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PCR-Restriction Fragment Length Polymorphism Analysis of the *ospC* Gene for Detection of Mixed Culture and for Epidemiological Typing of *Borrelia burgdorferi* Sensu Stricto

TOSHIYUKI MASUZAWA,* TETSURO KOMIKADO, AND YASUTAKE YANAGIHARA

Department of Microbiology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422, Japan

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Restriction fragment length polymorphism (RFLP) analysis of the outer surface protein C (*ospC*) gene amplicon was used for rapid screening for genetic variability within *Borrelia burgdorferi* sensu stricto species and for detection of multiple borreliae in culture. Primers for the *ospC* gene amplified a fragment of about 600 bp from *Borrelia* cultures. After cleavage of the amplified products by *Mbo*I and *Dra*I, eight different RFLP types were found among 13 *B. burgdorferi* sensu stricto strains from various sources and geographical areas, and three RFLP types were found among 10 representative isolates from skin biopsy specimens taken from patients residing on the eastern end of Long Island, New York (B. W. Berger, R. C. Johnson, C. Kodner, and L. Coleman, J. Clin. Microbiol. 30:359–361, 1992). These results suggested that the DNA organization of *B. burgdorferi* sensu stricto is heterogeneous not only globally but also within a localized geographical area and that the *ospC*-based typing approach could differentiate the *B. burgdorferi* sensu stricto. From the results obtained using mixed cultures of two different RFLP types of *B. burgdorferi* sensu stricto, contamination of at least 0.5% of different types of *Borrelia* cells in culture could be detected. This method could detect a multiple-*B. burgdorferi* sensu stricto infection in the bladders of mice experimentally infected with two different RFLP type strains. The present study showed that RFLP analysis of *ospC*-PCR products is a reliable method for epidemiological typing of *B. burgdorferi* sensu stricto and could be used for rapid detection of mixed *Borrelia* culture and multiple *B. burgdorferi* sensu stricto infections in animals, ticks, and patients.

Lyme disease is a multisystemic disorder (7, 32) caused by infection with *Borrelia burgdorferi* sensu lato (18), which is transmitted by ticks of the *Ixodes ricinus* complex (1). Members of *B. burgdorferi* sensu lato are classified into five species, *B. burgdorferi* sensu stricto, isolated in North America and Europe; *B. garinii* (2) and *B. afzelii* (8), isolated in Europe; *B. japonica* (19), isolated from *I. ovatus* in Japan; and *B. andersonii*, isolated from *I. dentatus* in North America (25). Furthermore, new genomic groups have been recognized among isolates in North America and Europe (30) and Japan (12, 26).

The outer surface protein C (OspC) of *B. burgdorferi* sensu lato is highly heterogeneous with the sequence identities of the deduced amino acid sequences among different species, ranging between 62 and 80% in sequences (11, 17), and 33 distinct restriction fragment length polymorphism (RFLP) types were identified among 76 Lyme disease *Borrelia* strains (22). *B. burgdorferi* sensu stricto is relatively homologous in comparison with *B. garinii* with regard to genetic, phenotypic, and immunological properties (13, 14, 20, 27, 30, 34–37). However, nine *ospC*-RFLP types were found within *B. burgdorferi* sensu stricto, and heterogeneity of the *ospC* gene sequence among strains belonging to the same species was confirmed (22).

Various epidemiological typing tools for *B. burgdorferi* sensu lato have been developed, such as RFLP analysis targeted to the rRNA genes, rRNA gene intergenic spacers, and flagellin gene, in addition to RFLP analysis of genomic DNA and DNA hybridization methods and serotyping systems based on reactivity with monoclonal antibodies (2, 5, 13, 14, 20, 23, 24, 30, 31, 34, 36, 37). These methods are available for determination of species but not for differentiation of strains belonging to the same species. Plasmid profile analysis by pulsed-field gel electrophoresis is a useful epidemiological typing tool for differentiation of isolates at the interspecies level (4, 16). However, this method requires large-scale cultures, and a long time is required for sample preparation and pulsed-field gel electrophoresis. *Borrelia* species cultured from natural sources such as ticks and wild mammals often include multiple *Borrelia* isolates (15, 28, 29), and the mixed *Borrelia* culture greatly hampers further analysis of isolates.

