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Development of Polymerase Chain Reaction Primer Sets for Diagnosis of Lyme Disease and for Species-Specific Identification of Lyme Disease Isolates by 16S rRNA Signature Nucleotide Analysis

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We have determined and compared partial 16S rRNA sequences from 23 Lyme disease spirochete isolates and aligned these with 8 sequences previously presented. The 16S rRNA signature nucleotide compositions were defined for each isolate and compared with the genomic species signature nucleotide sets previously established. To identify positions truly indicative of species classification which could serve as targets for polymerase chain reaction species-specific identification primers, 16S rRNA-based phylogenetic analyses were conducted. On the basis of the identified signature nucleotides, we designed polymerase chain reaction primer sets which (i) amplify all spirochete species associated with Lyme disease and (ii) differentiate between these species. The primer sets were tested on 38 *Borrelia* isolates associated with Lyme disease and were found to be sensitive and specific. All Lyme disease isolates tested were amplification positive. These primers allow for the rapid species identification of Lyme disease isolates.

Several studies have demonstrated that isolates of the causative agent of Lyme disease can be classified into three major genomic species (3, 6, 13–15, 20). The sensu stricto group contains isolates from both Europe and North America, whereas group 20047 contains non-North American isolates only (13–15, 20). The most recent group to be defined has been referred to as group 3 (14). The genetic distances between these groups were previously demonstrated to be significant, warranting species designation for each (13, 14). Recently, the three genomic species or subgroups of *Borrelia burgdorferi* have been assigned species status (3). The sensu stricto group retains the species designation *B. burgdorferi*, whereas the 20047 group has been designated *Borrelia garinii*. Although the separate species status for genomic group 3 has been agreed upon, a new species name for this group has not yet been assigned. Here, we utilize the nomenclature proposed by Baranton et al. (3) and will refer to genomic group 3 (14) as species group VS461.

Several methods have been proposed as a means for the typing or species identification of Lyme disease isolates. These include analysis of restriction fragment length polymorphism patterns of chromosomal DNA or of specific genes (1, 3, 11, 13, 20, 23), DNA-DNA hybridization (11, 20), oligonucleotide probes directed against 16S rRNA (15), multilocus enzyme electrophoresis (6), and 16S rRNA sequence analysis (1, 13, 14). Analysis of the reactivity profiles of isolates with various monoclonal antibodies has also been employed (5, 27). Although this approach has proven useful in delineating serotypes, its usefulness for the identification and differentiation of species is unclear. All of these approaches share the requirement for cultivation of the organism, which is not always successful. The development of a rapid method for species differentiation that can be applied directly to clinical samples is critical for defining potential correlations between the variable clinical presentations and the species of the infecting organism.

In this study, we define phylogenetically significant target sequences, based upon 16S rRNA signature nucleotide analysis, and exploit these targets in the development of polymerase chain reaction (PCR) primer sets which can be used to identify Lyme disease isolates as well as to identify the isolates to the species level. We have also identified a unique 16S rRNA gene restriction fragment length polymorphism pattern for the VS461 group which is distinct from those observed for *B. burgdorferi* and *B. garinii*.

MATERIALS AND METHODS

Bacteria and growth conditions, RNA isolation, and 16S rRNA sequencing. *Borrelia* isolates, described in Table 1, were cultivated in BSK II medium (4) at 34°C. Total cellular RNA isolation and reverse transcriptase primer extension sequencing were conducted as previously described (13). Between 900 and 1,400 bases of sequence were determined for each isolate. The 16S rRNA sequences for isolates CA2-87, Illinois 1, R-IP90, and VS219, which were used in the phylogenetic analyses, have been assigned GenBank accession numbers as indicated below. The GenBank accession numbers for other isolates used in tree construction have been previously presented (13, 14).

Phylogenetic analyses were accomplished by using the PHYLIP package written by Joseph Felsenstein (7) as previously described (13) by using masked sequences of 1,118 bases. The *Borrelia hermsii* HS1 16S rRNA sequence (GenBank accession no. M60968) was used as an outgroup.

