Detection and Typing of *Borrelia burgdorferi* Sensu Lato in *Ixodes ricinus* Ticks Attached to Human Skin by PCR

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**Summary**

Lyme disease is a multisystem disorder involving the skin, joints, heart, and nervous system (1, 6, 20, 21). The etiological agent is a spirochete species complex, *Borrelia burgdorferi* sensu lato, which is transmitted via infected ticks of the *Ixodes ricinus* species complex (5, 17). *B. burgdorferi* sensu lato has been found in other ticks, as well, like *Ixodes hexagonus, Ixodes canisuga,* and *Demodex reticulatus* (9–12), whose epidemiological relevance to human Lyme disease transmission is not entirely clear at the moment.

European *B. burgdorferi* sensu lato isolates have been divided into several genospecies based on phenotypic analysis, as well as sero- and genotyping, i.e., *B. burgdorferi* sensu stricto, the predominant species found in North America, *Borrelia afzelii,* and *Borrelia garinii* (3, 13, 16, 19, 25, 26). There is some evidence that this division is of relevance to the clinical presentation of Lyme disease in Europe: several studies have revealed an association between the presence of *B. burgdorferi* sensu stricto and arthritis and between *B. afzelii* and acrodermatitis chronica atrophicans (23). This picture, however, is still not complete, since other *B. burgdorferi* sensu lato isolates, VS116 and PotiB2, that cannot easily be attributed to the three known pathogenic species and whose pathological relevance is currently completely unclear have recently been described (18, 22). Accordingly, the prevalence of the different genospecies of *B. burgdorferi* sensu lato in infected ticks should be the prime determinant for the risk of acquiring Lyme disease and its clinical presentation.

We therefore analyzed live ticks (nymphal, larval, and adult stages), which had been found attached to human skin and submitted by patients during the period 1995 to 1996 from all parts of Lower Saxony, Germany, for the presence of four genospecies of *B. burgdorferi* sensu lato, i.e., *B. burgdorferi* sensu stricto, *B. afzelii,* *B. garinii,* and isolate VS116. (*B. afzelii* NE 632 [7] was kindly donated by L. Garn, Neuchatel, Switzerland, *B. garinii* N 34 was donated [2] by G. Baranton, Paris, France, *B. burgdorferi* sensu stricto B31 [4] was donated by W. Burgdorfer, Hamilton, Mont., and VS116 [18] was donated by O. Peter, Sion, Switzerland.) The ticks were identified to the species level by standard morphological criteria. Altogether, 2,421 ticks, of which the overwhelming majority belonged to the species *I. ricinus,* had been submitted for analysis (2,399 *I. ricinus,* 11 *I. hexagonus,* 6 *Rhiiccephalus sanguineus,* 2 *Dermacentor marginatus,* 1 *D. reticulatus,* 1 *Amblyomma cajennense,* and 1 *Argus reflexus* tick), but only some of these (1,654 ticks, all of the *I. ricinus* species, confirming that this tick is the predominant vector for human Lyme disease) were still alive on arrival and were included in this study.

To determine *Borrelia* infection of the ticks, the midgut of each tick was removed under a stereomicroscope and homogenized in a 0.9% NaCl solution (ca. 100 µl for larvae, 150 µl for nymphs, and 300 µl for engorged adults). Ten microliters of this homogenate was then quantitatively analyzed for the presence of borreliae by dark-field microscopy. One hundred fifty-three tick homogenates (9.3%) were found to be infected by *Borrelia* species, with numbers ranging from 1 to >3,000 borreliae per 10 µl aliquot. One hundred nineteen homogenates were further analyzed by PCR (see below); 34 homogenates were excluded from the analysis due to insufficient material (mainly larval stages of *I. ricinus*). The final sample collection consisted of borrelia-positive midgut homogenates from 61 adults, 57 nymphs, and 1 larval stage of *I. ricinus*.

To identify the pathogenic species of the *B. burgdorferi* sensu lato complex within these homogenates by PCR, we used the 16S rRNA PCR primer pairs originally described by Marconi and Garon (14, 15). The homogenates were first incubated with 200 µg of proteinase K per ml at 60°C overnight to release the DNA, and the reaction was stopped by 10 min of boiling. Five microliters of the supernatant was subjected to 40 cycles of PCR amplification (94°C for 1.5 min, 42 to 64°C for 2 min, 72°C for 2 min) using *Taq* polymerase, 1 µM oligonucleotide primers, and 1.5 mM MgCl₂ under standard conditions. PCR products were separated on a 1% (wt/vol) agarose gel and visualized under UV light after ethidium bromide staining. To analyze for the presence of inhibitory substances in PCR-negative homogenates, DNA equivalent to 20 genomes of *B. afzelii* (grown in BSK-H medium supplemented with 6% rabbit serum [Sigma] at 33°C) was added to one sample of the homogenate and amplified under the conditions specified above for *B. afzelii.* In our hands, the species-specific primer pairs for *B. burgdorferi* sensu stricto, *B. afzelii,* and *B. garinii* were quite effective, with a sensitivity of ca. 10 borreliae/PCR mixture (i.e., 50 µl for larvae, 150 µl for nymphs, and 300 µl for engorged adults). Ten microliters of the supernatant was subjected to 40 cycles of PCR amplification (94°C for 1.5 min, 42 to 64°C for 2 min, 72°C for 2 min) using *Taq* polymerase, 1 µM oligonucleotide primers, and 1.5 mM MgCl₂ under standard conditions. PCR products were separated on a 1% (wt/vol) agarose gel and visualized under UV light after ethidium bromide staining.

