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Polymerase Chain Reaction Analyses Identify Two Distinct Classes of *Borrelia burgdorferi*

P. A. ROSA,[1]* D. HOGAN,[1] AND T. G. SCHWAN[2]

Laboratory of Microbial Structure and Function[1] and Laboratory of Vectors and Pathogens,[2] Rocky Mountain Laboratories, National Institute of Allergy and Infectious Diseases, Hamilton, Montana 59840

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We sequenced homologous chromosomal loci from several North American and European isolates of the Lyme disease spirochete *Borrelia burgdorferi*, as well as from the relapsing fever spirochete *Borrelia hermsii*. Inter- and intraspecies sequence comparisons permitted the design of *B. burgdorferi*-specific polymerase chain reaction primers that detected all strains tested (n = 31) from diverse geographical and biological origins. Polymerase chain reaction "typing" with other unique sets of primers subdivided *B. burgdorferi* isolates into two groups: all North American isolates and a few European isolates made up one group, while the majority of the European and Asian isolates made up the second group. This classification may have a clinical correlate reflected in differences between "typical" Lyme borreliosis in North America and Europe.

The pathogenic spirochete *Borrelia burgdorferi* has received increasing scientific and public interest since its discovery almost a decade ago. It is the etiologic agent of Lyme disease (Lyme borreliosis), a potentially severe human illness with worldwide endemic foci (11, 23, 45). The clinical symptoms of Lyme disease vary among individuals and during the course of an infection, ranging from a relatively benign skin rash to severe arthritic, neurologic, and cardiac manifestations (34, 44, 46–48). Lyme disease appears to progress to neurologic and dermatologic manifestations more frequently in Europe, whereas arthritis is the more common late manifestation in the United States (43). The mechanisms underlying the pathologic changes accompanying Lyme disease remain ill defined.

Diagnosis of Lyme disease is not straightforward, primarily because of several characteristics of *B. burgdorferi* infections. (i) Most of the associated clinical symptoms are not unique to Lyme disease, (ii) strong, specific immunologic responses do not always accompany infection (14, 15, 26), and (iii) spirochetes generally cannot be directly detected or isolated from infected individuals (45).

Transmission of Lyme disease usually occurs by way of infected ticks of the *Ixodes ricinus* complex (10). Spirochetes are primarily detected in the mid-gut, and systemic infections with spirochetes in the mouthparts and salivary glands are rare (13). The mechanism by which spirochetes are transmitted following the bite of an infected tick remains debatable (7, 12, 13, 35, 51). Likewise, it is difficult to envision an efficient means by which ticks in endemic areas become infected, given the low concentration of spirochetes in the blood of the mammals on which they feed.

In order to investigate the transmission and pathogenesis of Lyme disease and to facilitate an accurate diagnosis, our goal has been to develop an assay specific for *B. burgdorferi* and capable of detecting a single organism. We have previously reported specific and sensitive amplification of *B. burgdorferi* DNA using the polymerase chain reaction (PCR) (16, 29, 30, 37) and a chromosomally encoded target sequence (36). As described, the PCR assay was species specific and detected 17 of 18 strains of *B. burgdorferi* tested. It was unclear whether the German isolate that was not detected by this assay was exceptional or representative of a class of somewhat more divergent European strains. The sensitivity of the PCR assay extended down to a few copies of *B. burgdorferi* DNA, even in the presence of a large excess of eukaryotic DNA. Hence, it appeared to be applicable to the analysis of infected tissues. However, hybridization with a probe homologous to the target sequence was needed to identify the specific amplification product, because multiple nonspecific fragments were generated from eukaryotic DNA.

In this study we refined the specificity of the PCR assay for *B. burgdorferi*. We successfully extended the reactivity of the assay to all strains of *B. burgdorferi* tested to date, including those of European and Asian origin. Likewise, we eliminated the nonspecific amplification of eukaryotic DNA and hence the need for the more laborious hybridization step. These modifications should substantially enhance the utility of this assay in both basic research and diagnostic applications.

Perhaps equally important, though, was the "typing" of *B. burgdorferi* strains by their differential primer reactivities in this PCR assay. All North American strains tested to date fall into a single reactivity group, whereas European strains fall into two groups, one of which is indistinguishable from the North American type. It is tantalizing to speculate that these two groups may be reflected clinically in the arthritic versus neurologic spectra of Lyme disease as seen in the United States and Europe.

MATERIALS AND METHODS

***Borrelia* strains and cultivation.** *Borrelia* cultures were maintained in BSKII medium (3) at 35°C and passaged twice a week. The biological and geographical origins of strains are described in Table 1. *B. burgdorferi* B31, ECM.NY.86, SH.2.82, CA.2.87, JD-1, HB19, G1, and G2 and *B. hermsii*, *B. coriaceae*, *B. parkeri*, *B. turicatae*, *B. anserina*, and *B. crocidurae* were described previously (36). *B. burgdorferi* 21721, 27985, 19535, 22921, 19678, 21305, 21343, 26815, 26816, and 20004 were isolated by and obtained from J. Anderson, Connecticut Agricultural Experiment Station, New Haven. *B. burgdorferi* 1325, 1579, 1352, and 245 were

* Corresponding author.

