Characterization of *Borrelia burgdorferi* strains isolated from Korea by 16S rDNA sequence analysis and PCR-RFLP analysis of *rrf* (5S)–*rrl* (23S) intergenic spacer amplicons

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Haenam strains of *Borrelia burgdorferi*, which had been isolated from *Ixodes granulatus* and *Apodemus agrarius* in Haenam, Korea, were characterized by PCR-RFLP analysis of *rrf* (5S)–*rrl* (23S) intergenic spacer amplicons and by sequence analysis of the 16S rRNA gene (rDNA). The *Mse*I and *Dra*I restriction patterns of the 5S–23S intergenic spacer amplicons of Haenam strains differed from those of other *B. burgdorferi sensu lato* strains. Furthermore, in the phylogenetic tree based on the 16S rDNA sequences, Haenam strains formed a distinctive cluster, clearly separated from the other members of *B. burgdorferi sensu lato*. These results suggest that, apart from *Borrelia garinii* and *Borrelia afzelii*, other genotypes of *B. burgdorferi sensu lato* exist in Korea and the Haenam strain is a newly identified one.

Keywords: *Borrelia burgdorferi*, 16S rDNA, *rrf* (5S)–*rrl* (23S) intergenic spacer gene, sequence analysis

INTRODUCTION

Borrelia burgdorferi, the causative spirochaete of Lyme disease, is transmitted to human and animals by *Ixodes* ticks. Lyme borreliosis now occurs in all parts of the world and is considered to be one of the most prevalent tick-borne infectious diseases in Europe and North America (Steere, 1989). Many Lyme disease spirochaetes have also been isolated in Korea, Japan, China and Russia (Park *et al.*, 1993; Anderson, 1989). Some of them have been classified as new species in the genus *Borrelia* (Kawabata *et al.*, 1993; Postic *et al.*, 1993; Fukunaga *et al.*, 1996).

Several methods have been used to characterize and identify the causative agents of Lyme disease. These are: protein analysis using monoclonal antibodies (Barbour *et al.*, 1985; Canica *et al.*, 1993), multilocus enzyme electrophoresis (Boerlin *et al.*, 1992), plasmid profiles (Barbour, 1988), 16S rDNA sequence analysis (Marconi & Garon, 1992) and PCR-RFLP analysis of 5S–23S intergenic spacer amplicons (Postic *et al.*, 1994). Using these methods, *B. burgdorferi sensu lato* is currently divided into 10 species. These are: *B. burgdorferi* (Johnson *et al.*, 1984), *Borrelia afzelii* (Canica *et al.*, 1993), *Borrelia garinii* (Baranton *et al.*, 1992), *Borrelia japonica* (Kawabata *et al.*, 1993), *Borrelia valaisiana* (Wang *et al.*, 1997), *Borrelia lusitaniae* (Le Fleche *et al.*, 1997), ‘*Borrelia andersonii*’ (Marconi *et al.*, 1995), *Borrelia turdi* and *Borrelia tanukii* (Fukunaga *et al.*, 1996) and ‘*Borrelia bissettii*’ (Postic *et al.*, 1998).

Recently we isolated two *Borrelia* strains from *Ixodes granulatus*, which is a rare species as tick vector for Lyme spirochaetes, and eight strains from *Apodemus agrarius* in the Haenam area, Korea. The PCR-RFLP

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Abbreviation: UPGMA, unweighted pair group method with arithmetic averages.

The GenBank accession numbers for the 5S–23S intergenic spacer sequences and 16S rDNA sequences of the Haenam strains are given in the text.

patterns of their 5S–23S intergenic spacer genes differed from those of the other Korean isolates which had been simultaneously isolated and identified as *B. afzelii* and *B. garinii*. The RFLP patterns of the genomic DNA probed by using the 23S rRNA gene (*NheI*–*PstI* and *StyI* fragments) were also different (Park *et al.*, 1998). In the present study, both PCR-RFLP analysis of 5S–23S intergenic spacer amplicons and comparative sequence analysis of 16S rDNA were performed for Haenam strains to determine their relationships with known species of the genus *Borrelia*. We also developed a PCR method for the rapid identification of the Haenam strains based on the 16S rDNA sequences.