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strain VS116, a novel species within the *B. burgdorferi* sensu lato complex which seems to be present in *I. ricinus* ticks in a remarkable proportion of cases (8). We therefore amplified a 1.5-kbp DNA fragment encompassing the almost complete 16S rRNA locus of strain VS116 and sequenced both strands (Fig. 1). Sequence analysis confirmed the presence of several single base changes in VS116 in comparison to the other genospecies of *B. burgdorferi* sensu lato, which allowed for the construction of a VS116-specific primer pair. Again, a VS116-specific amplification product was obtained with no apparent cross-binding to the other three genospecies or other bacterial genomes (as exemplified by PCRs with *E. coli*, *E. faecalis*, and *S. aureus*; data not shown) and a sensitivity similar to that of the other primer pairs used in this study. Oligonucleotide sequences and annealing temperatures are listed in Table 1.

### Nucleotide sequence accession numbers

16S rRNA sequences for the following strains have been submitted to GenBank: VS116, under accession no. AJ 225165, identical

### Table 1. Oligonucleotide primers used in this study

<table>
<thead>
<tr>
<th>Strain</th>
<th>Primer sequences (5′→3′)</th>
<th>Position(s)</th>
<th>Annealing, temp (°C)</th>
<th>Reference(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>B. afzelii</em></td>
<td>GCAATGCAAGTCACAAACGGA ATATAGTTTCCAACATAGC</td>
<td>5′-76 648–630</td>
<td>42</td>
<td>14, 15</td>
</tr>
<tr>
<td><em>B. garinii</em></td>
<td>GGATATGACATACATCT ATATAGTTTCCAACATAG</td>
<td>74–92 648–630</td>
<td>42</td>
<td>14, 15</td>
</tr>
<tr>
<td><em>B. burgdorferi</em></td>
<td>GGAATGACATACATCT ATATAGTTTCCAACATAG</td>
<td>74–92 648–630</td>
<td>42</td>
<td>14, 15</td>
</tr>
<tr>
<td>VS116</td>
<td>GCAATGCAAGTCACAAACGGA ATATAGTTTCCAACATAGC</td>
<td>39–58 603–610</td>
<td>64</td>
<td>This study</td>
</tr>
<tr>
<td><em>B. burgdorferi</em> sensu lato</td>
<td>ACGGCTGGAGCTGGGTCTAAA (P1) CTGATATCAGCATGCACACACGGCCC (P2)</td>
<td>39–58 603–610</td>
<td>64</td>
<td>This study</td>
</tr>
</tbody>
</table>

*In the 16S rRNA sequence of *B. burgdorferi* sensu strick (in nucleotides).
to the recently published sequence of Wang et al. (24), and M94496, under accession no. AJ 229044.

Of 119 samples tested by PCR, only 50 gave a positive result and 69 samples were PCR negative despite the microscopy-determined presence of borreliae. As shown in Table 2, about one in three samples contained inhibitory factors preventing proper PCR amplification, which accounted for 42% of the borrelia-positive homogenates with negative PCR results. Another 40.5% of these homogenates are probably accounted for by insufficient sensitivity of the PCR, since a considerable number of the positive homogenates contained only low numbers of borreliae (equivalent to 1 to 10 borreliae/PCR mixture) which were below or at the detection limits of this PCR procedure. However, two samples failed to produce a PCR product despite the microscopy-determined presence of high numbers of borreliae and the absence of inhibitory substances in the PCR mixture. This would indicate the presence of still other Borrelia species, either novel as-yet-undescribed species of the B. burgdorferi complex or non-B. burgdorferi species, which do not properly bind any of the species-specific or genus-specific primers.

The PCR results of the noninhibited samples are shown in Fig. 2. A genospecies identification was considered definite when both PCR mixtures (that with the genus-specific primers and that with the species-specific primers) were positive and yielded DNA fragments of the correct size. Several amplification products (at least two of each group, i.e., fragments specific for B. afzelii, B. garinii, and isolate VS116, as well as those reacting only with the genus-specific primers) were also partially sequenced, and this confirmed the presence of Borrelia 16S rRNA sequences in all cases tested. Samples reacting only with the genus-specific primers (14 of 50 or 28%) are indicated in Fig. 2 as “genus spec.” DNA sequence analysis could be performed for 7 of the 14 samples, and this revealed the presence of one additional B. garinii sequence and two novel partial 16S rRNA sequences of as-yet-unknown Borrelia species. Five of the six PCR fragments with novel 16S rRNA sequences were almost identical to each other but were rather distinct from the other B. burgdorferi sensu lato sequences. Their sequences are shown in Fig. 1 for sample M94496.

Our results show that B. burgdorferi sensu stricto was not identified once among 50 positive samples. This result confirms that this species is probably of minor importance in Germany. At least in our series, a prevalence of <1/50 indicates that in northern Germany only a very small percentage of the infecting ticks transmit B. burgdorferi sensu stricto, although this species is not absent in Europe (26). Notably, a recent report on the distribution and prevalence of B. burgdorferi sensu lato genospecies in field-collected I. ricinus ticks (8) indicated a higher incidence of B. burgdorferi sensu stricto than of B. afzelii (ca. 18% versus ca. 8%), which is in striking contrast to our own observations (0% versus 38%) (Fig. 2A). The reason for this discrepancy is unknown and may either reflect local vari-
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REFERENCES