis. One of three serum samples showed negative serological results by ELISA and WB analysis, but this patient had received short courses of antibiotics with her EM rashes and had remained seronegative, whereas the other two patients had seroconverted. The ability of antibiotics to block seroconversion without eliminating infection is well documented[11,17,20].

The two patients at the acute stage of disease presented with EM and flu-like symptoms after a tick bite episode. The patient in the latent stage was a 60-year-old man (patient 2) who was infected in the Amazon Forest. He presented with flu-like symptoms and EM at a tick bite site, and he was treated with 100 mg doxycycline twice a day for 15 days. About eight months later, he presented with a relapsing episode of knee arthritis. At that time, serological tests performed for *B. burgdorferi* had positive results, and the PCR targeting the *flgE* gene confirmed the diagnosis of borreliosis.

In Brazil, patients who receive inadequate treatment at the acute stage or are not diagnosed until the latent stage exhibit relapsing symptoms in approximately 75% of the cases[27,35]. Delay in diagnosis or misdiagnosis leads to improper treatment and an unfavorable outcome. Unfortunately, because Brazilian physicians are not used to identifying this emerging zoonosis, most of the cases are diagnosed at the latent stage of the disease.

The reasons for why there are differences between LD and Brazilian borreliosis are still unclear. Brazilian researchers speculate that the etiologic agent of Brazilian borreliosis could either be a different *Borrelia* species or a genetically modified *B. burgdorferi sensu stricto*[38]. It is common knowledge that borreliosis can be caused by different species of *Borrelia*. Recently, STANEK & REITER[30] reviewed all of the identified species/genospecies of *Borrelia*. According to the authors, 10 years after the discovery of spirochaetes as agents of LD in 1982 in the United States of America, three genomic species had diverged from the phenotypically heterogeneous strains of *B. burgdorferi* that were isolated in North America and Europe: *B. afzelii*, *B. burgdorferi sensu stricto* and *B. garinii*. In the 1990s, another seven genospecies were described, including species from Asia, North America and Europe. From 2000 to 2010, another eight species were delineated. Of these 18 genomic species, *B. afzelii*, *B. burgdorferi* and *B. garinii* are the confirmed agents of localized, disseminated and chronic manifestations of Lyme borreliosis. *B. spielmanii* has been detected in early skin disease, and *B. bissettii* and *B. valaisiana* have been detected in specimens from single cases of Lyme borreliosis. The clinical role of other *Borrelia* remains to be determined. Therefore, it is possible that a new "Brazilian species/ genospecies/variant" of *Borrelia* is circulating in Brazilian patients with localized, disseminated and chronic manifestations of borreliosis. In fact, the Brazilian geographic conditions and fauna biodiversity could have contributed to the development of a genetically modified spirochete, which is adapted to survive in the environmental conditions found in Brazil[12,28,33,34]. This would explain the distinct attributes of Brazilian borreliosis, as well as the difficulty of culturing the microorganism in BSK II medium, the low sera reactivity to *B. burgdorferi* and the failure of PCR methods using primers targeting the gene of the outer membrane proteins and periplasmic flagella.

In conclusion, for the first time, we show that Brazilian borreliosis is caused by microorganisms possessing the flagellar hook gene (*flgE*), reinforcing the presence of a *Borrelia* species in the country.

RESUMO

Amplificação do gene *flgE* fornece evidência para a existência de uma borreliose brasileira

Introdução: Os sintomas da borreliose brasileira se assemelham às manifestações clínicas da Doença de Lyme (DL), porém, existem diferenças epidemiológicas e laboratoriais entre essas enfermidades. *Primers* normalmente utilizados para diagnosticar a DL não conseguiram detectar cepas de borrelia no Brasil. **Objetivo:** O objetivo desse trabalho foi identificar a borrelia brasileira usando um gene conservado que sintetiza o gancho flagelar (flgE) da *Borrelia burgdorferi sensu lato*. **Método:** Três pacientes com eritema migratório e epidemiologia positiva foram recrutados para o estudo. Amostras de sangue foram coletadas, e o DNA foi extraído por kits comerciais. **Resultados:** O gene *flgE* foi amplificado a partir do DNA de todos os pacientes selecionados. Após o sequenciamento, essas amostras positivas revelaram homologia de 99% para *B. burgdorferi*. **Conclusão:** Estes resultados reforçam a existência de borreliose no Brasil. No entanto, não está claro se esta borreliose é causada por uma variante geneticamente modificada da *B. burgdorferi sensu stricto* ou por uma nova espécie de *Borrelia* spp.

