RESEARCH LETTER

Molecular detection of Borrelia bissettii DNA in serum samples from patients in the Czech Republic with suspected borreliosis

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Introduction

From the time of the discovery of the causative agent of Lyme borreliosis (LB), a large number of borrelia isolates have been obtained from various vertebrate species, including humans. Fourteen species of spirochetes from the Borrelia burgdorferi sensu lato (sl) complex are well established and recognized globally today and they are B. burgdorferi sensu stricto (ss), Borrelia afzelii, Borrelia andersonii, Borrelia bissettii, Borrelia californiensis, Borrelia garinii, Borrelia japonica, Borrelia lusitaniae, Borrelia spielmani, Borrelia tsutsugamushi, Borrelia turdi, Borrelia valaisiana (for references, see Postic et al., 2007) and the recently described Borrelia carolinensis (Rudenko et al., 2009). For a long time, only three of them were considered to be the causative agents of LB, i.e. B. burgdorferi ss (North America and Europe), B. afzelii and B. garinii (Europe and Asia). However, the presence of B. valaisiana, B. lusitaniae, B. spielmani and B. bissettii has been confirmed in some samples of human origin (Picken et al., 1996; Rijpkema et al., 1997; Ryffel et al., 1999; Wang et al., 1999; Collares-Pereira et al., 2004; Rudenko et al., 2008). The pathogenic potential of B. valaisiana was suspected among patients with Erythema migrans (EM) based on PCR (Rijpkema et al., 1997). The association of B. valaisiana with chronic clinical symptoms was also shown (Ryffel et al., 1999). Molecular analysis of spirochete isolates identified as B. lusitaniae from a Portuguese patient suggests a clinical pattern for B. lusitaniae different from the other Borrelia spp. examined so far (Collares-Pereira et al., 2004). Although the role of B. spielmani as a causative agent of LB was confirmed by reported cases of patients with EM in the Netherlands, Germany, Hungary and Slovenia (Maraspin et al., 2006), no direct evidence of pathogenic potential of B. bissettii in humans has been presented. The results of our previous studies showed an interesting distribution of different Borrelia species in serum samples collected in several hospitals located in the south of the Czech Republic. The highest prevalence was found for B. afzelii (43%), followed by cases with mixed infection (24%), then cases of B. garinii infection.
causative agents of LB. The results of the study presented different from those traditionally considered as species that causes LB in Europe.

Almost identical results were published in 2008 by an Italian group of scientists. Their PCR-based analysis of serum samples collected from patients with LB symptoms showed that out of the PCR-positive serum samples, 50% were positive for B. afzelii, 18% for B. garinii and 23% for mixed infection. Nine percent of the samples were not identified to the species level (Santino et al., 2008). These data support the fact that European patients were infected with B. burgdorferi sl species, different from those traditionally considered as causative agents of LB. The results of the study presented here support the statement that B. bissettii might be another species that causes LB in Europe.

**Materials and methods**

**Patients and clinical samples**

The group under investigation was heterogeneous in terms of symptoms. The samples were taken from patients with an LB diagnosis and from those with nonspecific symptoms. The serum samples were collected at the Department of Infectious Diseases of the Regional Hospital in České Budějovice (Czech Republic). All samples were examined by enzyme-linked immunosorbent assay (ELISA) [VIR-ELISA anti-Borrelia IgM/IgG (Viro-Immun Labor, Germany)] and Western Blot [ID Blot Borrelia IgM and ID Blot Borrelia IgG<sup>®</sup> (DPC, Germany)] for Lyme immunoglobulin M (IgM) and immunoglobulin G (IgG) antibodies at the Department of Clinical Parasitology of the Regional Hospital, and the results were interpreted using CDC criteria (<http://www.cdc.gov/mmwr/preview/mmwrhtml/00038469.htm>). The need for molecular typing of spirochetes appeared after the general serological tests gave unclear results in the analysis of some samples. These serum samples from patients (12 in total) were analyzed using molecular biology techniques. The processing involved direct DNA purification, flagellin gene amplification and amplification of the 5S–23S rRNA intergenic spacer region (IGS), analysis of PCR products, direct sequencing, cloning and sequencing in cases when the direct sequencing of PCR products indicated the presence of different sequences, sequencing of individual recombinants (48 per sample), sequence analysis with DNASTAR software, database searches using the BLAST programs of the NCBI (Bethesda) and phylogenetic analysis.

**PCR primers**

The primers for amplification of the flagellin gene and the 5S–23S rRNA IGS were described previously (Postic et al., 1994; Fukunaga et al., 1996; Clark et al., 2005).