PCR. Primers were synthesized on an Applied Biosystems Inc. DNA synthesizer and deblocked by standard methods. Primer sequences are listed in Table 2. The LD primer set, which was designed to amplify all species associated with Lyme disease, generates an amplification product of 357

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TABLE 1. *Borrelia* isolates

Borrelia isolate(s)	Geographic origin	Source[a]
B31, SH-2-82, 25015	New York	*Ixodes dammini*
NY1-86, NY13-87	New York	Human skin
CA2-87, CA3-87, CA12	California	*Ixodes pacificus*
CA13	California	*Ixodes neotomae*
21721	Wisconsin	*Ixodes dammini*
Illinois 1	Illinois	Mouse
21343	Texas	White-footed mouse
1352	Texas	*Amblyomma americanum*
3028	Texas	Human pus
Veery	Connecticut	Veery bird
27985	Connecticut	*Ixodes dammini*
297	Connecticut	Human CSF
20004, 20047, 153	France	*Ixodes ricinus*
IP2A	France	Human CSF
R-IP90	Khzabarovsk, Russia	*Ixodes persulcatus*
R-IP21, R-IP3	Leningrad, Russia	*Ixodes persulcatus*
J1	Japan	*Ixodes persulcatus*
G25, VS102, VS461, VS219	Sweden	*Ixodes ricinus*
B023	Germany	Human skin
G2, G1	Germany	Human CSF
FRG, N34, PBi	Germany	*Ixodes ricinus*
ECM1, UM01	Sweden	Human skin
VS116	Switzerland	*Ixodes ricinus*

[a] CSF, cerebral spinal fluid.

bases. Primer sets for the differentiation between *B. burgdorferi* (BB primer set), *B. garinii* (BG primer set), and group VS461 (VS461 primer set) generate amplification products of 574, 574, and 591 bp, respectively. *Borrelia* DNA was isolated as described by Barbour et al. (5). All PCR components were obtained from the GeneAmp kit and used as recommended by the supplier (Perkin-Elmer Cetus). A total of 50 pmol of the appropriate primer set and various amounts of template DNA were used in each 100-μl reaction mixture. PCRs were performed with a Perkin-Elmer Cetus thermocycler by denaturing for 1 min at 94°C, annealing for 30 s at 47°C, and extending for 1.5 min at 72°C for either 25 or 35 cycles. Products were visualized by electrophoresis of 10% of the reaction volume in 1.5% agarose gels in TAE buffer (40 mM Tris-acetate, 2 mM EDTA [pH 8.5]) containing ethidium bromide at 0.5 μg/ml. To determine the sensitivity of detection, PCR with serial dilutions of template DNA ranging from 500 ng to 0.5 fg was performed. Detection sensitivity was also assayed by hybridization analysis of 10 μl of the reaction volume with the Mini-fold II slot blotter (Schleicher & Schuell) according to the instructions of the manufacturer. The DNA was fixed to the membrane via UV cross-linking with a StrataLinker (Stratagene) set at 120,000 μJ/cm^2 for 30 s. Hybridizations were performed as previously described (14). Blots of the genomic species-specific amplification products were probed with the 16S-A probe, and blots of the Lyme disease-specific amplification products were probed with the 16S-B probe (Table 2).

Oligonucleotides were 5′ end labeled by standard methods with [γ-^{32}P]ATP (6,000 Ci/mmol). Blots were washed once with 2× SSC (1× SSC is 0.15 M NaCl, 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS) for 10 min (at room temperature), once with the same buffer at 37°C for 10 min, and once with 0.1× SSC–0.1% SDS at 42°C for 1 h. Blots were exposed to Kodak XAR-5 film with intensifying screens at −70°C for 2 h.

Nucleotide sequence accession numbers. The 16S rRNA sequences for isolates CA2-87, Illinois 1, R-IP90, and VS219, which were used in the phylogenetic analyses, have been assigned the GenBank accession numbers M89935 to M89938, respectively.