TABLE 1. Reactivities of *B. burgdorferi* isolates with PCR primers

PCR primer reactivity[a]			Strain	Origin	
a	b	c		Biological	Geographical
+	−	+	B31[b]	Tick (*Ixodes dammini*)	New York
+	−	+	Sh-2-82	Tick (*Ixodes dammini*)	New York
+	−	+	21721	Tick (*Ixodes dammini*)	Wisconsin
+	−	+	27985	Tick (*Ixodes dammini*)	Connecticut
+	−	+	JD-1	Tick (*Ixodes dammini*)	Massachusetts
+	−	+	CA-2-87	Tick (*Ixodes pacificus*)	California
+	−	+	1325	Tick (*Amblyomma americanum*)	Texas
+	−	+	1579	Tick (*Amblyomma americanum*)	Texas
+	−	+	1352	Tick (*Amblyomma americanum*)	Texas
+	−	+	245	Tick (*Amblyomma maculatum*)	Texas
+	−	+	532	Flea (*Ctenocephalides felis*)	Texas
+	−	+	19535	White-footed mouse	New York
+	−	+	22921	White-footed mouse	New York
+	−	+	19678	White-footed mouse	New York
+	−	+	21305	White-footed mouse	Connecticut
+	−	+	21343	White-footed mouse	Wisconsin
+	−	+	26815	Chipmunk	Connecticut
+	−	+	26816	Vole	Rhode Island
+	−	+	HB19[b]	Human blood	Connecticut
+	−	+	NY-1-86	Skin (EM[c])	New York
+	−	+	ECM-NY-86	Skin (EM)	New York
+	−	+	20004[b]	Tick (*Ixodes ricinus*)	France
+	− (+)	+	IP2	Cerebrospinal fluid	France
−	+	+	K-48	Tick (*Ixodes ricinus*)	Czechoslovakia
−	+	+	G25	Tick (*Ixodes ricinus*)	Sweden
−	+	+	Ip-3	Tick (*Ixodes persulcatus*)	USSR
−	+	+	Ip-21	Tick (*Ixodes persulcatus*)	USSR
−	+	+	G1	Cerebrospinal fluid	Germany
−	+	+	G2[b]	Cerebrospinal fluid	Germany
−	+	+	P/GU	Skin (EM)	Germany
−	+	+	J-1	Tick (*Ixodes persulcatus*)	Japan

[a] Primer sets a, b, and c are identical to those identified as such in Fig. 3.
[b] Strain for which the PCR target sequence has been determined, as shown in Fig. 2.
[c] EM, Erythema migrans.

isolated by and obtained from J. Rawlings, Texas State Department of Health, Austin. *B. burgdorferi* NY-1-86 was isolated by and obtained from A. MacDonald, Southampton (New York) Hospital. *B. burgdorferi* (or purified DNA from) IP2 (G. Baranton), K-48 (E. Kmety), G25 (G. Stiernstedt), P/GU (V. Preac-Mursic), and J-1 (Kenji) were obtained from R. Johnson, University of Minnesota, Minneapolis. *B. burgdorferi* Ip-3 and Ip-21 (V. Kryuchechnikov) were obtained from A. Barbour. The names in parentheses represent the persons who isolated the strains.

DNA isolation. Total genomic *Borrelia* DNA was prepared as described previously (36).

Cloning. Genomic Southern analyses of G2 DNA (data not shown) demonstrated that a *Bgl*II fragment of approximately 1.6 kb contained sequences homologous to the B31 insert of clone 2H1 (36). Therefore, *Bgl*II-digested total genomic DNA from *B. burgdorferi* G2 was separated on an agarose gel, and the 1.5- to 2-kb DNA fraction was extracted from the gel (Gene-Clean; Bio 101, La Jolla, Calif.), ligated into phosphatase-treated, *Bam*HI-digested pUC18, and transformed into *Escherichia coli* DH5α. Thirty recombinant colonies were picked and screened by hybridization with the *B. burgdorferi* insert from clone 2H1 (36) that was radiolabeled with [α-^{32}P]dATP (New England Nuclear, Boston, Mass.) by random priming (Boehringer Mannheim, Indianapolis, Ind.) (17). Three positive colonies were identified, and recombinant plasmids were prepared by alkaline lysis (28). Restriction digests with *Hin*dII and *Eco*RI demonstrated identical inserts of approximately 1.6 kb in all three clones.

Sequencing. The complete sequence of the G2 insert (clone designated G2-48) was determined by the dideoxy-chain termination technique (38) with double-stranded plasmid DNA, [^{35}S]dATP (New England Nuclear), and a commercial sequencing kit (Sequenase; U.S. Biochemicals, Cleveland, Ohio). Initial sequences were obtained with M13 primers from flanking vector sequences at each end. Primers to insert sequences were then used to extend the sequence across both strands.