In this study, we developed a method that allows rapid screening from genetic variability within a relatively homologous species, *B. burgdorferi* sensu stricto. Our method can detect the mixed *Borrelia* culture by RFLP analysis of *ospC* gene amplicon.

MATERIALS AND METHODS

Strains and cultivation. Table 1 shows the sources of strains of *B. burgdorferi* sensu stricto isolated from various geographical areas. Table 2 shows strains isolated from erythema migrans lesions of patients in Southampton, New York, an area in which Lyme disease is endemic in the United States (6). Passage numbers of strains used were less than 10, except for high-passage strain B31 (passage number unknown). Low-passage strains 297 and NCH-1 have been maintained by passage in ddY mice, and reisolates from bladders of infected mice were used for experimental infection. These uncloned *Borrelia* strains were cultivated at 32°C in Barbour-Stoenner-Kelly II medium (3).

PCR and RFLP analysis. Oligonucleotide primers CF1 (forward) 5′-AAGT GCGATATTAATGAC-3′ and CR2 (reverse) 5′-GATCTTTCTGCCACAACA G-3′ were chosen on the basis of the previously published *ospC* gene sequence (10) and amplified an approximately 600-base amplicon. A *Borrelia* sample precipitated from 500 μl of culture was resuspended with 100 μl of PBS and 50 μl of 1% Triton X-100 and subsequently boiled for 5 min. The PCR was set up with a final mixture containing each deoxynucleotide triphosphate at 200 μM, 1.5 mM $MgCl_2$, 10 mM Tris-HCl (pH 8.3), 0.01% (wt/vol) gelatin, and 2.5 U of *Taq* polymerase (Takara, Kyoto, Japan) as well as 0.4 μM each primer in a volume of 100 μl overlaid with light mineral oil. Aliquots of 5 μl of heated bacterial suspension were amplified by 30 cycles under the following conditions: denaturation at 92°C for 30 s, annealing at 41°C for 30 s, and extension at 72°C for 90 s.

* Corresponding author. Mailing address: Department of Microbiology, School of Pharmaceutical Sciences, University of Shizuoka, Shizuoka 422, Japan. Phone: (054)-264-5710. Fax: (054)-264-5715. E-mail: masuzawa@ys7.u-shizuoka-ken.ac.jp.

TABLE 1. OspC-RFLP patterns of *B. burgdorferi* sensu stricto isolated from various geographic areas

Strain	Source	Origin	CF1-CR2 product (bp)	Size of restriction fragments (bp) *Mbo*I and *Dra*I[b]
297	Human spinal fluid	Connecticut	600	510 + 90
ALA-4-10-88	*I. pacificus*	California	600	510 + 90
B31	*I. dammini*	New York	604[a]	266 + 146 + 142 + 50[a]
Buco-2-10-89	*I. pacificus*	California	604[a]	266 + 146 + 142 + 50[a]
TXGW	Human skin	Texas	604[a]	266 + 146 + 142 + 50[a]
HB19	Human blood	Connecticut	604[a]	253 + 149 + 142 + 50 + 10[a]
HUM-7-8-14	*I. pacificus*	California	600	250 + 150 + 90 + 52 + 50
MMI	White-footed mouse	Minnesota	600	260 + 210 + 90 + 52
MMT1	*I. dammini*	Minnesota	600	260 + 210 + 90 + 52
NCH-1	Human skin	Wisconsin	600	220 + 200 + 100 + 48 + 46
NEV-5-4-88	*I. pacificus*	California	600	220 + 200 + 100 + 48 + 46
SM-1-6-88	*I. pacificus*	California	600	260 + 195 + 100 + 48
SON-3-1-89	*I. pacificus*	California	600	230 + 205 + 145 + 30

[a] Product size and fragment size were estimated from previously published *ospC* sequence (11, 17, 22).
[b] Fragments were double cut.