**DNA purification, PCR amplification, cloning and sequencing**

Total DNA of all samples was purified using the DNeasy Blood and Tissue kit (Qiagen) strictly according to the manufacturer’s recommendations (<http://www.qiagen.com>). The MasterTaq Kit (Eppendorf, Germany) that contained recombinant Taq DNA polymerase from *Escherichia coli* DH1 and a special 5 × TaqMaster PCR enhancer was used for amplification of spirochete sequences. Because of the high A/T content in the B. burgdorferi genome, a special *Borrelia* dNTP mixture was used. The *Borrelia* dNTP mixture was prepared by mixing 10 mM ATP, 10 mM dTTP, 10 mM dCTP and 10 mM dGTP at a ratio of 26 : 26 : 14 : 14, respectively. The *Borrelia* dNTP mixture was used at a final concentration of 0.2 mM. Amplifications were made in a 25-μL reaction volume and the final concentration of the rest of the components were in accordance to the recommendations of the manufacturer (<http://www.eppendorf.com>). The PCR conditions for amplification of the flagellin gene and the 5S–23S rRNA IGS were similar: 95 °C for 5 min, 30 × (94 °C for 30 s, 52 °C for 30 s and 68 °C for 1 min), followed by a final extension step at 72 °C for 5 min. Amplifications were conducted in 0.2-mL thin-wall PCR tubes using the Mastercycler® ep system with a 96-well thermoblock and heated lid technology. Reactions were set up in a separate area with all precautions (supplies, equipment, and personnel safety items, pre- and postamplification activities). Negative (no template) and positive (*B. burgdorferi* B31 DNA) controls were added to each amplification reaction. The total PCR products of the expected size were cut from the gel and purified using the MinElute Gel Extraction kit (Qiagen). Cloning into the pCR<sup>®</sup>4-TOPO<sup>®</sup> vector for sequencing (Invitrogen) and the subsequent transformation was performed strictly according to the manufacturer’s recommendation. This step became necessary when the direct sequence of PCR products showed the presence of more than one sequence in some samples. Forty-eight individual recombinants from transformation of each sample were selected from LB/ampicillin plates and submitted for sequencing. Sequences were conducted in both directions, using M13Forward (−20) and M13Reverse vector-specific primers.

**Nucleotide sequence accession numbers**

Sequences of the partial flagellin gene and the 5S–23S rRNA IGS from different *B. burgdorferi* sl species amplified and isolated from the serum samples of LB patients have been deposited in GenBank and were given the following accession numbers: partial flagellin gene sequences (FJ231330–FJ231346); 5S–23S rRNA IGS (FJ431126–FJ431142).
Phylogenetic analysis

Sequences of the flagellin gene and the 5S–23S rRNA IGS of all 13 control species of the *B. burgdorferi* ss complex were downloaded from GenBank and used in phylogenetic analysis. Both control and sample sequences were adjusted in size and appropriately concatenated. The alignment of concatenated sequences was performed using CLUSTAL X (version 1.81) (Thompson *et al.*, 1997). Identical sequences were excluded from the analysis. Phylogenetic reconstruction of the molecular data was performed using the maximum parsimony heuristic search, performed in PAUP* 4.0, beta version 10, by implementing the tree-bisection-reconnection (TBR) algorithm. Gaps were treated as missing characters. Branch supports were calculated by bootstrap analyses (500 replicates). The results were confirmed using the maximum likelihood (ML) method.

Results

Molecular typing of LB spirochetes in serum samples collected in the hospital (107 serum samples in total) became necessary when the general immunological tests gave unclear results in the case of 12 patients. None of the patients from the group recalled any recent tick bites. Sample collection, ELISA, Western blot and spirochete cultivation were conducted at the Regional Hospital of České Budějovice (Table 1). Serum samples (400–600 μL) were forwarded to our laboratory for the molecular analysis. Partial sequences of the spirochete flagellin gene (495–591 nt dependent on the PCR primers used) and the 5S–23S rRNA IGS (247–256 nt species-dependent size) were amplified from total DNA purified from serum samples, as the cultivation of spirochetes in the hospital was unsuccessful. Direct sequencing of PCR products with the same primers showed the presence of more than one sequence in four samples. As the coinfection with different *Borrelia* species is known (see Discussion), we applied the cloning procedure as a sequence separation step for codetection of different species obviously present in these human samples. Forty-eight individual recombinants from each sample were selected after the cloning/transformation procedure and sequenced in both directions. Flagellin sequences were adjusted in size to controls available in GenBank and yielded 488-nt-long internal amplicons for all species (487 nt in case of *B. bissettii* species). The sequences of 5S–23S rRNA IGS amplicons were ‘framed’ by PCR primers at the 5’- and 3’-ends. Phylogenetic analysis of each locus showed the separation of spirochete sequences isolated from human samples into three groups, clustering with control *B. burgdorferi* B31, *B. garinii* 20047 and *B. bissettii* DN127. Four out of 12 samples were confirmed to carry single *B. burgdorferi* ss [patients no. 5 (p5Bbss), no. 6 (p6Bbss), no. 8 (p8Bbss) and no. 15 (p15Bbss)], three samples – single *B. bissettii* [patients no. 3 (p3Bbis), no. 11 (p11Bbis) and no. 13 (p13Bbis)], one sample – single *B. garinii* [patient no. 20 (p20Bg)] and four samples contained more than one spirochete species. From four coinfected samples, three were defined as a double infection with *B. burgdorferi* ss and *B. bissettii* [patients no. 2 (p2Bbss and p2Bbis), no. 9 (p9Bbss and p9Bbis) and no. 16 (p16Bbss and p16Bbis)] and one as a triple infection with *B. burgdorferi* ss, *B. bissettii*, and *B. garinii* [patient no. 1 (p1Bbss, p1Bbis and p1Bgar)]. Sequences of *flagellin* gene and 5S–23S rRNA IGS were used for calculation of nucleotide sequence similarity values. The similarity matrix