The sequences of the homologous fragments in *B. burgdorferi* HB19 and 20004 and *B. hermsii* were obtained by dideoxy-chain termination sequencing (38) of single-stranded, PCR-generated DNA with [^{35}S]dATP (New England Nuclear) and commercial sequencing reagents (Sequenase). Single-stranded DNA was generated by PCR amplification from a double-stranded PCR fragment (oligonucleotides 88 to 107 and 1537 to 1556; see Fig. 2) by using only one or the other primer. Approximately 100 ng of purified double-stranded DNA was the template for 25 cycles with a single primer. PCR samples were phenol-chloroform extracted, precipitated, resuspended, and purified free from primers and nucleotides (Ultrafree-MC 30,000

a - CGAAG ATACT AAATC TGT

a'- GATCA AATAT TTCAG CTT

b - GATAA AAACG AAGAT AATCG

b'- ACTAG GATCT GTGGA TATTC

c - CCAAC TTTAT CAAAT TCTGC

c'- AGGAT CTATT CCAAA ATC

d - AGAAC AATAC AGCTC AGATG

d'- GGATC TATTC CTAAA TCAGA G

e - AAGCT TAATT AGAAC CAAAC

e'- CTTCC TACAC ATAGC TGATG C

f - TGCAG AAACA CCTTT TGAAT

f'- AATCA GTTCC CATTT GCA

FIG. 1. Nucleotide sequences of the PCR primers.

NMWL filter unit; Millipore Corp., Bedford, Mass.) before both the single-strand PCR amplification and the sequencing.

PCR. Oligonucleotides were synthesized with a SAM One DNA Synthesizer (Biosearch, San Raphael, Calif.). Taq polymerase and PCR reagents (Perkin Elmer-Cetus, Norwalk, Conn.) were used with an automated DNA thermal cycler (Perkin Elmer-Cetus). A total of 30 pmol of each primer was used per 100-μl reaction. Unless otherwise specified, 100 ng of total genomic DNA was amplified for 25 cycles under the following conditions: 94°C, 1 min; 37°C, 30 s; 60°C, 1 min. A total of 10 μl per reaction was analyzed on 1.5% agarose–0.5× TBE gels under standard electrophoresis conditions (28). PCR amplification of the *osp* locus was done under the following conditions: 94°C, 1 min; 37°C, 30 s; 60°C, 3 min. Samples were analyzed on 0.8% agarose–0.5× TBE gels. Nested PCR conditions are specified in the legend to Fig. 4. The nucleotide sequences of the PCR primers are shown in Fig. 1.

Nucleotide sequence accession numbers. The GenBank numbers for the nucleotide sequences reported here are M58429 (for strain 20004), M58430 (for *B. hermsii*), M58431 (for G2), M58432 (for HB19), and M58433 (for B31).

RESULTS

PCR target sequence comparison among North American and European *B. burgdorferi* isolates. The previously described *B. burgdorferi* PCR target sequence (36) was derived from the prototype North American tick isolate B31 (11). A 1.6-kb *Bgl*II fragment containing the homologous segment of DNA from the nonreactive European isolate G2 (from human cerebrospinal fluid [20]) was cloned and sequenced to facilitate the design of more broadly reactive PCR primers. The published B31 sequence corresponds to nucleotides 139 to 520 of the cloned G2 fragment (Fig. 2). Strains B31 and G2 exhibited approximately 90% nucleotide identity over this region. The positions of the original PCR primers (nucleotides 147 to 164 and 500 to 520, previously designated A and C, respectively) are underlined on the B31 sequence. The lack of reactivity of these primers with G2 DNA is easily understood, given the degree of mismatch between the B31 and G2 sequences at these positions.

This segment of G2 DNA encodes a single large open reading frame (434 amino acids) that apparently begins upstream of the 5′ end of the cloned fragment and terminates at nucleotide 1302 (Fig. 2). Subsequent to this and throughout all other reading frames are multiple translational stop codons. Immediately following the translational stop codon at the 3′ end of the open reading frame is an 11-bp inverted repeat capable of forming a stem-loop structure that represents a perfect (*rho*)-independent transcriptional terminator. A similar motif is not found at any other position throughout this sequence. It is likely that this sequence represents the 3′ end of a chromosomally encoded structural gene. The large G2 open reading frame encompasses the shorter sequenced region from B31 DNA.

The nucleotide sequences of the homologous segments of DNA from two additional *B. burgdorferi* isolates, HB19 (human blood, North America) and 20004 (tick, Europe), were obtained by direct sequencing of their amplified PCR products (Fig. 2). They exhibited 97.4 and 99.5% nucleotide identity, respectively, to B31. Hence, a second European isolate resembles the North American isolates more closely than it resembles G2.

Extensive amplification of *B. hermsii* DNA with a set of G2 primers (nucleotides 88 to 107 and 1537 to 1556, G2 sequence, Fig. 2) under low-stringency conditions resulted in a discrete fragment of approximately 1,700 bp (data not shown). Direct sequencing of this PCR fragment demonstrated that it was homologous to the *B. burgdorferi* target sequence, with which it shared approximately 70% nucleotide identity (Fig. 2).