METHODS

Bacterial strains and DNA extraction. Ten Haenam strains (HN-6, HN-8, HN-11, HN-12, HN-13, HN-14, HN-15, HN-16, HN-18 and HN-19) were used for the characterization. Two strains (HN-6 and HN-8) were isolated from *I. granulatus* and the others were isolated from *A. agrarius*. Three reference strains (*B. burgdorferi* B31^T, *B. garinii* IP89 and *B. afzelii* VS461^T) were used for the PCR-RFLP analysis of the 5S–23S intergenic spacer amplicon as a direct comparison with the Haenam strains. Also, nine reference strains (*B. burgdorferi* B31^T, *B. garinii* IP89, *B. afzelii* VS461^T, *B. japonica* HO14^T, *B. valaisiana* VS116^T, *B. lusitaniae* PotiB2^T, '*B. bissettii*' DN127^T, '*B. andersonii*' 21123 and *B. hermsii* HS1) were used for the specific PCR identification of Haenam strains. These spirochaetes were cultivated at 32 °C in BSKII medium (Barbour, 1984). DNA was extracted by a modification of the method previously described by Barbour & Garon (1987). Briefly, a pellet from a 5 ml culture was suspended in 650 µl TE (10 mM Tris pH 8.0, 100 mM EDTA). RNase (10 µl, 10 mg ml⁻¹) and lysozyme (20 µl, 10 mg ml⁻¹) were added to the suspension. After 30 min incubation at 37 °C, SDS (0.5%) was added and the suspension further incubated at 65 °C for 10 min. The DNA was extracted four times with equal volumes of phenol and once with an equal volume of chloroform. The DNA was precipitated by adding 0.1 vol. 3 M sodium acetate and 2 vols absolute ethanol, washed with 70% ethanol and resuspended in TE pH 8.0.

PCR-RFLP analysis of the 5S–23S intergenic spacer. PCR was performed by using a specific primer set (forward, 5'-CTGCGAGTTCGCGGGAGA-3'; and reverse, 5'-TCC-TAGGCATTACCATTA-3') to amplify 254 bp DNA as described by Postic *et al.* (1994). Template DNA (50 ng) and 20 pmol of each primer were added to a PCR mixture tube (AccuPower PCR PreMix; Bioneer), which contained 1 U *Taq* DNA polymerase, 250 µM of each dNTP, 50 mM Tris/HCl pH 8.3, 40 mM KCl, 1.5 mM MgCl₂ and gel loading dye. The volume was then adjusted to 20 µl with distilled water. The reaction mixture was subjected to 30 cycles of amplification (1 min at 94 °C, 1 min at 52 °C and 2 min at 72 °C) followed by 5 min extension at 72 °C (Perkin-Elmer Cetus, model 9600 thermocycler). Endonucleases *MseI* and *DraI* (Promega) were used to cleave the PCR products as recommended by the manufacturer. The restriction fragments were electrophoresed on a 16% acrylamide gel for 3 h at 100 V.

Nucleotide sequencing of the 5S–23S intergenic spacer gene. The PCR products were electrophoresed on a 2% agarose

Fig. 1. Primers (B1–B6) used for the amplification of 16S rDNA in this study. H1 and H2 are specific primers for Haenam strains.

gel and purified with a QIAEX II Gel Extraction kit (Qiagen). Purified PCR product (20 ng) was ligated with 50 ng pGEM-T vector (Promega) at 16 °C for 18 h and used for the transformation of *Escherichia coli* XL-1 Blue. Recombinant DNA was extracted by using a High Pure Plasmid Isolation kit (Boehringer Mannheim). The purified DNA was sequenced using an Applied Biosystems 373A automatic sequencer and BigDye Terminator Cycle Sequencing kit (PE Applied Biosystems) with forward and reverse sequencing primer (M13) (New England Biolabs). At least two clones were sequenced. For the sequencing reaction, 500 ng purified DNA, 3.2 pmol primer and 8 µl BigDye Terminator RR mix (PE Applied Biosystems) were mixed, and the final volume adjusted to 20 µl by adding distilled water. The reaction was performed using 5% (v/v) DMSO for 30 cycles of 15 s at 95 °C, 10 s at 50 °C and 4 min at 60 °C.