*Mbo*I (Takara) and *Dra*I (Takara) were used to cleave the PCR amplicon according to the manufacturer's protocol. Electrophoresis was carried out in 8% polyacrylamide gels for 2 h at 100 V. pBR322 DNA-*Msp*I digest (New England Biolabs Inc., Beverly, Mass.) was used as a molecular mass marker.

Sensitivity test for detection of multiple *Borrelia* culture. Samples were made by mixing cultured cells (10^8 cells/ml) of strains B31 and 297 at ratios of 10:90 to 0.5:99.5. Cell numbers in cultures were determined by dark-field microscopy with a Petroff-Hauser chamber according to the counting method of Stoenner (33). The mixed culture samples were subject to the *ospC*-PCR-RFLP analysis.

Experimental infection with *Borrelia* species. Five-week-old C3H/HeN mice were purchased from SLC (Hamamatsu, Japan). Six mice were inoculated subcutaneously into the right and left hind footpad with 0.05 ml each of *B. burgdorferi* sensu stricto 297 and NCH-1 culture containing 5×10^4 cells, respectively. The other two mice of each group were inoculated with the same number of cells of strain 297 or NCH-1. At day 14 after inoculation, bladders were removed from mice and were frozen at −20°C. The frozen bladder in a 1.5-ml microtube was ground with a pestle (Micropistille; Eppendorf, Hamburg, Germany) and DNA was extracted with a Wizard Genomic DNA purification kit (Promega Co., Madison, Wisc.) according to the manufacturer's instructions. PCR and subsequent RFLP analysis were carried out using DNA extracted from the bladder as templates.

RESULTS AND DISCUSSION

We designed OspC primers to amplify a fragment of about 600 bp from *Borrelia* cultures. PCR amplification of the *ospC* gene generated a fragment of about 600 bp from all isolates tested (Table 1). *Mbo*I and *Dra*I were used for subsequent RFLP analysis. After cleavage by the enzymes, 13 *B. burgdorferi* sensu stricto isolates were classified into eight different RFLP patterns (Fig. 1 and Table 1). *ospC* sequences are highly heterogeneous, and sequence homology among strains belonging to *B. burgdorferi* sensu stricto, *B. afzelii*, and *B. garinii* ranges from 76.9 to 85.7%, 83.5 to 91.2%, and 80.7 to 99.0%, respectively (22). Isolates belonging to *B. burgdorferi* sensu stricto showed at least nine patterns on RFLP analysis of the *ospC* gene amplicon (22). The sizes of fragments generated were in good agreement with the expected fragment sizes from the previously reported *ospC* gene sequence (11, 17, 22). These results suggested that RFLP analysis of the *ospC* gene amplicon can be applied for further classification and differentiation of isolates belonging to *B. burgdorferi* sensu stricto. However, there were no correlations between RFLP pattern, source of organism, and geographic origin of source. Liveris et al. (21) developed a molecular typing method by PCR-RFLP analysis

TABLE 2. OspC-RFLP patterns of *B. burgdorferi* sensu stricto isolated from patients in Southampton, New York

Patient no.	CF1-CR2 products (bp)	Size of restriction fragments (bp) *Mbo*I and *Dra*I[a]	RFLP type
18	600	260 + 210 + 90 + 50	MMI
2	600	510 + 90	297
6	600	510 + 90	297
7	600	510 + 90	297
8	600	510 + 90	297
10	600	510 + 90	297
17	600	266 + 146 + 142 + 50	B31
19	600	266 + 146 + 142 + 50	B31
21	600	266 + 146 + 142 + 50	B31
22	600	266 + 146 + 142 + 50	B31

[a] Fragments were double cut.