Universal and subtype-specific PCR primers for *B. burgdorferi*. This comparison of the PCR target sequence among distinct *B. burgdorferi* isolates as well as *B. hermsii* provides information useful in optimization of the PCR assay. Primers specific for regions of the target sequence identical among *B. burgdorferi* strains yet divergent from *B. hermsii* (such as nucleotides 292 to 311 and 401 to 418; underlined on the G2 sequence, Fig. 2) provided the basis for a widely reactive but *B. burgdorferi*-specific assay. An example of the amplification of B31 and G2 DNA, but not *B. hermsii* DNA, with such primers is shown in Fig. 3c. Primers specific for *B. hermsii* sequences (nucleotides 140 to 163 and 396 to 496; underlined on the *B. hermsii* sequence, Fig. 2) showed no amplification of *B. burgdorferi* DNA (Fig. 3d). Primers derived from sequences unique to B31 (nucleotides 147 to 164 and 500 to 520; underlined on the B31 sequence, Fig. 2) or G2 (nucleotides 139 to 158 and 476 to 495; underlined on the G2 sequence, Fig. 2) were specific for DNA from the respective *B. burgdorferi* strain (Fig. 3a and b).

PCR amplification of genomic DNA from 31 different *B. burgdorferi* isolates demonstrated that the primers to sequences conserved between B31 and G2 reacted with all strains tested (PCR primer set c, Table 1). These included 21 North American isolates, 9 European isolates, and 1 Japanese isolate from ticks (six species), rodents (three species), and humans (three different tissues). Hence, this PCR assay detects *B. burgdorferi* of very broad biological and geographic origins. These primers did not react with *B. hermsii*