RESULTS AND DISCUSSION

Alignment of the 16S rRNA sequences determined here with others previously presented (13, 14) allowed for the identification and analysis of 16S rRNA signature nucleotides. Table 3 lists the nucleotide identities at these positions. Variability at some of the previously identified signature positions suggested that some of these positions may not accurately reflect the species of an isolate. To determine the species of isolates which were variable at these positions, we determined nearly complete 16S rRNA sequences for several isolates and constructed phylogenetic trees. Upon tree construction, isolates VS219 and Illinois 1 grouped with, but peripherally to, *B. burgdorferi* isolates (Fig. 1). The identification of isolates VS219 and Illinois 1 as *B. burgdorferi* was highly supported by bootstrap analyses. Multilocus enzyme electrophoresis studies also identified isolate VS219 as *B. burgdorferi* (6). These approaches demonstrate that all isolates investigated here reside in one of the three currently identified species groups. The species-specific signature set can now be defined by positions 91, 92, 140, and 630, with position 630 in itself being sufficient to differentiate between species. Our analysis of the isolates studied so far would indicate this, but prospective application to additional isolates which have been identified to the species level by other approaches would be helpful in confirming these data.

Previous studies have demonstrated the utility of PCR as a tool for the identification of the Lyme disease spirochetes (8, 10, 12, 16, 19, 21, 22). In general, the primer sets described offer a rapid and effective means for detection. Primers sets directed against the *osp* locus (12, 17, 19), various chromosomal loci (8, 21), and the flagellin gene have been designed (10). The genetic stabilities of these targets

TABLE 2. PCR primers and oligonucleotide probe sequences

Primer set	Plus strand primer (5′-3′)	Minus strand primer (5′-3′)
LD[a]	ATGCACACTTGGTGTTAACTA (819–842)	GACTTATCACCGGCAGTCTTA (1153–1173)
BB	GGGATGTAGCAATACATTC (74–92)	ATATAGTTTCCAACATAG (630–648)
BG	GGGATGTAGCAATACATCT (74–92)	ATATAGTTTCCAACATAGT (630–648)
VS461	GCATGCAAGTCAAACGGA (59–76)	ATATAGTTTCCAACATAGT (630–648)
16S-A	GAATTTTACAATCTTTCGACC (416–436)	
16S-B	GGGGAATAATTATCTCTAAC (1009–1028)	

[a] The *Borrelia* 16S rRNA sequences possess a 3-base deletion relative to the *E. coli* sequence in the LD target site.

TABLE 3. Nucleotide identities of 16S rRNA variable and signature positions in Lyme disease spirochetes

Isolate(s)[a]	Nucleotide identity at the following 16S rRNA positions[b]:								
	91	92	140	183	187	278	630	649	979
B31, SH-2-82, 20004, 1352, IP2A, CA2-87, CA3-87, CA12, CA13, 21721, 21343, Veery, 3028, NY1-86, NY13-87, 27985, 297	U	C	U	G	G	A	C	G	C
25015, Illinois 1	U	C	U	A	G	G	C	G	C
R-IP3, R-IP21, VS461, J1	U	U	U	G	A	G	G	G	C
G2, G1, G25	C	U	C	A	A	G	A	A	U
VS102, R-IP90	C	U	C	A	A	G	A	G	C
VS219	U	C	U	G	A	A	C	G	C

[a] The identities of the 16S rRNA positions for isolates VS116, FRG, N34, 20047, 153, PBi, UM01, and ECM1 were not determined.
[b] *E. coli* numbering.