Nucleotide sequencing of the 16S rRNA gene (rDNA). A primer set [B1 (5'-CAGTGCCTTAAAGCATGC-3'; position 40–58 in *B. burgdorferi* B31^T) and B6 (5'-CAACC-ATGCAGCACCTGTA-3'; 1053–1035)] was used to amplify 16S rDNA (1013 bp). Target DNAs were amplified in a 20 µl PCR mixture tube (AccuPower PCR PreMix; Bioneer). Template DNA (50 ng) and 20 pmol of each primer were added and adjusted to a volume of 20 µl with distilled water. The reaction mixture was subjected to 30 cycles of amplification (30 s at 95 °C, 30 s at 58 °C and 45 s at 72 °C), followed by 5 min extension at 72 °C. Cloning and sequencing were carried out by the same method as above except that the sequencing primers used were: B1, B2 (5'-CGACCTTCTTCATTACGC-3'; 416–398), B3 (5'-GC-AGCTAAGAATCTTCCGCAATGG-3'; 350–373), B4 (5'-AAGTTCGCCTTCGCCTCCGGTA-3'; 735–714), B5 (5'-TGTAAGGGTGAATCTGTTG-3'; 681–700) and B6 (Fig. 1).

Sequence alignment and the phylogenetic tree. Sequences of 16S rDNA were aligned using the multiple alignment algorithm in the MegAlign package (Windows version 3.12e; DNASTAR). All positions with alignment gaps were excluded from the pairwise sequence comparison. A phylogenetic tree was constructed using MEGA software (Kumar *et al.*, 1993). UPGMA (unweighted pair group method with arithmetic averages) and neighbour-joining trees were created based on the Jukes–Cantor distance estimation method and pairwise deletion. Bootstrap analysis was performed with 100 replications.

Nucleotide sequence accession numbers. The GenBank accession numbers of the 5S–23S intergenic spacer sequences of the Haenam strains are AF058705 (HN-6) and AF058706 (HN-8). The accession numbers of the 16S rDNA sequences are AF055416 (HN-6), and AF055418–AF055421 (HN-8, HN-14, HN-19 and HN-13, respectively). To compare the members of *B. burgdorferi sensu lato*, the 16S rDNA sequences of reference strains available from GenBank were compared with those of the Haenam strains. These were: *B.*

Fig. 2. *MseI* (a) and *DraI* (b) restriction patterns of the amplified 5S–23S intergenic spacer DNA fragments from Haenam strains and three *B. burgdorferi sensu lato* strains (*B. burgdorferi* B31^T, *B. garinii* IP89 and *B. afzelii* VS461^T). The restriction patterns of the Haenam strains differ from those of the reference strains. Lanes: M₁, marker DNA (25 bp DNA ladder); 1, *B. burgdorferi* B31^T; 2, *B. garinii* IP89; 3, *B. afzelii* VS461^T; 4, HN-6; 5, HN-8; 6, HN-11; 7, HN-12; 8, HN-13; 9, HN-14; 10, HN-15; 11, HN-16; 12, HN-18; 13, HN-19; M₂, marker DNA (10 bp DNA ladder).