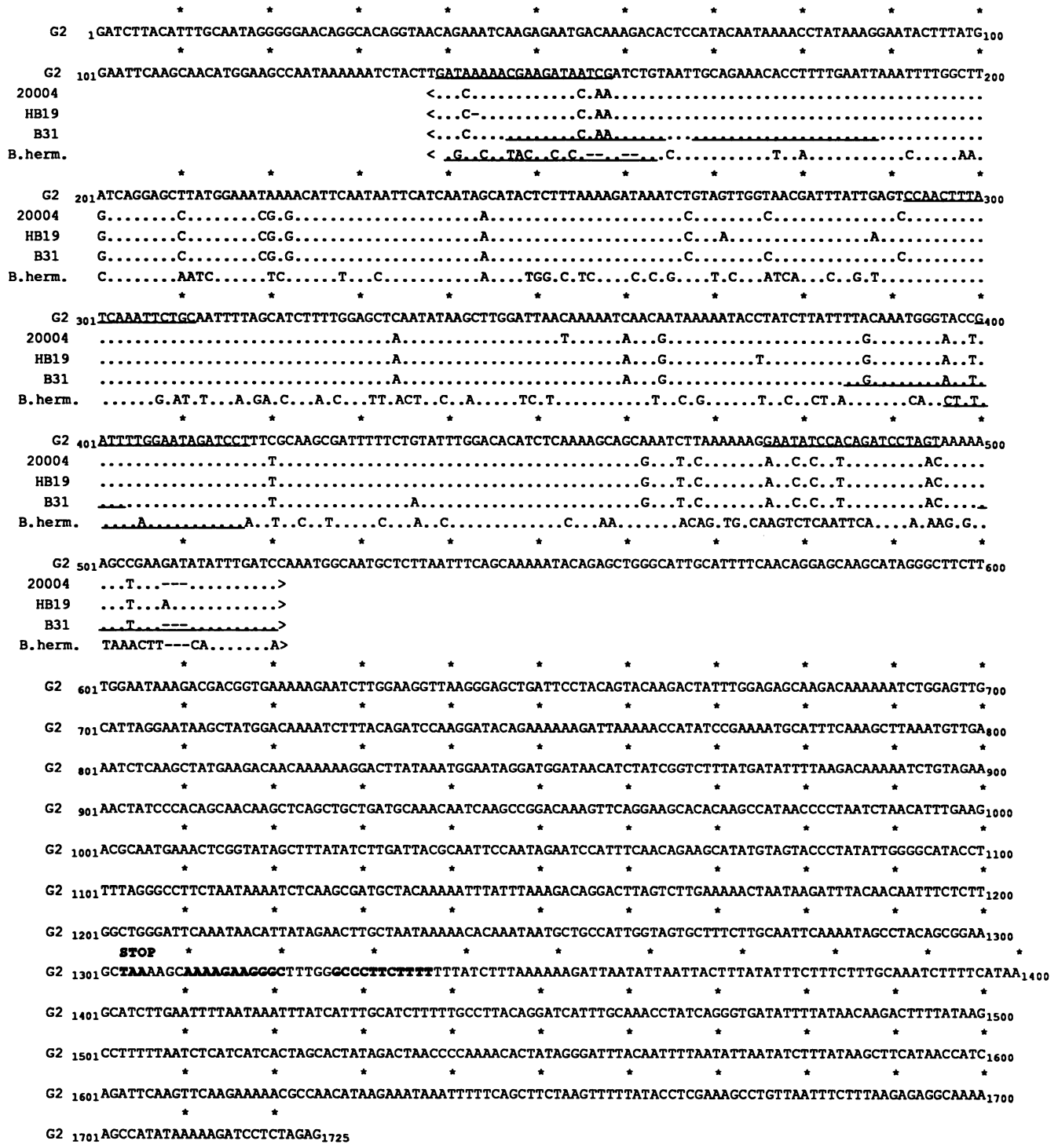

FIG. 2. PCR target sequence comparisons among *Borrelia* strains. The 1,725-bp nucleotide sequence shown is from European *B. burgdorferi* isolate G2. Nucleotides 139 to 520 represent the segment of this DNA homologous to the previously described PCR target sequence (36). The sequences of the homologous segments of DNA from *B. burgdorferi* 20004, HB19, and B31 and the relapsing fever spirochete *B. hermsii* (B. herm.) are given below the corresponding G2 sequence. Dots indicate identity with the G2 sequence; letters indicate the actual sequence where it differs from G2. Underlined sequences indicate the positions of PCR primers described in the legends to Fig. 3 to 5 and Table 1. Nucleotides shown in boldface type represent putative transcriptional and translational stop signals following the single long open reading frame.

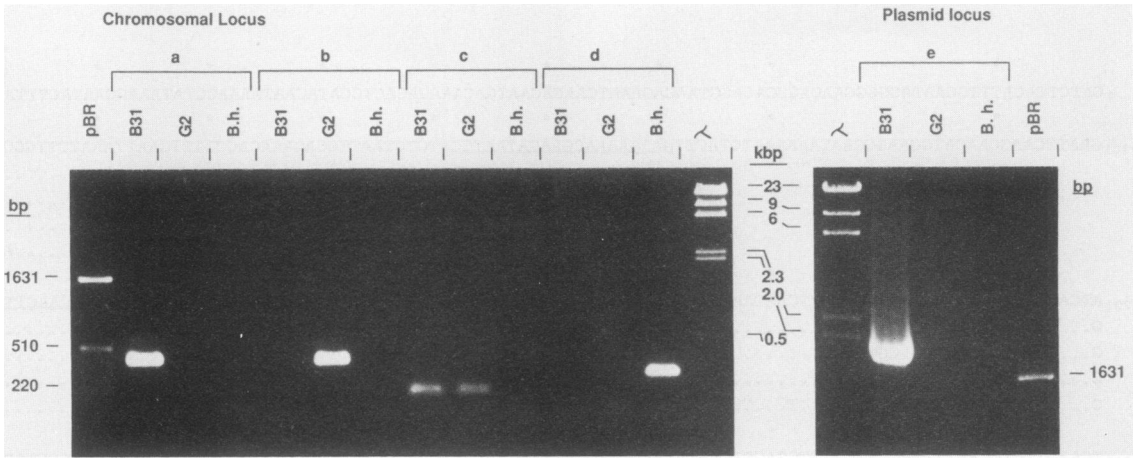

FIG. 3. Universal and subtype-specific primers for *B. burgdorferi*. The template DNA in each PCR is indicated at the top of the lane. Letters grouping lanes refer to the different PCR primers used in the reactions. Primer set a represents nucleotides 147 to 164 and 500 to 520 of the corresponding B31 sequence in Fig. 2. Primer set b represents nucleotides 139 to 158 and 476 to 495 and primer set c represents nucleotides 292 to 311 and 401 to 418 of the G2 sequence in Fig. 2. Primer set d represents nucleotides 140 to 163 and 396 to 416 of the *B. hermsii* (B. h.) sequence in Fig. 2. Primer set e represents nucleotides 1 to 20 and 1896 to 1915 of the *osp* locus of *B. burgdorferi* B31 (8). The 3′ primers of a given set were synthesized as the complementary strand to the sequence shown. Lambda and pBR322 markers were run in the outside lanes, and the lengths of their respective fragments are indicated.

(Fig. 3c), other members of the genus *Borrelia*, or *Treponema pallidum* (data not shown).

PCR amplification of *B. burgdorferi* with primers to sequences unique to either B31 or G2 (Fig. 2) subdivided *B. burgdorferi* isolates into two groups. B31 primers detected all North American and some European isolates (PCR primer set a, Table 1). G2 primers exhibited a "mirror-image" pattern of reactivity (PCR primer set b, Table 1); that is, previously nonreactive European and Asian isolates were amplified, whereas none of the North American nor the two previously detected European strains (20004 and IP2) were amplified. Uncloned strain IP2 showed a faint signal after 10 additional cycles of amplification with G2 primers. However, cloned lines derived from IP2 reacted with only the B31 primers. This presumably reflects a mixture of both "types" of *B. burgdorferi* in the original uncloned isolate, with the B31 type predominating, since the nature of the primers would preclude the binding of a single DNA sequence to both sets. Neither the B31 nor G2 primers were reactive with *B. hermsii* (Fig. 3a and b, respectively), other members of the genus *Borrelia*, or *T. pallidum* (36; data not shown).