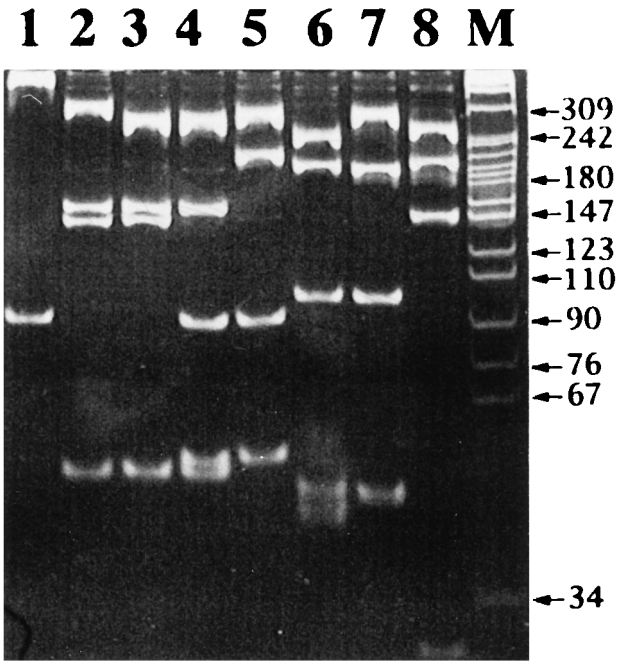

FIG. 1. PCR-RFLP analysis of eight representative types observed among 13 isolates of *B. burgdorferi* sensu stricto from various geographical areas. PCR amplification was carried out with *ospC*-specific primers, CF1 and CR2, and products were digested with *Mbo*I and *Dra*I. Lanes: 1, 297; 2, B31; 3, HB19; 4, HUM; 5, MMI; 6, NCH-1; 7, SM; 8, SON. Molecular size markers (in base pairs) are indicated on the right.

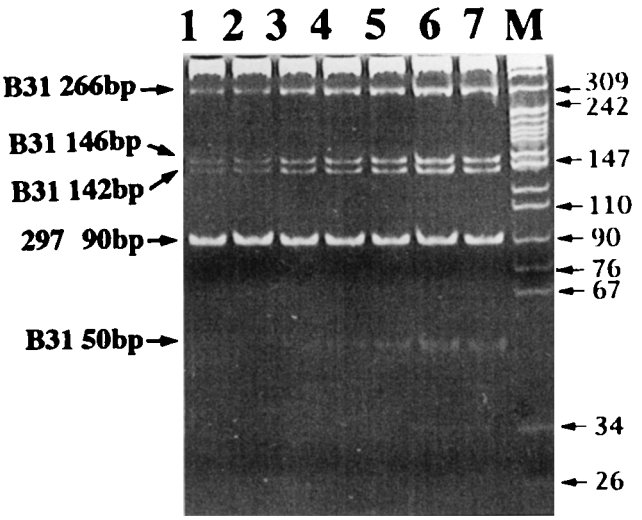

FIG. 2. Detection of multiple *B. burgdorferi* sensu stricto cultures by PCR-RFLP analysis. Lanes: 1, mixture of strains B31 and 297 at a ratio of 0.5:99.5; 2, 1:99; 3, 2:98; 4, 3:97; 5, 4:96; 6, 5:95; 7, 10:90. The sizes of the fragments are indicated on the left, and molecular size markers (in base pairs) are on the right.

