Detection of *Borrelia burgdorferi sensu lato* DNA by PCR in serum of patients with clinical symptoms of Lyme borreliosis

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*Borrelia burgdorferi*; PCR; serum samples; diagnosis.

Abstract

Lyme borreliosis is a disease caused by spirochaetes belonging to the genospecies complex *Borrelia burgdorferi sensu lato* (s.l.) transmitted by *Ixodes* ticks. At present, serology remains the main diagnostic tool for laboratory diagnosis of Lyme borreliosis. Recently, the PCR technique has been applied for diagnosis of *B. burgdorferi* s.l., but, until now, a reliable, easy-to-perform and sensitive method has not been described. Here we present a new PCR-based method for the detection of both *B. burgdorferi* s.l. and *Borrelia* genospecies DNAs in serum samples collected from patients showing Lyme disease symptoms. Of 265 serum samples of patients included in this study, 7.5% were positive, 1.9% was borderline and 90.6% were negative for antibodies against *B. burgdorferi* by enzyme-linked immunosorbent assay and Western blotting. The *B. burgdorferi* s.l. 16S rRNA gene was detected by PCR in all serum-positive and in two borderline samples. None of the serum-negative samples nor serum samples collected from healthy subjects gave positive PCR reactions. Of PCR-positive serum samples, 50% gave a positive reaction for *Borrelia afzelii*, 18% for *Borrelia garinii* and 23% for two *Borrelia* species. Two samples (9%) were not identified to species level. The new protocol could be considered to be reliable as neither false-positive nor false-negative reactions were recorded, and to be sensitive as it detects DNA from one bacterial cell.

Introduction

Lyme borreliosis is an infectious disease caused by spirochaetes belonging to the genospecies complex *Borrelia burgdorferi sensu lato* (s.l.) transmitted by *Ixodes* ticks.

Three genospecies, *Borrelia afzelii*, *Borrelia garinii* and *B. burgdorferi sensu stricto* (s.s.), are widely distributed in Europe causing human borreliosis (van Dam et al., 1993). Each species is correlated with distinct clinical manifestations: *B. garinii* is predominantly associated with neurologic symptoms, *B. afzelii* with late skin manifestations and *B. burgdorferi* s.s. with arthritis. Other *Borrelia* genospecies, such as *Borrelia valaisiana*, have been isolated from ticks in Europe and are involved in human Lyme borreliosis (Escuredo et al., 2000). In addition, *Borrelia spielmani* has been isolated from a patient affected by erythema migrans (Wang et al., 1999) and *Borrelia lusitaniae* has been isolated from a patient for the first time in 2004 in Europe (Collares-Pereira et al., 2004).

At present, borreliosis is diagnosed mainly on the basis of clinical symptoms and serological tests. These tests, based on demonstration in human serum of anti-*B. burgdorferi* s.l. IgG and IgM antibodies, are usually carried out by enzyme-linked immunosorbent assay (ELISA) and Western blot tests (Aguero-Rosenfeld et al., 2005). Unfortunately, serological tests have a poor reliability for the identification of different *Borrelia* genospecies, such as *B. afzelii*, *B. garinii* and *B. burgdorferi* s.s. Misdiagnosis (both false-positive and false-negative) is frequent due to technical reasons (Niscigorska et al., 2003).

The PCR technique has been applied for the diagnosis of *B. burgdorferi* s.l. in human serum samples (Schmidt, 1997). Although the recovery of *B. burgdorferi* s.l. DNA in clinical samples represents an appealing alternative tool, the low number of spirochetes in blood, plasma and serum could represent a serious problem for PCR reliability (Aguero-Rosenfeld et al., 2005).

In order to develop a reliable PCR-based method, widely applicable to the identification of various *Borrelia*
Materials and methods

Bacterial isolates and culture conditions

*Borrelia burgdorferi* B31, *B. afzelii* and *B. garinii*, kindly provided by the Department of Infectious, Parasitic and Immune-Mediated Diseases, Istituto Superiore di Sanità, Rome, Italy, were used in this study. All strains were cultured in BSKII medium (Sigma Chemical Co., St. Louis, MO) at 34 °C. Growth was checked by dark-field microscopy.