PCR amplification with primers flanking the plasmid-encoded *osp* operon, using sequences from the B31 strain (nucleotides 1 to 20 and 1896 to 1915 [8]), similarly did not amplify G2 DNA (Fig. 3e). Amplification of the same panel of 31 strains with these primers gave a reactivity pattern identical to that of the B31-type primers for the chromosomal target locus (PCR primer set a, Table 1). That is, they reacted with all North American strains and European strains 20004 and IP2. Hence, PCR typing with both chromosomal and plasmid-derived target sequences subdivides *B. burgdorferi* strains into the same two groups.

***B. hermsii*-specific PCR primers.** Amplification of *Borrelia* DNA with primers derived from *B. hermsii* sequences that were divergent from *B. burgdorferi* (nucleotides 140 to 163 and 396 to 416; underlined on the *B. hermsii* sequence in Fig. 2) demonstrated specificity for the North American relapsing fever spirochetes *B. hermsii*, *B. parkeri*, and *B. turicatae* (Fig. 4). No reactivity was detected with *B. coriaceae*, *B. crocidurae*, or *B. anserina*, the agents of epizootic bovine abortion (putative), Mediterranean relapsing fever, and avian spirochetosis, respectively (22, 24). Likewise, these primers did not react with B31 or G2 DNA (Fig. 4) or any of the remaining 29 *B. burgdorferi* isolates tested (data not shown).

PCR amplification using "nested" primers. Although PCR amplification of bacterial DNA with most primers generates only a single specific fragment, extensive amplification of highly complex eukaryotic DNA frequently results in multiple nonspecific products. As shown in Fig. 5, lanes 2 and 9, 15 cycles of amplification with 200 ng of *B. burgdorferi* DNA

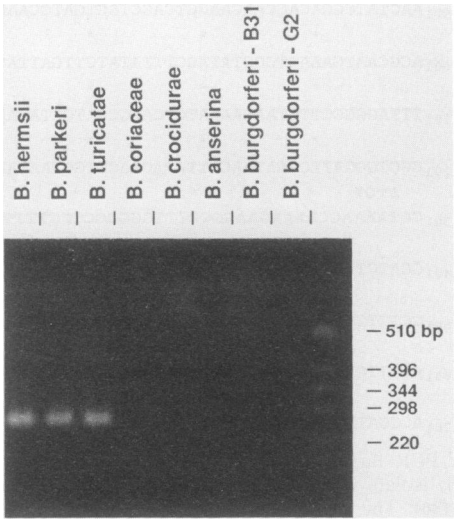

FIG. 4. PCR amplification of *Borrelia* DNA with *B. hermsii*-specific primers. The template DNA in each PCR is indicated at the top of each lane. Primer set d (Fig. 3) was used in all reactions and represents nucleotides 140 to 163 and 396 to 416 of the *B. hermsii* sequence in Fig. 2. The mobilities of pBR322 markers are indicated on the right.

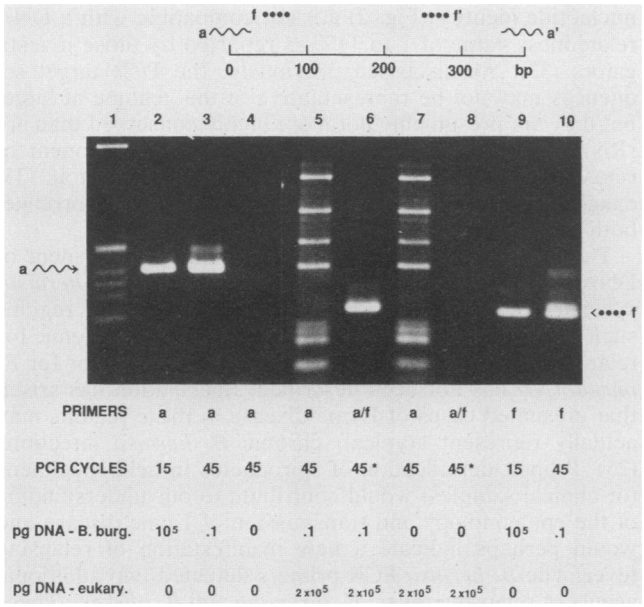

FIG. 5. PCR amplification using nested primers. The relative positions of the PCR primers on the DNA target sequence are diagrammed at the top. Primer set a represents nucleotides 147 to 164 and 500 to 520 on the B31 sequence in Fig. 2. Primer set f represents nucleotides 168 to 187 and 386 to 403 on the B31 sequence in Fig. 2. The total number of cycles for which a sample was amplified is indicated. Samples analyzed in lanes 6 and 8 were initially amplified for 30 cycles with primer set a. Five microliters of this reaction was then added to a new PCR containing primer set f and subsequently amplified 15 more cycles (45 cycles total). The quantities of *B. burgdorferi* (B. burg.) and mouse eukaryotic (eukary.) DNA in each reaction are indicated at the bottom. Arrows indicate the mobilities of the PCR products of the respective primers.