burgdorferi B31^T (U03396), *B. burgdorferi* 297 (X85204), *B. garinii* 20047^T (D67018), *B. garinii* PBi (X85199), *B. afzelii* VS461^T (U78151), *B. afzelii* HT61 (D67019), *B. japonica* HO14^T (L40597), ‘*B. bissetti*’ DN127^T (L40596), ‘*B. andersonii*’ 21038^T (L46701), *B. turdi* Ya501 (D67022), *B. turdi* Ac502 (D67024), *B. tanukii* Hk501 (D67023), *B. tanukii* OR2eL (D67020), *B. lusitaniae* PotiB1 (X98226), *B. lusitaniae* PotiB2^T (X98228), *B. lusitaniae* PotiB3 (X98229), *B. valaisiana* VS116^T (X98232), *B. valaisiana* UK (X98233), *B. valaisiana* Am501 (D67021), *B. hermsii* HS1 (M60968) and finally *B. anserina* ES-1 (U42284). The 5S–23S intergenic spacer sequences of reference strains available from GenBank were also used for comparison purposes. These were: *B. burgdorferi* B31^T (L30127), *B. garinii* 20047^T (L30119), *B. garinii* NT29 (L30130), *B. afzelii* VS461^T (L30135), *B. japonica* HO14^T (L30128), *B. bissetti* DN127^T (L30126), *B. bissetti* CA55 (L30124), *B. bissetti* 25015 (L30122), ‘*B. andersonii*’ 21133 (L30120), *B. turdi* Ya501 (D84407), *B. tanukii* Hk501 (D84404), *B. lusitaniae* PotiB2^T (L30131), *B. lusitaniae* PotiB3 (L30132), *B. valaisiana* VS116^T (L30134), *B. valaisiana* UK (L30133) and *B. valaisiana* Am501 (D84402).

PCR for the identification of Haenam strains. The 16S rDNAs (305 bp) were amplified with the newly designed specific primers for Haenam strains [H1 (5′-GTCACACTGGAACTGAGAC-3′; position 306–324 in *B. burgdorferi* B31^T) and H2 (5′-GCTATTTTATGCATAGACTTAC-3′;

610–589)] (Fig. 1) in a 20 µl PCR mixture tube (AccuPower PCR PreMix; Bioneer). Template DNA (50 ng) and 20 pmol of each primer were added and the volume adjusted to 20 µl. The reaction mixture was subjected to 25 cycles of amplification (20 s at 94 °C, 15 s at 59 °C and 30 s at 72 °C), followed by 5 min extension at 72 °C.

RESULTS

PCR-RFLP analysis and sequence analysis of the 5S–23S intergenic spacer amplicon

The 254 bp fragment of 5S–23S intergenic spacer genes was amplified from the Haenam strains and cleaved by two different restriction enzymes, *MseI* and *DraI*. The *MseI* restriction pattern (150, 58, 24 and 22 bp, Fig. 2a) and *DraI* restriction pattern (173 and 81 bp, Fig. 2b) of Haenam strains were identical, but differed from those of previously reported *B. burgdorferi sensu lato* strains (Postic *et al.*, 1994, 1998; Masuzawa *et al.*, 1996) (Table 1).

The nucleotide sequences of amplified 5S–23S intergenic spacer DNAs (254 bp) from Haenam strains were determined and compared to those of other *B. burgdorferi sensu lato* strains. The sequence similarity of the Haenam strains ranged from 99.6 to 100 % (data

Table 1. *DraI* and *MseI* restriction fragments of 5S–23S intergenic spacer amplicons

Strain	Amplicon size (bp)	Restriction fragment sizes (bp) with*	
		<i>DraI</i>	<i>MseI</i>
<i>B. burgdorferi</i> B31 ^T	254	144, 53, 29, 28	108, 51, 38, 29, 28
<i>B. garinii</i> 20047 ^T	253	201, 52	108, 95, 50
NT29	253	144, 57, 52	108, 57, 50, 38
<i>B. afzelii</i> VS461 ^T	246	174, 52, 20	108, 68, 50, 20
<i>B. japonica</i> HO14 ^T	236	No restriction site	108, 78, 50
' <i>B. andersonii</i> ' 21133	266	No restriction site	120, 67, 51, 28
' <i>B. bissettii</i> ' DN127 ^T	257	144, 53, 33, 27	107, 52, 38, 33, 27
CA55	226	144, 53, 29	107, 52, 38, 29
25015	253	173, 53, 27	107, 52, 34, 27, 17, 12, 4
<i>B. turdi</i> Ya501	248	144, 81, 23	107, 51, 38, 21, 16, 8, 7
<i>B. tanukii</i> Hk501	245	173, 52, 20	174, 51, 20
<i>B. valaisiana</i> VS116 ^T	255	203, 52	175, 50, 23, 7
<i>B. lusitaniae</i> PotiB1, PotiB2 ^T	257	145, 83, 29	108, 81, 39, 29
PotiB3	255	146, 109	108, 79, 52, 16
Haenam strains			
HN-6	254	173, 81	150, 58, 24, 22
HN-8	254	173, 81	150, 58, 24, 22
HN-11	254	173, 81	150, 58, 24, 22
HN-12	254	173, 81	150, 58, 24, 22
HN-13	254	173, 81	150, 58, 24, 22
HN-14	254	173, 81	150, 58, 24, 22
HN-15	254	173, 81	150, 58, 24, 22
HN-16	254	173, 81	150, 58, 24, 22
HN-18	254	173, 81	150, 58, 24, 22
HN-19	254	173, 81	150, 58, 24, 22