(i.e., mutation rates), however, have not been defined. In contrast, rRNA genes have been shown to be highly conserved (for a review, see reference 28) and hence are attractive targets for both diagnosis and species-specific identification. In the present study, PCR primers were designed to exploit the 16S rRNA signature positions common to the three spirochete species associated with Lyme disease. The LD primer set may have potential in the diagnosis of Lyme disease. Signature nucleotides unique to each species were also exploited to develop species-specific primers. With the LD primer set, all isolates were amplified to roughly equivalent levels. Amplification products were not detected when DNAs from other *Borrelia* species were used as templates (Fig. 2). All PCR amplification results are summarized in Table 4. Titration of the input amount of template DNA used in the PCR reactions (35 cycles) and subsequent agarose gel electrophoresis of 10% of the reaction volume revealed that a template input of 0.05 pg was sufficient to visualize the amplification products. The presence of a large excess of nonspecific DNA (300 ng of human liver DNA) had no effect on the sensitivity of detection, and cross-amplification of human DNA sequences was not observed (data not shown). Southern blot analyses with the 16S-B probe confirmed that the amplification products were the result of amplification of *Borrelia* DNA. Slot blot hybridization analysis of 10% of the amplification reaction volume increased the sensitivity level by 10-fold to an input of 5 fg of sample DNA (2 genome equivalents). Signal was not observed when either primers or template DNA was omitted from the reactions. The 16S rRNA genes from *B. hermsii*, *Borrelia coriaceae*, and *Borrelia anserina*, which exhibit 16S rRNA sequence similarity values of 96.7, 96.6, and 96.4%, respectively, compared with that of the *B. burgdorferi* type strain B31 (13), were not amplified by the LD set. These *Borrelia* species are the organisms most closely related to the Lyme disease spirochetes (13, 18). The species-specific primer sets, BB and BG, also did not exhibit cross-amplification of other *Borrelia* species. Minor amplification of

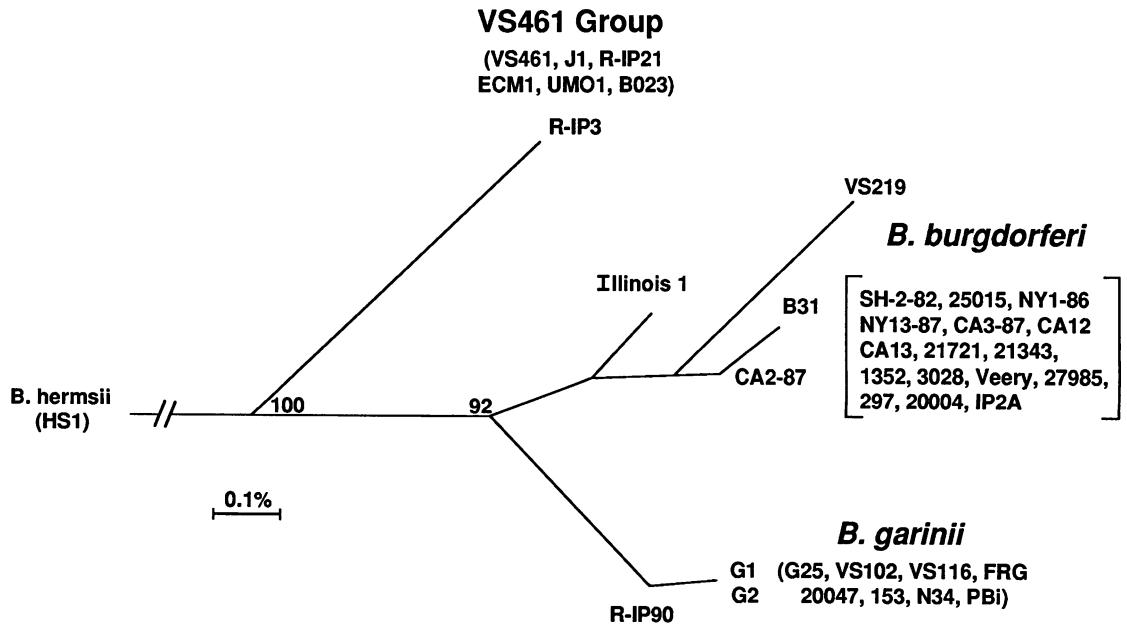

FIG. 1. Phylogenetic relationships of *Borrelia* isolates associated with Lyme disease. A phylogenetic tree was constructed by using masked sequences of 1,118 nucleotides as described in the text. The scale bar indicates a sequence similarity difference value of 0.1%. The species or genomic species affiliations of isolates, not incorporated into the tree, are indicated in parentheses or brackets, adjacent to the appropriate branches. The numbers at the major branch nodes indicate the results of bootstrap analyses. *B. hermsii* (HS1) served as the outgroup.