Patient samples

A total of 265 human serum samples were collected during January 2005 and April 2007 at the Policlinico Umberto I Hospital, Sapienza University of Rome, from patients presenting clinical manifestations of Lyme borreliosis, including early manifestations (erythema migrans, fever, malaise, fatigue, skin rash, arthralgia, myalgia) or later manifestations of Lyme disease (severe arthritic, neurologic and cardiac manifestations). Twenty serum samples collected from patients showing symptoms of Lyme borreliosis, including early manifestations (erythema migrans, fever, malaise, fatigue, skin rash, arthralgia, myalgia) or later manifestations of Lyme disease (severe arthritic, neurologic and cardiac manifestations). Twenty serum samples collected from patients showing symptoms of Lyme borreliosis, including early manifestations (erythema migrans, fever, malaise, fatigue, skin rash, arthralgia, myalgia) or later manifestations of Lyme disease (severe arthritic, neurologic and cardiac manifestations).

Table 1. PCR primer sets used in this study

<table>
<thead>
<tr>
<th>Primer set</th>
<th>Sequence (5’–3’)</th>
<th>Amplimer (bp)</th>
<th>Annealing temperature (°C)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>LD</td>
<td>ATGCACACTTGGTGTTAACTA GACTTATACCGGCACTGCTT</td>
<td>357</td>
<td>50</td>
<td>Marconi &amp; Garon (1992)</td>
</tr>
<tr>
<td>BB</td>
<td>GGGATGTAGCAATACACTC ATATAGTTCCCACTAG</td>
<td>574</td>
<td>50</td>
<td>Marconi &amp; Garon (1992), erratum (1993)</td>
</tr>
<tr>
<td>BG</td>
<td>GGGATGTAGCAATACACTT ATATAGTTCAACATAGT</td>
<td>574</td>
<td>44</td>
<td>Marconi &amp; Garon (1992)</td>
</tr>
<tr>
<td>BA</td>
<td>GCATGCAAGTCAACAGGGA ATATAGTTCCCAACATAGC</td>
<td>591</td>
<td>46</td>
<td>Marconi &amp; Garon (1992), erratum (1993)</td>
</tr>
<tr>
<td>BV</td>
<td>GCAAGTCCAAACCGGGATGTAGT GTATTTATAGCTAGATATAT</td>
<td>549</td>
<td>49</td>
<td>Liebisch et al. (1998)</td>
</tr>
</tbody>
</table>


Serological tests

The presence of specific IgM and/or IgG antibodies against *B. burgdorferi s.l.* was determined by ELISA (recomWell IgM/IgG, Mikrogen, Neuried, Germany) and Western blot (ViraBlot IgM/IgG, Viramed Biotech AG) tests. Recombinant proteins specific for *B. burgdorferi s.l.* were used as positive control.

ELISA tests were considered negative, positive or borderline when values were lower than 20, higher than 24 or between 20 and 24 U mL⁻¹, respectively, as suggested by the manufacturer.

Detection of *B. burgdorferi s.l.* DNA by PCR

*Borrelia* DNA was extracted from human serum samples according to the following experimental protocol (Protocol A): 100 μL of human serum samples was incubated in the presence of 200 μL ammonium hydroxide (0.7 M) at 100 °C for 5 min in a 1.5-mL tube, followed by 10 min at 100 °C with the tube open. *Borrelia* DNA precipitation was obtained by adding 2 volumes of ethanol and 0.1 volume of 3 M sodium acetate. The samples were centrifuged at 12,000 g for 15 min and DNA rinsed with 70% ethanol. After centrifugation, DNA samples were air dried, suspended in 100 μL Tris-EDTA (TE) buffer and stored at −70 °C until use. In comparative experiments *Borrelia* DNA was extracted from human serum samples following the protocol of Guy & Stanek (1991) (Protocol B). PCRs were performed using 16S rRNA gene PCR primers described by Marconi & Garon (1992) and Liebisch et al. (1998). In particular, the primer sets LD, BB, BG, BA and BV were specific for *B. burgdorferi s.l.*, *B. burgdorferi s.s.*, *B. garinii*, *B. afzelii* and *B. valaisiana*, respectively (Table 1). PCR reactions were performed in a reaction volume of 25 μL containing 12.5 pmol of appropriate primer set and 1 μL of bacterial DNA extracted from human serum samples as described above. Companion PCR experiments were carried out using DNA extracted from human serum samples containing 1 bacterium mL⁻¹, and 100% specificity, as neither false-negative nor false-positive reactions were detected.
as template DNA extracted from *Borrelia* strains, i.e. *B. burgdorferi* B31, *B. afzelii*, *B. garinii*. *Borrelia* strains were cultured in BSK-II medium for 3 days at 34 °C. After incubation, bacterial cells were treated for DNA extraction according to the protocol outlined above (Rosa & Schwan, 1989).

