Rapid Typing of *Borrelia burgdorferi* Sensu Lato Species in Specimens from Patients with Different Manifestations of Lyme Borreliosis

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To further investigate the pathogenic potential of different *Borrelia burgdorferi* genospecies, specimens from 27 patients with different manifestations of Lyme borreliosis were analyzed by PCR and reverse line blotting (RLB). In samples from Lyme arthritis patients, *B. burgdorferi* sensu stricto was predominantly identified, while in patients with neuroborreliosis or acrodermatitis, *Borrelia garinii* and *Borrelia afzelii*, respectively, were exclusively detected. The results demonstrate that PCR-RLB is a valuable tool for epidemiological and pathogenetic studies of Lyme borreliosis.

Since the causative agent of Lyme disease was discovered in 1982 (3) and subsequently described as a unique species (8), molecular studies have led to the description of different genomic groups constituting a complex named *Borrelia burgdorferi* sensu lato (19). Of the 10 different genospecies of *B. burgdorferi* which have been characterized to date, only *B. burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii* have been identified as pathogenic in humans. Based on serological data and PCR-based typing analysis, there is growing evidence that these species possess different organotropisms and may preferentially cause distinct clinical manifestations of Lyme borreliosis (1, 2, 16). Therefore, the identification of the causative species in patients with Lyme borreliosis appears to be of epidemiological, pathogenetic, and diagnostic importance.

Recently, Rijpkema et al. (14) described the genotyping of *B. burgdorferi* strains in Dutch *Ixodes ricinus* ticks by amplifying the intergenic spacer region between 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (*rrfA* and *rrfB*) genes and subsequent characterization of amplicons by hybridization to multiple species-specific oligonucleotide probes immobilized on a membrane. This hybridization format, designated reverse line blotting (RLB), allows the simultaneous identification of gene polymorphisms at a PCR-amplified DNA locus within a single procedure.

The present study aimed at employing this hybridization assay for the rapid and easy genotyping of *B. burgdorferi* species in European patients with Lyme disease.

A total of 27 patients with different clinical manifestations of Lyme borreliosis (Table 1) as diagnosed by experienced physicians were investigated (9). In all of the patients, *B. burgdorferi* DNA could be detected by PCR. All but two patients were seropositive for specific immunoglobulin G antibodies against

B. burgdorferi in both full-antigen enzyme-linked immunosorbent assays and Western blotting (DPC Biermann, Bad Nauheim, Germany).

For PCR analysis, clinical specimens, including urine, cerebrospinal fluid (CSF), synovial fluid, and skin biopsy specimens, were obtained prior to antibiotic treatment of the patients. DNA was prepared by alkaline lysis, and a nested PCR targeting the *ospA* gene was performed according to our previously published protocol (13). In addition, a second PCR using primer sets targeting a sequence of the spacer region between chromosomally encoded rRNA genes (*rrfA*-*rrlB* spacer) (12) was carried out as follows: outer PCR with 25 cycles, annealing at 52°C for 1 min, inner PCR with 40 cycles, and annealing at 50°C for 1 min (PTC 100; Biozym, Hessisch Oldendorf, Germany). For the visualization of amplicons, primers of the inner PCR were labeled with 5'-digoxigenin (TIB Molbiol, Berlin, Germany). Appropriate positive and negative controls were included in each experiment, and precautionary measures to avoid contamination were taken as described earlier (13, 15).