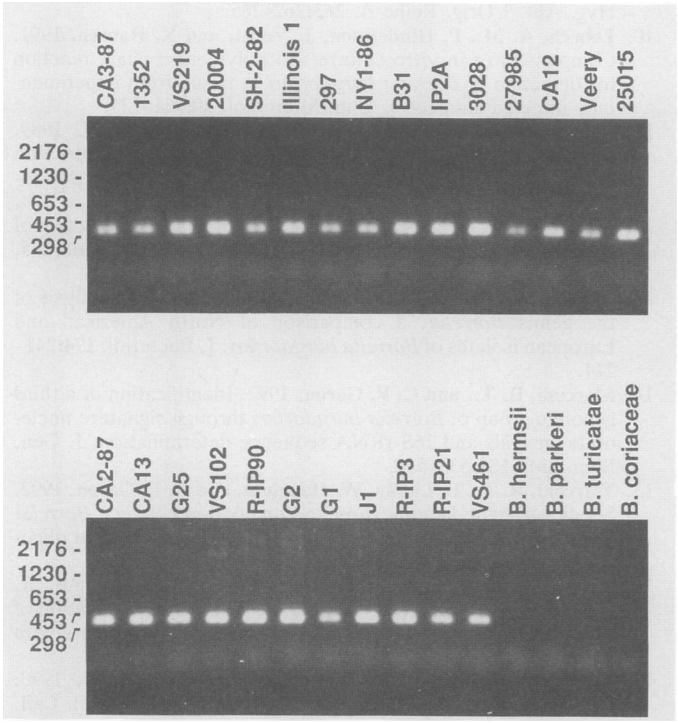

FIG. 2. Amplification with the LD primer set. Twenty-five amplification cycles were performed, and 10% of the reaction volume was analyzed by agarose gel electrophoresis in a 1.5% gel. All methods are described in the text.

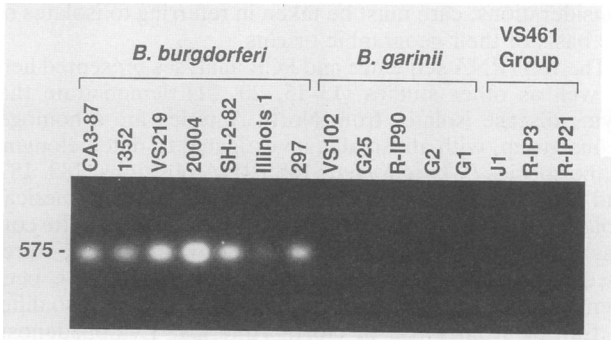

FIG. 3. Amplification specificity of the BB primer set with Lyme disease isolates belonging to each of the three species groups. The species group to which each isolate belongs is indicated above the panel. Twenty-five amplification cycles were performed, and samples were electrophoresed as described in the text.

Borrelia parkeri and *Borrelia turicatae* DNA was observed with the VS461 primer set. However, since *B. parkeri* and *B. turicatae* were not amplified by the LD set, they are easily identified as non-Lyme disease-causing organisms.