* Exact restriction fragment sizes of both Haenam strains and reference strains (Postic *et al.*, 1994, 1998; Masuzawa *et al.*, 1996) were determined from the sequences.

not shown). The sequences of HN-6, HN-14 and HN-19 were identical. The sequences of HN-8, HN-11, HN-12, HN-13, HN-15, HN-16 and HN-18 strains were also identical and differed by only one nucleotide from the former sequence (HN-6 strain). The sequences of Haenam strains were compared to those of 12 reference strains from GenBank. The sequence similarities of Haenam strains to other strains of *B. burgdorferi sensu lato* were 70.1–88.2% (data not shown). Haenam strains showed relatively high similarity (87.8 and 88.2%) with *B. burgdorferi* B31^T. The sequence similarities of the 5S–23S intergenic spacer sequences of Haenam strains to *B. valaisiana*, *B. lusitaniae* and *B. tanukii* were 71.0–85.8%, 70.1–70.5% and 80.4–80.8%, respectively.

16S rDNA sequence analysis

The 1013 bp fragment of 16S rDNA from five Haenam strains (HN-6, HN-8, HN-13, HN-14 and HN-19) was amplified and characterized. The sequences were

aligned and compared with those of 21 *Borrelia* reference strains by using the multiple alignment algorithm in the MegAlign package (Windows version 3.12e; DNASTAR). Sequence similarity of the Haenam strains ranged from 99.5 to 100%. In general, they showed 97.6–99.5% similarity with 19 reference strains of *B. burgdorferi sensu lato*. A particularly high level of similarity of 16S rDNA sequences was observed in *B. valaisiana* (99.0–99.5%), *B. tanukii* (98.9–99.4%) and *B. lusitaniae* (98.7–99.1%). *B. valaisiana* was found to be similar to *B. tanukii* (99.0–99.6%).

A phylogenetic tree was constructed by the UPGMA and neighbour-joining methods. In the phylogenetic tree created by the UPGMA method, Haenam strains formed a distinctive cluster, separated from other strains of *B. burgdorferi sensu lato*. Haenam strains were closer to *B. valaisiana* and *B. tanukii* than to *B. afzelii* and *B. garinii*, which had been previously isolated and reported in Korea (Park *et al.*, 1993) (Fig.

Fig. 3. Phylogenetic tree of *B. burgdorferi sensu lato* strains. The tree was constructed by the UPGMA method using MEGA software. ●, Haenam strains. Bootstrap analysis was performed with 100 replications. The scale bar represents 0.5% divergence.

3). In the phylogenetic tree created by the neighbour-joining method, Haenam strains again formed a distinctive cluster separated from other strains of *B. burgdorferi sensu lato* (data not shown).

Identification of Haenam strains by PCR

Three signature nucleotides for Haenam strains in the sequences of 16S rDNA were found. These are C324,

G589 and T836 (numbering of *B. burgdorferi* B31^T). These nucleotides differentiate Haenam strains from other strains of *B. burgdorferi sensu lato*. Among these nucleotides, C324 and G589 were used for the specific primers (H1 and H2) to amplify 305 bp DNA from Haenam strains. The targeted PCR products were observed only from the Haenam strains, suggesting that these primers can be used specifically to identify Haenam strains (Fig. 4).