PCR amplifications were performed using a Perkin-Elmer Cetus thermocycler by denaturing the template DNA for 1 min at 94 °C, with extension for 1.5 min at 72 °C for 35 cycles. Annealing temperatures ranged from 44 to 50 °C for the different primer sets as detailed in Table 1. Amplification products were visualized after electrophoresis with 10 μL of the PCR reaction volume in 1.0% agarose gels in TAE buffer [40 mM Tris-acetate, 2 mM EDTA (pH 8.5)] containing ethidium bromide at 0.5 pg ml⁻¹.

In order to confirm the PCR-based species attribution, amplimers obtained with species-specific primer sets were digested with HindIII and SacII restriction enzymes. Table 2 shows restriction fragments consistent with species-specific primer sets were positive (data not shown).

To determine PCR sensitivity, serum samples from two healthy subjects were pooled and divided into 1-mL aliquots. Each aliquot was infected by 10-fold serial dilutions of *B. burgdorferi* B31 live cells. DNA was extracted from 100 μL infected serum samples following the methods described above (protocols A and B) and PCRs were performed using the LD (*B. burgdorferi* s.l.) primer set.

### Results

To confirm PCR reliability, preliminary experiments were carried out using DNA samples extracted from reference *Borrelia* strains. As expected, PCRs performed using extracted DNA from cultured strains and LD and genospecies-specific primer sets were positive (data not shown).

To test PCR sensitivity, *Borrelia* DNA was extracted from serial dilutions of *B. burgdorferi* B31 culture in serum samples following both protocols A and B. PCRs were performed using the BB primer set (Table 2). Using DNA extracted following Protocol A, PCR reactions were positive on serum samples infected with 10⁵, 10⁴ and 10³ CFU ml⁻¹; following Protocol B, PCR was positive on serum sample infected with 10⁵ UFC ml⁻¹. As PCRs were performed using 1 μL of extracted DNA, the sensitivity was equal to 1 and 100 bacterial cells for protocols A and B, respectively.

In order to investigate the possibility of increasing the sensitivity of Protocol A, a further set of experiments were carried out. The amount of serum to be treated was raised to 200 μL and the volume of TE to suspend *Borrelia* DNA was reduced to 25 μL. The results did not indicate a significant increase in PCR sensitivity (data not shown).

Of the 265 patient samples included, 20 (7.5%) were positive, five (1.9%) were borderline and 240 (90.6%) were negative for antibodies against *B. burgdorferi* by ELISA and Western blotting.

Twenty seropositive, five borderline and nine seronegative samples, as well as 20 serum samples collected from healthy subjects were analyzed for the presence of *B. burgdorferi* s.l. DNA by PCR (Table 3). *Borrelia burgdorferi* s.l. DNA was found in all 20 seropositive and in two of the five borderline serum samples. All nine seronegative as well all 20 serum samples from healthy subjects gave negative PCRs.

PCR reactions with species-specific primers for *B. burgdorferi* s.s., *B. afzelii*, *B. garinii* and *B. valaisiana* were carried out on the 22 *B. burgdorferi* s.l. PCR-positive serum samples. Fifteen samples (68%) contained DNA from a single *Borrelia* genospecies, and five samples (23%) showed the presence of DNA from two different genospecies. The genospecies attribution could not be made for two serum samples (9%) positive for *B. burgdorferi* s.l. as PCRs performed with genospecies-specific primers were negative (Fig. 1). None of the serum sample was positive for *B. burgdorferi* s.s.

In order to confirm the PCR-based species attribution, amplimers obtained with species-specific primer sets were digested with HindIII and SacII restriction enzymes. Results showed restriction fragments consistent with species-specific 16S rRNA gene sequences (data not shown).