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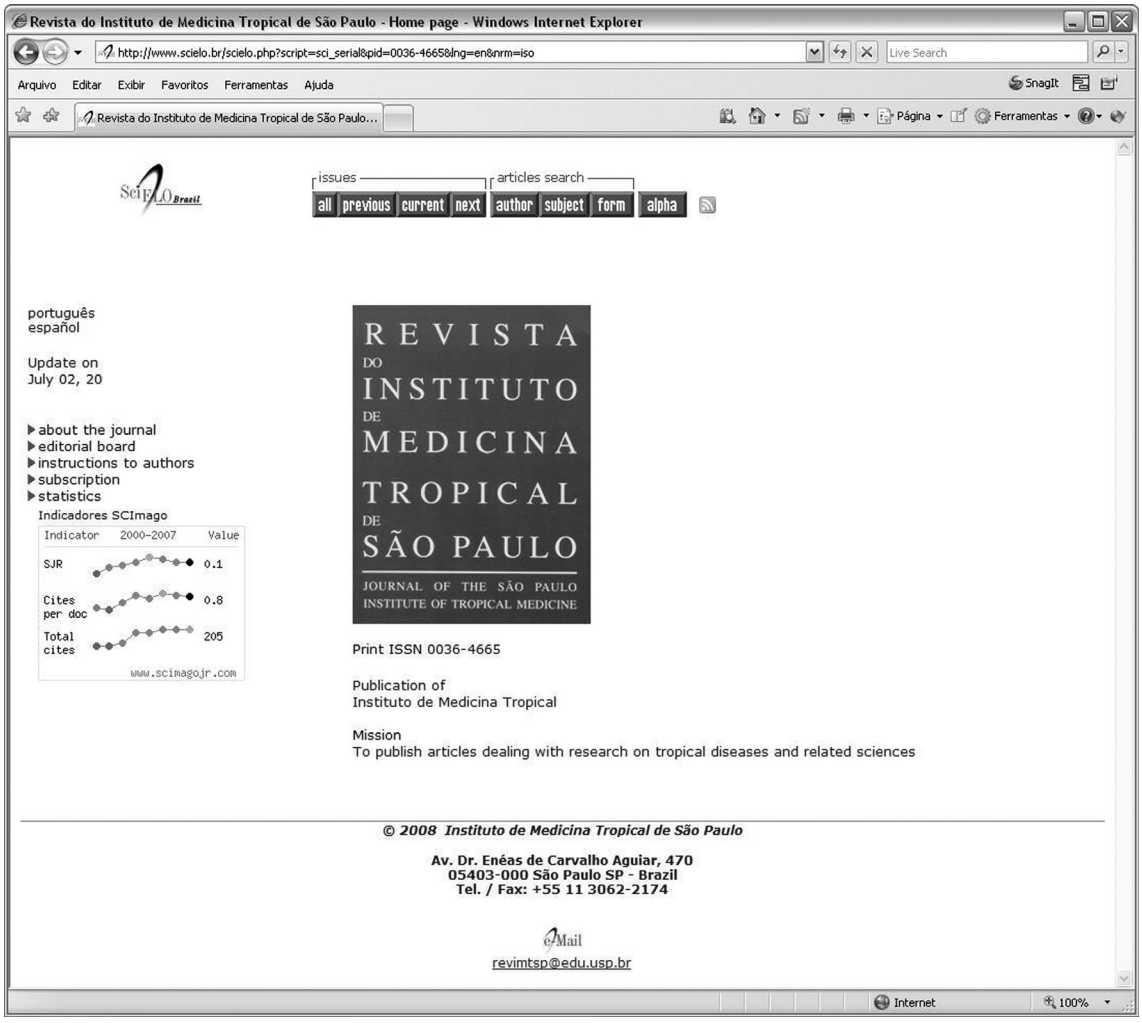

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