resulted in a single fragment of the appropriate size. In this case, two different sets of primers (designated a and f) were used in two separate reactions. A similar pattern was seen with 0.1 pg of *B. burgdorferi* DNA (approximately 50 genome equivalents) after 45 cycles of amplification (Fig. 5, lanes 3 and 10). However, after 45 cycles of amplification in the presence of 200 ng of eukaryotic (mouse) DNA, multiple fragments were generated and no difference was seen between eukaryotic DNA alone or with 0.1 pg of *B. burgdorferi* DNA (Fig. 5, lanes 5 and 7, respectively). As demonstrated previously (36), a hybridization step with an internal *B. burgdorferi* probe is necessary in such instances to demonstrate a specific *B. burgdorferi* fragment. This step can be eliminated, however, with two sequential PCRs by using nested primers (29), as follows.

Thirty cycles of PCR amplification were performed with the external pair of primers (set a); 5% of this reaction was placed in a new reaction mixture containing only the internal primers (set f), and this reaction was amplified 15 additional cycles. As shown in Fig. 5, when such a protocol was followed with 0.1 pg of *B. burgdorferi* DNA in the presence of 200 ng of eukaryotic DNA, only a single fragment of the appropriate size was generated (Fig. 5, lane 6; compare with lane 9 or 10). Significantly, when the same procedure was followed with 200 ng of eukaryotic DNA alone without any *B. burgdorferi* DNA, there was no amplified product (Fig. 5, lane 8). Hence, this protocol resulted in the specific amplification of only the *B. burgdorferi* fragment, and nonspecific products of eukaryotic DNA were eliminated. Hybridization with an internal probe is no longer necessary since the *B. burgdorferi* fragment can be visualized directly. Similar results were obtained with other sets of nested primers and with human and tick (eukaryotic) DNAs (data not shown).

DISCUSSION

Results of these studies permit a direct assessment of the relatedness of spirochetes isolated from distinct biological and geographical origins. Nucleotide sequence comparisons of a chromosomally encoded PCR target locus demonstrated 0.5 to 10% mismatch among four different *B. burgdorferi* isolates. Although limited, these data are compatible with previous estimates of genetic relatedness among *B. burgdorferi* isolates (21, 39) and demonstrate more diversity between European than between North American *B. burgdorferi* isolates. Such diversity has been described previously in protein and plasmid profiles (4–6, 9, 40–42, 52) and was further substantiated at the nucleotide level when a large number of *B. burgdorferi* isolates were surveyed with different PCR primers (discussed below).

B. hermsii exhibits approximately 70% nucleotide sequence identity with *B. burgdorferi* at this locus. Previous estimates of genetic relatedness between these two *Borrelia* species were considerably lower than this (30 to 60%) (21, 39). An element not accounted for in those measurements was the contribution of the plasmid contents of both species to the extent of DNA reannealing. Since these extrachromosomal elements may be present in multiple copies per cell, their variability could exaggerate the apparent genetic differences between *Borrelia* species.

Alternatively, a higher conservation of sequence in this region relative to the entire genome could reflect evolutionary constraints imposed by the nature of the putative protein which this DNA encodes. Computer analysis of European Molecular Biology Laboratory and National Biomedical Research Foundation data bases revealed no significant homology between the deduced amino acid sequence and any known proteins. However, whether this DNA locus encodes a protein, and if so, the function of that protein, is not relevant to its suitability as a target sequence in a PCR assay. The conservation of this sequence among *B. burgdorferi* isolates, yet its distinction from *B. hermsii*, constituted the basis for its selection. The validity of these criteria for a broadly reactive but *B. burgdorferi*-specific assay was further substantiated in this study.

A comparison of the PCR target sequence between two distinct *B. burgdorferi* strains of European and North American origins permitted the design of primers that are reactive with all strains of *B. burgdorferi* tested to date. Considering the broad geographic origins and biological sources of these strains, this demonstrates that this target sequence is well conserved in *B. burgdorferi*. However, it is sufficiently divergent from the homologous sequence in *B. hermsii*, the most closely related non-Lyme spirochete, to easily allow the design of primers that are species specific.