RLB was done according to the protocol described by Rijpkema et al. (14). For the characterization of PCR products, one probe which reacted with all genomic groups and three sequence-specific oligonucleotides (SSO) (TIB Molbiol) for the distinct detection of *B. burgdorferi* sensu stricto, *B. garinii*, and *B. afzelii* were used. In addition to the SSO complementary to the *rrfA*-*rrlB* ribosomal DNA spacer region (14), the following SSO within the *ospA* gene have been designed by comparison to nucleotide sequences from the EMBL-GenBank database: *B. burgdorferi* sensu lato, 5'-ACTAATGTTTTGCCATC TTCTTTGAA-3' (nucleotides 306 to 331); *B. burgdorferi* sensu stricto, 5'-ATTAGATCGTACTTGCCGTCCTTTGTT-3' (nucleotides 140 to 165); *B. garinii*, 5'-GTTCCAGAACCGTTGT TTTTATCAGA-3' (nucleotides 201 to 206); and *B. afzelii*, 5'-GATTTTCATCTGTTGATGTTTTGTCTT-3' (nucleotides 352 to 377).

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TABLE 1. Patient characteristics and molecular typing results of *rrfA-rrlB* spacer PCR and RLB in clinical samples from 27 patients with different manifestations of Lyme borreliosis

Patient no.	Age (yr)	Sex ^a	Clinical manifestation	Duration of disease (mo)	Western Blot ^b	Clinical specimen	Identified <i>B. burgdorferi</i> genospecies
2	40	F	Arthritis	7	+	Urine	<i>B. burgdorferi</i> sensu stricto
3	30	F	Arthritis	7	+	Urine	<i>B. burgdorferi</i> sensu stricto
4	63	F	Arthritis	1	+	Urine	<i>B. burgdorferi</i> sensu stricto
5	66	M	Arthritis	115	+	Urine	<i>B. burgdorferi</i> sensu stricto
6	32	M	Arthritis	5	+	Urine	<i>B. burgdorferi</i> sensu stricto
7	52	F	Arthritis	16	+	Urine	<i>B. burgdorferi</i> sensu stricto
8	58	M	Arthritis	117	+	Synovial fluid	<i>B. burgdorferi</i> sensu stricto
9	29	F	Arthritis	17	+	Urine	<i>B. burgdorferi</i> sensu stricto
10	59	M	Arthritis	6	+	Urine	<i>B. burgdorferi</i> sensu stricto
11	50	F	Arthritis	27	+	Urine	<i>B. garinii</i>
12	52	F	Arthritis	7	+	Urine	<i>B. garinii</i>
13	56	F	Arthritis	3	+	Urine	<i>B. afzelii</i>
14	40	M	Neuroborreliosis	18	+	Urine	<i>B. garinii</i>
15	50	M	Neuroborreliosis	1	+	Urine	<i>B. garinii</i>
16	65	F	Neuroborreliosis	26	+	Urine	<i>B. garinii</i>
17	52	M	Neuroborreliosis	21	+	Urine	<i>B. garinii</i>
18	38	M	Neuroborreliosis	19	+	Urine	<i>B. garinii</i>
19	9	F	Neuroborreliosis	1	—	Urine	<i>B. garinii</i>
20	33	M	Neuroborreliosis	1	—	Urine	<i>B. garinii</i>
21	11	F	Neuroborreliosis	4	+	CSF	<i>B. garinii</i>
22	64	F	Neuroborreliosis	6	+	Urine	<i>B. garinii</i>
23	53	F	Neuroborreliosis	10	+	Urine	<i>B. garinii</i>
24	32	F	Acrodermatitis	5	+	Skin biopsy specimens	<i>B. afzelii</i>
25	56	F	Acrodermatitis	16	+	Skin biopsy specimens	<i>B. afzelii</i>
26	28	M	Acrodermatitis	5	+	Skin biopsy specimens	<i>B. afzelii</i>
27	50	F	Acrodermatitis	32	+	Skin biopsy specimens	<i>B. afzelii</i>
	35	M	Acrodermatitis	14	+	Skin biopsy specimens	<i>B. afzelii</i>

^a F, female; M, male.^b Immunoglobulin G. +, positive; —, negative.