The distribution of *Borrelia* genospecies in relation to clinical manifestations is shown in Table 4. Of particular note, 16 patients had early clinical manifestations and six late clinical manifestations.

### Table 2. PCR sensitivity of *Borrelia burgdorferi* B31 DNA detection in human serum samples

<table>
<thead>
<tr>
<th>Serum sample</th>
<th>DNA used as PCR template (μL)</th>
<th>BB primer set PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protocol A</td>
<td>Protocol B</td>
<td></td>
</tr>
<tr>
<td>100 000</td>
<td>100</td>
<td>1 +</td>
</tr>
<tr>
<td>10 000</td>
<td>100</td>
<td>1 +</td>
</tr>
<tr>
<td>1000</td>
<td>100</td>
<td>1 +</td>
</tr>
<tr>
<td>100</td>
<td>100</td>
<td>1 +</td>
</tr>
<tr>
<td>10</td>
<td>100</td>
<td>1 +</td>
</tr>
<tr>
<td>1</td>
<td>100</td>
<td>1 +</td>
</tr>
<tr>
<td>None</td>
<td>100</td>
<td>– –</td>
</tr>
</tbody>
</table>

The 16S rRNA gene BB primer set specific for *B. burgdorferi* s.s. was employed (see Table 1).

### Table 3. PCR detection of *Borrelia burgdorferi* s.l. DNA in human serum samples

<table>
<thead>
<tr>
<th>Subjects</th>
<th>Serological tests</th>
<th>PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. tested</td>
<td>Positive</td>
</tr>
<tr>
<td>With clinical manifestations</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Negative</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>Borderline</td>
<td>5</td>
<td>2</td>
</tr>
<tr>
<td>Healthy subjects</td>
<td>20</td>
<td>0</td>
</tr>
<tr>
<td>Positive control</td>
<td>1</td>
<td>1</td>
</tr>
</tbody>
</table>

*Positive, > 24 U mL⁻¹; negative, < 20 U mL⁻¹; borderline, between 20 and ≥24 U mL⁻¹.*
Detection of *Borrelia burgdorferi* by PCR

**Table 4. Distribution of Borrelia genospecies in human serum samples in relation to clinical manifestations**

<table>
<thead>
<tr>
<th>Genospecies</th>
<th>Clinical manifestation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Early</td>
</tr>
<tr>
<td><em>B. afzelii</em></td>
<td>7</td>
</tr>
<tr>
<td><em>B. garinii</em></td>
<td>4</td>
</tr>
<tr>
<td><em>B. afzelii</em> and <em>B. garinii</em></td>
<td>3</td>
</tr>
<tr>
<td><em>B. afzelii</em> and <em>B. valaisiana</em></td>
<td>–</td>
</tr>
<tr>
<td><em>B. garinii</em> and <em>B. valaisiana</em></td>
<td>1</td>
</tr>
<tr>
<td>Unknown</td>
<td>1</td>
</tr>
<tr>
<td>Total</td>
<td>16</td>
</tr>
</tbody>
</table>

**Fig. 1.** *Borrelia burgdorferi* DNA genospecies recovered in human serum samples.

**Discussion**

The aim of the present study was to improve the PCR-based protocol for identification of *B. burgdorferi* s.l. from human serum samples and to compare the diagnostic value of PCR with that of serology. At present, serology remains the main diagnostic tool for laboratory diagnosis of Lyme borreliosis. However, in the early phase of infection, the level of antibodies against *B. burgdorferi* is low. False-negative results occur primarily during the first weeks of infection (Hofmann, 1996). Culturing *B. burgdorferi* from body fluids is difficult because of the long incubation period, the high cost of culture medium and small proportion of positive results (Aguero-Rosenfeld et al., 2005). Therefore, the need for a reliable diagnostic method is evident (Hofmann, 1996). As PCR has been considered as a sensitive tool for identification of fastidious microorganisms that are difficult to culture, attempts have been made to apply this technique to detect *Borrelia* DNA in human serum (Niscigorska et al., 2003).