<table>
<thead>
<tr>
<th>Patient#</th>
<th>Diagnosis</th>
<th>Clinical symptoms, diagnosis and laboratory analysis of selected LB patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>p1</td>
<td>F48.8</td>
<td>Long-lasting fatigue, myalgia, subfebrile, flu-like syndrome, occasional tremor in arms and hands</td>
</tr>
<tr>
<td>p2</td>
<td>S80.0 M25.5</td>
<td>Weakness, arthralgia, myalgia</td>
</tr>
<tr>
<td>p3</td>
<td>A69.2 M25.5</td>
<td>Weakness, arthralgia, myalgia</td>
</tr>
<tr>
<td>p5</td>
<td>M54.1</td>
<td>Headache, myalgia, tremor in hands</td>
</tr>
<tr>
<td>p6</td>
<td>R50</td>
<td>Weakness, headache, flu-like syndrome</td>
</tr>
<tr>
<td>p8</td>
<td>G93.8</td>
<td>Vascular cerebral apoplexy, fatigue, weakness, headache</td>
</tr>
<tr>
<td>p9</td>
<td>A69.2 M25.5</td>
<td>Weakness, arthralgia, myalgia, headache</td>
</tr>
<tr>
<td>p11</td>
<td>A69.2</td>
<td>Arthralgia, tiredness</td>
</tr>
<tr>
<td>p13</td>
<td>R50 M25.5</td>
<td>Flu-like symptoms, fatigue, arthralgia</td>
</tr>
<tr>
<td>p15</td>
<td>R50</td>
<td>Somnolency, myalgia, flu-like syndrome, headache</td>
</tr>
<tr>
<td>p16</td>
<td>R50 M25.5</td>
<td>Weakness, arthralgia, fatigue</td>
</tr>
<tr>
<td>p20</td>
<td>R50</td>
<td>Fatigue, subfebrile, headache, depression</td>
</tr>
</tbody>
</table>

*F48.8, other specified neurotic disorders; R50.9, fever, unspecified; G56.0, Carpal tunnel syndrome (nerve disorder); S80.0, contusion, knee; M25.5, pain in joint; A69.2, Lyme disease; M54.1, neuritis or radiculitis; R50, fever of unknown origin; G93.8, other specified disorders of the brain.*
referred considerable conservation in case of flagellin sequences. The similarity values within each species were high, ranging from 99% to 100% for B. bissetii species (Fig. 1b). In case of B. burgdorferi ss, the amplicons from human serum revealed higher similarity to flagellin from the European B. burgdorferi GeHo strain (99.4–100%) than to B. burgdorferi B31 (99.0–99.6%) (Fig. 1a). Seven types of flagellin from different B. garinii strains were described previously. Flagellin type IIa is prevalent in Europe/Russia, but was also detected in Japan. Type Iic was identified in Russia, Japan and China. Types IIb, IId–Iig are restricted to Japan only. Amplicons from human serum showed 99.4–99.6% similarity to the flagellin type IIa prevalent in Europe (Fig. 1c). The similarity matrix revealed a greater variation in 5S–23S rRNA IGS. The level of similarity of IGS from the Czech samples and other strains isolated in European countries (Slovakia, Germany, Switzerland and the Czech Republic) ranged from 98.6% to 100%, while in comparison with B. burgdorferi B31, it was only 97.7–99.1% (Fig. 2a). When amplicons from human samples were compared with B. bissetii IGS, the value was < 98%, showing 96.0–97.6% similarity to both North American strains DN127 and 25015 (Fig. 2b). In case of B. garinii sequences, both amplicons from human serum samples showed 99.2% similarity to B. garinii 20047 (Fig. 2c). Flagellin and 5S–23S rRNA IGS were concatenated as they belonged to the specific patient, aligned using CLUSTAL X and phylogenetic analysis was conducted in PAUP* by implementing the TBR algorithm. The distance matrix for phylogenetic analysis was generated from the aligned partial sequences of flagellin gene (488 nt) and the 5S–23S rRNA spacer (247–256 nt) of 13 control species from B. burgdorferi sl complex and the spirochete sequences isolated from human samples. The final tree contained 772 total characters; 566 characters were constant and 100 characters were parsimony-informative. Branch supports were calculated by
bootstrap analyses (500 replicates) (Fig. 3). Results were confirmed using the ML method.