Fig. 4. Amplification of 16S rDNA from *B. burgdorferi sensu lato* strains with the Haenam-specific primers (H1 and H2). The 305 bp DNAs were amplified only from the Haenam strains (lanes 1–5). Lanes: M, marker DNA (ϕ X174/RF DNA/HaeIII fragments); 1, HN-6; 2, HN-8; 3, HN-13; 4, HN-14; 5, HN-19; 6, *B. burgdorferi* B31^T; 7, *B. garinii* IP89; 8, *B. afzelii* VS461^T; 9, *B. japonica* HO14^T; 10, *B. valaisiana* VS116^T; 11, *B. lusitanae* PotiB2^T; 12, '*B. bissettii*' DN127^T; 13, *B. andersonii* 21123; 14, *B. hermsii* HS1.

DISCUSSION

According to previous reports, among the currently recognized species of the genus *Borrelia*, *B. afzelii* and *B. garinii* have been isolated and identified in Korea (Park *et al.*, 1993). *B. afzelii* and *B. garinii* were also found to be the principal *Borrelia* species in Russia, China and Japan (Sato *et al.*, 1996; Li *et al.*, 1998; Kawabata *et al.*, 1993; Postic *et al.*, 1993). Other species, such as *B. japonica*, *B. turdi* and *B. tanukii*, have been isolated in Japan (Fukunaga *et al.*, 1996; Kawabata *et al.*, 1993). It is therefore not surprising that *Borrelia* species other than *B. afzelii* and *B. garinii* were identified in Korea.

However, the Haenam strains showed several interesting characteristics, firstly concerning their tick vector. There is a relationship between *Borrelia* species and their vectors. For example, *B. japonica* is associated with *Ixodes ovatus*. *B. turdi* and *B. tanukii* were isolated from *Ixodes turdus* and *Ixodes tanuki* in Japan, and '*B. andersonii*' was associated with *Ixodes dentatus* in the US. *B. burgdorferi sensu lato* has so far been isolated from heart and kidney of *A. agrarius* and *Ixodes* ticks (*Ixodes nipponensis* and *Ixodes persulcatus*). Interestingly, Haenam strains were isolated from *I. granulatus* and *A. agrarius* in the Haenam area, Korea. *I. granulatus* is a rare species of tick vector for Lyme spirochaetes. Its relationship with *Borrelia* has previously been reported only in China (Zhang, 1992).

Secondly, these strains showed RFLP patterns using a 23S rRNA gene probe that differed from those of the other isolates, which had been differentiated and identified as *B. afzelii* and *B. garinii* (Park *et al.*, 1998). In this study, Haenam strains were further characterized by PCR-RFLP analysis of the 5S–23S gene, which is a simple, rapid diagnostic tool (Postic *et al.*, 1994). The *Mse*I and *Dra*I restriction patterns of Haenam strains described in this paper were markedly different from those of other *B. burgdorferi sensu lato*

strains reported by other investigators (Postic *et al.*, 1994, 1998; Masuzawa *et al.*, 1996) (Table 1).

To determine the topology of Haenam strains in the phylogenetic relationships among the *Borrelia* species, we also used 16S rDNA sequence analysis (Marconi & Garon, 1992; Le Fleche *et al.*, 1997; Wang *et al.*, 1997; Postic *et al.*, 1998). Haenam strains formed a distinctive cluster, separated from other strains of *B. burgdorferi sensu lato*. Haenam strains were closer to *B. valaisiana* and *B. tanukii* than to *B. afzelii* and *B. garinii*, which had been previously isolated and reported in Korea (Park *et al.*, 1993). Furthermore, signature nucleotides in the 16S rDNA sequences of Haenam strains enabled us to design specific primers for the rapid identification and differentiation of Haenam strains from other members of *B. burgdorferi sensu lato*. PCR, with the primers described, can be used for the study of the vector, the reservoir and the geographical distribution of the Haenam strains.

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