The SSO were bound to activated Biodyne C membranes (Pall Europe Ltd., Portsmouth, United Kingdom). The heat-denatured and labeled amplicons were hybridized for 60 min at 48°C (ribosomal DNA RLB) or 64°C (*ospA* RLB), respectively. Colorimetric detection of bound amplicons was performed with the DIG DNA nonradioactive labeling and detection kit (Boehringer Mannheim), using an alkaline phosphatase-conjugated anti-digoxigenin antibody.

For evaluation of our PCR and RLB protocols, the following low-passage *B. burgdorferi* sensu lato reference strains were used: ZS7 (kindly provided by M. M. Simon, Max Planck Institute, Freiburg, Germany), B31 and LW2 (*B. burgdorferi* sensu stricto), PBi and A (*B. garinii*; kindly provided by U. Göbel, Institute of Microbiology, Charité, Berlin, Germany), and PKo (*B. afzelii*; kindly provided by B. Wilske, Max von Pettenkofer Institute, Munich, Germany). *Borrelia hermsii* (kindly provided by U. Göbel) served as the specificity control.

The results of initial experiments showed that in urine specimens from uninfected persons spiked with 10-fold serial dilutions of different *B. burgdorferi* sensu lato strains, ≥ 300 borreliae/sample could be detected by the outer PCR while a sensitivity of ≥ 3 borreliae/sample, corresponding to approximately 15 fg of DNA per sample, could be achieved by nested PCR. Strains of the different species were detected with similar sensitivities, and there was no difference in sensitivity between the PCR protocols.

In a second step, experiments were performed to characterize PCR products in spiked samples by RLB. In all experiments, the hybridization assay confirmed the positive PCR

results, but the sensitivity was not enhanced by RLB. The reference strains could be attributed to the anticipated genomic groups only by hybridization of *rrfA-rrlB* spacer PCR products (Fig. 1). In contrast, RLB hybridization of the amplicons obtained by *ospA* PCR could reliably identify only *B. afzelii* but could not always differentiate between amplicons from *B. burgdorferi* sensu stricto and those from *B. garinii*. These data were confirmed by DNA sequencing of the outer PCR product (performed by TIB Molbiol). DNA of *B. hermsii* could be amplified by *rrfA-rrlB* spacer PCR, but the amplicons could not be hybridized by RLB. There was no amplification of *B. hermsii* DNA by the *ospA* PCR.

In a prospective investigation, 20 urine specimens, 5 skin biopsies, 1 synovial fluid specimen, and 1 CSF sample from Lyme borreliosis patients were analyzed. Urine samples were preferentially used because they were easy to obtain without invasive procedures and earlier studies have shown that *B. burgdorferi* DNA is detectable in urine specimens from untreated patients with Lyme arthritis or neuroborreliosis (13). All samples were positive with both PCR assays. Since the *ospA* assay was shown to give unspecific results in our initial experiments, only the *rrfA-rrlB* spacer PCR and RLB were performed for genotyping of *B. burgdorferi* species in clinical samples.

In 9 of the 12 PCR-positive clinical samples (8 urine samples and 1 synovial fluid sample) from patients suffering from Lyme arthritis, *B. burgdorferi* sensu stricto could be identified, while in 2 urine samples *B. garinii* was detected and in 1 urine sample *B. afzelii* was detected. In contrast, in the 10 PCR-positive

FIG. 1. Representative example of RLB hybridization assay. Four species-specific probes targeting the *rrfA-rrlB* spacer gene were applied in vertical lines on a Biotyne C membrane in concentrations ranging from 12.5 to 100 pmol (*Bbsl*, *B. burgdorferi* sensu lato; *Bbss*, *B. burgdorferi* sensu stricto; *Bg*, *B. garinii*; *Ba*, *B. afzelii*). Labeled PCR amplicons were applied in horizontal lines. The upper part of the membrane contained positive controls from spirochetal cultures (two lines each for different *B. burgdorferi* strains); the lower part contained urine specimens from patients with different manifestations of Lyme disease. Lines 1 and 2, strain ZS7; lines 3 and 4, strain Pko; lines 5 and 6, strain PBI; line 7, patient 2 (Lyme arthritis); line 8, patient 3 (Lyme arthritis); line 9, patient 13 (neuroborreliosis); line 10, patient 5 (Lyme arthritis); line 11, patient 6 (Lyme arthritis). Negative controls ($2\times$ SSPE [$1\times$ SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 , and 1 mM EDTA {pH 7.7}]–0.1% sodium dodecyl sulfate) were applied to the membrane between the numbered lines.