The species-specific primer sets described here type all Lyme disease isolates tested into one of the three recognized species groups. The detection sensitivity of the species-specific primer sets was equivalent to that of the LD set and consistent with that previously reported by others (8, 10, 22). Figure 3 depicts an ethidium bromide-stained gel in which representative isolates of the three genomic species were tested for amplification with the BB primer set. Isolate Illinois 1 was amplified to a lesser degree than other isolates that were amplification positive. However, sequence analysis of the primer target sites indicated that it was of the same sequence as other isolates belonging to the species *B. burgdorferi*, and subsequent PCR experiments have demonstrated that it is amplified to a level equivalent to that of other isolates. The BG and VS461 primer sets were also found to amplify in a species-specific fashion. Cross-amplification between species with the species-specific primers did not occur even when a large input (0.7 μg) of template DNA was used. The species identities inferred from use of these primer sets are consistent with those inferred from 16S rRNA sequence analysis (13, 14), multilocus enzyme electrophoresis (6), or DNA-DNA hybridization studies (3, 20). Other primer sets directed against an uncharacterized chromosomal locus which type isolates into two subgroups have been described elsewhere (21). However, those primers did not differentiate between *B. garinii* and VS461 isolates. The three-species grouping scheme is also supported by multilocus enzyme electrophoresis studies (6) and DNA-DNA hybridization studies (3, 20). Phylogenetic analyses have demonstrated that *B. garinii* and group VS461 are no more closely related to each other than *B. burgdorferi* is to either of these species (14).

TABLE 4. Summary of PCR amplification results

Sources of template DNA	Result with the following PCR primer sets:			
	LD	BB	BG	VS461
B31, SH-2-82, 25015, NY1-86, NY13-87, CA2-87, CA3-87, CA12, CA13, 21721, Illinois 1, 21343, 1352, 3028, Veery, 27985, 297, 20004, IP2A, VS219	+	+	−	−
R-IP90, G25, VS102, G2, G1, FRG, VS116, 20047, N34, PBi, 153	+	−	+	−
R-IP21, R-IP3, J1, VS461, ECM1, B023, UM01	+	−	−	+
B. hermsii (HS1, YOR), *B. anserina*, *B. coriaceae*	−	−	−	−
B. parkeri, *B. turicatae*	−	−	−	+/−

The separation of and distinction between *B. garinii* and group VS461 is also supported by the restriction fragment length polymorphism patterns of the rRNA genes. We determined the *Eco*RV restriction fragment length polymorphism patterns of the rRNA genes by using nick-translated pKK3535, which carries the *rrnB* operon from *Escherichia coli*, as a probe. Isolates belonging to the VS461 group were found to exhibit a unique pattern distinct from those observed for *B. burgdorferi* and *B. garinii* (13, 20); the former have hybridizable bands of 6, 3, and 0.85 kbp (data not shown), providing further evidence for the existence of three distinct species. Hence, although the studies of Rosa et al. (21) were important in that they demonstrated the heterogeneous genomic makeup of the Lyme disease spirochetes, the primers described are not suitable for the differentiation of species.

PCR primers which distinguish European from North American Lyme disease isolates have also been described elsewhere (16). Since all three of the species associated with Lyme disease have been isolated in Europe, a geographic distinction between isolates is relevant only in regard to North America. The results of typing studies are supportive of this conclusion (3, 6, 13–15, 20, 21). In light of these

considerations, care must be taken in referring to isolates on the basis of their geographic origins.

The 16S rRNA sequence and PCR analyses presented here as well as other studies (13–15, 20, 21) demonstrate that Lyme disease isolates from North America are a homogeneous group, with all isolates investigated to date belonging to the species *B. burgdorferi*. 16S rRNA positions (183, 187, and 649), which are highly conserved in North American isolates, are less conserved in European isolates. In contrast, European isolates belonging to each of the three species *B. burgdorferi*, *B. garinii*, and VS461 have been identified. The clinical symptoms of Lyme disease also differ in Europe from those in North America. Lymphadenosis benigna cutis and acrodermatitis chronica atrophicans are common in Europe (2, 26) yet rare in the United States (9). Arthritic complications, on the other hand, are more common in North America than in Europe (24). The clinical symptoms in North America appear to be a subset of those observed in Europe (25). It is conceivable that the specific manifestations in Europe may be related to infection with *B. garinii* or VS461 isolates, i.e., a Europe-specific species. The PCR primer sets presented here may prove useful in assessing whether such a correlation exists.

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