Here we present a simple, easy-to-perform, and reliable PCR protocol for the identification of *Borrelia* from human serum samples. The protocol involves a simple method to extract *Borrelia* DNA from human serum samples. Several PCR methods do indeed yield acceptable results when *Borrelia* DNA is extracted from skin biopsy of patients with Erythema chronicum migrans (ECM) or synovial fluid in patients with Lyme arthritis (Aguero-Rosenfeld et al., 2005).

In the present study, the extracted DNAs were used to perform PCRs using different primer sets to identify *Borrelia* to genospecies level. We have chosen to employ the previously described primer sets that amplify the 16S rRNA gene. It is well known that these primer sets are specific for *B. burgdorferi* s.l. and *B. burgdorferi* genospecies (Liebisch et al., 1998; Marconi & Garon, 1992). Our protocol has been shown to be more sensitive than a previously published method, giving a positive PCR reaction using DNA from 1 as compared with 10³ bacterial cells (Portnoi et al., 2006). Sensitivity similar to that for our protocol has been reported by Joss et al. (2008), who described a real-time PCR method to detect *Borrelia* from serum samples. However, it is well known that real-time PCR is costly, laborious to perform and, moreover, requires the use of sophisticated apparatus.

The protocol proposed shows 100% reliability: no false-positive or false-negative reactions were recorded. In fact, our protocol recognized as positive only those serum samples positive by ELISA and Western blotting and two serum samples from subjects with early symptoms that give borderline reactions in serological tests. This result confirms the poor reliability of serological tests in early *Borrelia* infections (Riesbeck & Hammas, 2007). Moreover, the protocol we have described allowed us to identify at species level *Borrelia* DNA in human serum samples. To confirm the reliability of the proposed method, we have also analyzed the PCR amplimers obtained by digesting them with restriction enzymes. As expected, DNA fragments obtained by restriction enzymes were consistent with PCR amplification and confirmed the PCR-based *Borrelia* identification.

In the present study, 7.5% of the patients with suspected Lyme borreliosis had antibodies against *B. burgdorferi* in blood serum. *Borrelia burgdorferi* DNA was detected by PCR in all serum samples from seropositive patients (100%) and from two (22%) seronegative subjects. Of note, 72.7% of patients with positive PCR showed symptoms referable to early borreliosis, while only 27.3% of PCR-positive patients showed late borreliosis. Moreover, two borderline patients manifesting early symptoms as erythema migrans were PCR positive. These results are in agreement with those of Guy & Stanek (1991), underlining that the PCR method can contribute to the reliability of diagnosis during early stages of infection.

In the present study, the frequency of recovery of *Borrelia* DNA was equal to 8.3% (22 positive of 265 serum samples). Variable frequency of recovery values have been reported in the past, ranging from 76 to 3.8% (Oksi et al., 1999, 2001; Kondrusik et al., 2004; Chmielewska-Badora et al., 2006). These discrepancies are probably due to the frequency of infection and to the different protocols used in performing
PCR reactions, underlining the need to develop a reliable PCR protocol.

Regarding genospecies identification, *B. afzelii* was the most frequent species (50%, 11 of 22 serum samples) while *B. burgdorferi s.s.* DNA was not recovered. Interestingly, 23% of the samples were positive for two different *Borrelia* species. Several studies indicate that different *Borrelia* species may be associated with specific reservoir hosts and with distinct clinical manifestations of Lyme disease (van Dam et al., 1993; Balmelli & Piffaretti, 1995). Therefore, *Borrelia* identification at genospecies level in patients with Lyme borreliosis appears to be of epidemiological, pathogenetic and diagnostic importance.

Mixed infections with two or more *Borrelia* species are frequent in ticks (Santino et al., 1998; Santino et al., 2003) and have been reported also in human patients (Ruzic-Sabljic et al., 2005). The present study indicates that human patients with Lyme borreliosis can simultaneously harbor different *B. burgdorferi s.l.* strains and that these infections are mainly double infections with the presence of *B. garinii* and *B. afzelii* or *B. valaisiana*.

In conclusion, the protocol described here could be usefully employed in PCR-based *Borrelia* identification from human serum samples.

**Acknowledgements**

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Borrelia burgdorferi sensu lato strains isolated from specimens obtained simultaneously from two different sites of infection in individual patients. J Clin Microbiol 43: 2194–2200.