Other investigators have recently reported PCR detection of *B. burgdorferi* in clinical and tick specimens, using target sequences derived from the genes encoding the outer surface proteins (Osp) of North American strain B31 (27, 31, 32). Not all strains of *B. burgdorferi* were detected in these assays, and not all presumed-positive samples were amplified. We found that only 2 of 10 European and Asian *B. burgdorferi* strains were amplified using primers derived from sequences flanking the *osp* operon of strain B31. Using

primers derived from the sequence of the *osp* operon of European strain G2 (35a), only four of the remaining eight European and Asian isolates were amplified (data not shown). Hence, unlike the chromosomal PCR target sequence, a comparison of the *osp* loci of two *B. burgdorferi* strains is not sufficient to allow detection of all strains. Substantial antigenic, structural, and phase variation has been described in the immunodominant Osp proteins and may provide *B. burgdorferi* with a means of avoiding the host immune response (5, 6, 9, 40, 52). Therefore, we feel that the *osp* genes probably do not represent an optimal *B. burgdorferi* PCR target because of a lack of sequence conservation among strains. Wallich et al. (50) have reported the sequence of the flagellin gene of *B. burgdorferi* and demonstrated sensitive amplification of *B. burgdorferi* DNA with primers derived from this sequence. However, given the fundamental structural nature of the flagellin protein and the conservation of a homologous gene in organisms as diverse as *Salmonella typhimurium* and *Bacillus subtilis* (50), the specificity of these primers for *B. burgdorferi* remains to be demonstrated.

The elimination of nonspecific amplification of eukaryotic DNA with nested primers (29) significantly enhances the PCR assay. First, hybridization is not necessary to identify the specific *B. burgdorferi* fragment. This usually means a substantial reduction in the time needed to analyze samples. Second, because nonspecific products are not competing for the polymerase, it more efficiently amplifies the *B. burgdorferi* target sequence. This results in greater sensitivity of the assay with fewer cycles of amplification.

We do not yet know the significance of the *B. burgdorferi* subtypes that we have defined by selective PCR reactivity. There does not appear to be any correlation between the biological source (tick versus human) and the type of isolate. It is recognized, however, that Lyme disease in Europe more frequently progresses to neuroborreliosis and a severe dermatologic disorder known as acrodermatitis chronica atrophicans, whereas in the United States, arthritis is a more common manifestation of late Lyme disease (1, 2, 19, 43, 49). This could reflect differences in the pathogenic features of the indigenous spirochetes. We do not believe that the PCR target sequence has any bearing on pathogenicity. Rather, it is probably an indication of a previous divergence within *B. burgdorferi* that may now be manifested clinically. Although in general this typing distinguishes European from North American isolates, two *B. burgdorferi* strains from France were included with the North American group. Interestingly, one of these strains, an uncloned isolate from a human, showed evidence of a mixture of both types of *B. burgdorferi*. Hence, one cannot assume that human isolates are clonal.

On the basis of DNA hybridization studies and restriction endonuclease patterns of rRNA genes, Postic et al. (33) have recently proposed that *B. burgdorferi* is actually composed of two species. European strains IP2 and G25 were included in their studies (as well as ours) and were classified as members of the original *B. burgdorferi* prototype B31 species and the proposed new species, respectively. These data are in agreement with our division of *B. burgdorferi* into two groups and, when comparable, the typing of strains. However, the interpretation that these groups make up two separate species is not supported by our sequence analyses, which demonstrate approximately 90% nucleotide identity at a chromosomal locus among members of these two groups (Fig. 2). Likewise, sequence comparisons at this locus between *B. burgdorferi* and *B. hermsii* (approximately 70% nucleotide identity; Fig. 2) are not compatible with a DNA relatedness value of 2 to 11% as reported by those investigators (33). As discussed previously, the PCR target sequences may not be representative of the genome at large, but they are presumably not more highly conserved than are rRNA genes, which encode a fundamental component of ribosomes. It appears that the analyses of Postic et al. (33) exaggerate apparent genomic differences among borreliae, both within and between species.

The nucleotide sequence of the homologous segment of DNA from *B. hermsii* also allowed us to design *B. hermsii*-specific primers. This has particular relevance in regions such as the northwestern United States that are endemic for relapsing fever but in which a competent tick vector for *B. burgdorferi* has not been described. Speculation has arisen that presumed cases of Lyme disease in these regions may actually represent atypical, chronic *B. hermsii* infections (25). Proper identification of spirochetes in field specimens (or clinical samples) would contribute to our understanding of the epidemiology and transmission of Lyme disease and would perhaps indicate a new manifestation of relapsing fever. The *B. hermsii* PCR primers detected two additional agents of relapsing fever, *B. turicatae* and *B. parkeri*. These spirochetes were initially classified as different species of *Borrelia* by the criterion that each had a unique tick vector, but more recent estimates of genetic relatedness indicate that they probably make up a single species (21). The *B. hermsii*-specific primers did not cross-react with any other *Borrelia* species tested, including *B. crocidurae*, a distinct species of spirochete that causes Mediterranean relapsing fever (18).

In this report we presented data that provide the basis for a broadly reactive, *B. burgdorferi*-specific PCR assay. The applicability of this assay to the detection of *B. burgdorferi* in infected mammalian and arthropod tissues is apparent and is being undertaken. We also demonstrated that all *B. burgdorferi* isolates tested to date can be divided into two groups. The relationship between this typing of *B. burgdorferi* and the clinical manifestations of disease remains to be explored.

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