**Discussion**

The Czech Republic is a region where LB is endemic. Recent clinical surveys showed that 65% of Lyme disease patients had skin lesions, caused by *B. afzelii*, 12% suffered from neuroborreliosis, caused by *B. garinii*, and 9% had musculoskeletal disorders, caused by *B. burgdorferi* (D. Janovská, B. Macková, M. Vondrová, and D. Hulínska, Abstr. Tick-Borne Infect. Dis. Other Zoonoses, p. 19, 2001). This is in agreement with reports from the neighboring states of Slovakia and Germany (Gern et al., 1999; Kurtenbach et al., 2001), and with the results on prevalence of *Borrelia* species in *Ixodes ricinus* ticks in Europe (59.2%, 21.1% and 10.5%, respectively) (Derdakova et al., 2003). Nevertheless, there is always a group of patients with an LB diagnosis in whom clinical symptoms or serological responses vary considerably. This could have been influenced by possible co-infections, pre-existing disease, prompt initiation of antibiotic treatment or an apparent lack of immune response despite disseminated disease (Strle et al., 1997). Alternatively, it could be related to the causative agent that has never been thought to be involved in Lyme disease. Perhaps, the negative serological findings in the human samples under investigation might have been connected to the use of the *B. afzelii* antigen (the most prevalent local species) and its antigenic profile. This antigen might be drastically different from that of *B. bissettii*. This and other factors may be the reason why direct detection of the pathogen or pathogen DNA in clinical samples continues to be the ultimate confirmatory method for the diagnosis of LB. Members of the *B. bissettii* DN127 genomic group had been isolated previously from ixodid ticks and small reservoir hosts only, suggesting that this species may not be pathogenic to humans. Nevertheless, a decade ago, Strle et al. (1997) presented clinical findings wherein some patients from Slovenia had LB that was caused by *Borrelia* species with genotypic and phenotypic similarities to strain 25015. This strain is a member of the DN127 genomic group of *B. bissettii*. These results confirmed that *B. bissettii* shares with *B. burgdorferi* ss the distinction of being present in both the Old and the New World. Strain 25015 was previously
found to be infectious, but nonpathogenic, using a mouse model of LB (Anderson et al., 1990). However, a later study using a different murine model showed that this strain was mildly arthritogenic (Fikrig et al., 1992). Moreover, Schneider et al. (2008) reported the potential pathogenesis of *B. bissettii* to cause human LB in a mouse model. It was proved that two Colorado isolates of *B. bissettii* (CO-Bb) induced lesions of the bladder, heart and femorotibial joint 8 weeks after inoculation into mice (Schneider et al., 2008). Isolation of *B. bissettii* from patients in Slovenia (Picken et al., 1996; Strle et al., 1997; Strle, 1999), the detection of *B. bissettii* in cardiac valve tissue of the patient with endocarditis and aortic valve stenosis in the Czech Republic (Rudenko et al., 2008) and now the detection of *B. bissettii* in the serum samples of patients with symptomatic borreliosis or chronic borrelial infection all provide strong supporting evidence that this species is involved in human LB in Europe. However, the small number of patients confirmed to be infected with *B. bissettii* does not allow any conclusions to be made yet on the association of this species with organotropism or species-specific clinical manifestations. Unfortunately, an unambiguous assignment of ‘syndrome-genospecies’ is not possible due to the presence of more than one *Borrelia* species in the sample. It is not unusual that the vector or the reservoir host harbors more than one *Borrelia* species, but data on the presence of multiple spirochete species in patients with LB symptoms have been limited. Nevertheless, Demaerschalck et al. (1995) used species-specific PCR analysis to show that the prevalence of coinfection may be higher than expected; out of 18 patients examined with neuroborreliosis, eight were shown to have multiple infection. Moreover, it was confirmed by culture
that a patient had bilateral facial palsy and meningitis caused by coinfection with *B. garinii* and *B. afzelii* (Oksi et al., 1995).

In conclusion, the detection of *B. bissettii* in serum samples as a single species and as a part of coinfection was confirmed by phylogenetic analysis of concatenated spirochete sequences from two *Borrelia* genomic loci – the flagellin gene and the 5S–23S rRNA IGS. The presence of *B. bissettii* as a single strain in patients with Lyme disease symptoms strongly supports that *B. bissettii* might be a causative agent of Lyme disease in Europe.

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**References**


association between clinical manifestations and presence of 