samples from patients suffering from neuroborreliosis (9 urine specimens and 1 CSF sample), only *B. garinii* (10 of 10) could be identified. The five PCR-positive skin biopsies from patients with acrodermatitis were shown to contain DNA from *B. afzelii* exclusively (Table 1 and Fig. 1). Double or pluri-infections were not observed.

The present study demonstrates that *rrfA-rrlB* spacer PCR-RLB, but not *ospA* PCR-RLB, is a reliable and rapid laboratory method for the distinct detection of *B. burgdorferi* sensu lato species in both bacterial cultures and clinical specimens from patients with Lyme borreliosis.

The utility of rRNA spacer regions for typing bacterial species, including *B. burgdorferi*, has been demonstrated in several studies (12, 14). While rRNAs are a very highly conserved class of molecules, the spacer regions, like the one between 23S rRNA (*rrlA* and *rrlB*) and 5S rRNA (*rrfA* and *rrfB*) genes used in our experiments, accumulate higher degrees of sequence

variation between related species than do coding genes because these sequences do not produce a functional gene product. With the *rrfA-rrlB* spacer PCR-RLB protocol optimized for our requirements as described above, we were able to rapidly and simultaneously detect all three pathogenic *B. burgdorferi* species with high sensitivity in clinical specimens from patients with different clinical manifestations of Lyme disease.

Since it has been possible to identify different species of *B. burgdorferi*, studies have been performed to link the different clinical manifestations of Lyme borreliosis to infections with certain species. In these studies, an exclusive association between the late cutaneous manifestation acrodermatitis chronica atrophicans and *B. afzelii* infection (1, 2, 16, 20) was demonstrated. Reports of CSF isolates from patients suffering from neuroborreliosis were rather heterogeneous, but *B. garinii* strains were much more often involved than *B. burgdorferi* sensu stricto or *B. afzelii* (4, 10, 21). Lyme arthritis has been shown to be a frequent manifestation of *B. burgdorferi* sensu stricto infection in North America (11), but molecular analyses of clinical specimens obtained from European Lyme arthritis patients gave controversial results. Based on *ospA* sequence variation analysis, it has previously been reported that in Europe *B. burgdorferi* sensu lato strains causing Lyme arthritis are very heterogeneous with no prevalence of certain genospecies (6, 18). In contrast, more recently published studies demonstrated a preponderance of *B. burgdorferi* sensu stricto DNA in joint samples from Lyme arthritis patients (7, 17).

To our knowledge, this is the first study that simultaneously detected the three human pathogenic species of *B. burgdorferi* sensu lato by means of direct molecular typing by RLB in clinical samples from patients showing the major dermatologic, neurologic, and rheumatologic features of Lyme disease. Our results corroborate earlier reports of the organotropism of *B. burgdorferi* species. Moreover, we demonstrated that European Lyme arthritis is preferentially, but not exclusively, caused by *B. burgdorferi* sensu stricto.

Multiple infections were not detected in the samples analyzed in our study. In contrast, Demaerschalk et al. (5) found that pluri-infections are common in Lyme disease based on results obtained by species-specific PCR targeting of *ospA*. These varying results may be due to differences in study populations and methodology, particularly the gene target used. Further investigations are needed to clarify the prevalence of pluri-infections in Lyme borreliosis.

In conclusion, the methodology described represents a powerful tool for epidemiological studies and to further investigate the association between infections with *B. burgdorferi* sensu lato species and clinical manifestations of Lyme disease.

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