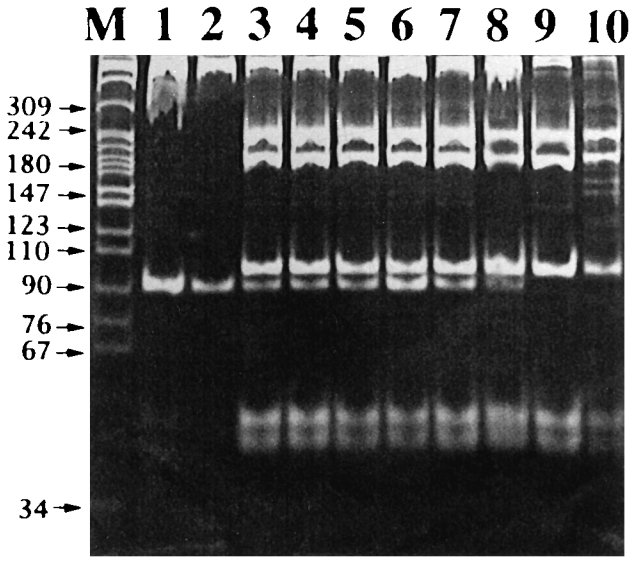

FIG. 3. Demonstration of multiple *B. burgdorferi* sensu stricto infections with two strains, 297 and NCH-1, by PCR-RFLP analysis. DNA extracted from the bladders of mice infected with *Borrelia* species was used as template. Lanes 1 and 2, DNA from mice infected with strain 297 alone; 3 to 8, DNA from mice coinfected with strain 297 and NCH-1; 9 and 10, DNA from mice infected with strain NCH-1 alone. Molecular size markers (in base pairs) are indicated on the left.

of 16S-23S rRNA genes, and 93 *B. burgdorferi* sensu stricto isolates could be classified by this method into three distinct RFLP types. On the other hand, eight different RFLP types of *ospC* amplicon were found among 13 strains of *B. burgdorferi* sensu stricto. These findings suggested that RFLP analysis of the *ospC* gene was superior to the PCR-RFLP method applied to 16S-23S rRNA spacer for typing capacity. Since 8 and 14 RFLP types were found in *B. afzelii* and *B. garinii*, respectively (22), this RFLP typing method might be applicable to strains belonging to *B. afzelii* and *B. garinii.*

Table 2 shows RFLP results for isolates from skin biopsy specimens taken from patients residing on the eastern end of Long Island, New York (3). We found three RFLP types designated MMI type, 297 type, and B31 type, among 10 representative patient isolates. Hughes et al. reported that 31 *Borrelia* isolates from 19 patients were classified into three groups based on the plasmid profiles. This suggested that DNA organization of *B. burgdorferi* sensu stricto is heterogeneous within a localized geographical area (15a). The *ospC* gene-based typing approach could differentiate the *B. burgdorferi* sensu stricto as well as plasmid profile analysis. However, there was no relationship between typing results based on *ospC*-RFLP and plasmid profiles (15a).

We applied our method to cultures containing multiple *Borrelia* species. As shown in Fig. 2, 270-, 150-, and 145-bp fragments derived from *ospC* amplicon of strain B31 and a 90-bp fragment derived from strain 297 were detected in all samples prepared. This result indicated that at least 0.5% of different types of *Borrelia* cells (approximately 5×10^5 cells/ml) as contaminants in culture (approximately 10^8 cells/ml) could be detected by this method. Furthermore, to evaluate the usefulness of the method, we attempt to detect multiple-species infections in mice. Figure 3 shows the results of PCR-RFLP using DNA extracted from the bladders of mice experimentally infected with *Borrelia* species as template. DNA from mice coinfected with strains 297 and NCH-1 showed fragments of 220, 200, 100, 48 and 46 bp originating from strain NCH-1 and 90 bp originated from strain 297 (lanes 3 to 8). On the other hand, DNA from mice infected with strain 297 or NCH-1 alone showed only one RFLP pattern corresponding to each *Borrelia* strain (297, lanes 1 and 2; NCH-1, lanes 9 and 10). Simultaneous infection of *I. ricinus* ticks, reservoir mice, and humans with two distinct *B. burgdorferi* sensu lato species has been reported (9, 28, 29). Although various molecular biological methods can detect multiple-species infections in reservoir animals, vector ticks, and patients, this method seems to be superior in its simplicity, high sensitivity, and high typing capacity. Although the genetic exchange is proposed to be mediated by lateral transfer of *ospC* sequence from the evidence of high levels of *ospC* gene diversity (17, 22), it is not clear whether such recombination events play a major role in the evolution of the considerable heterogeneity of OspC. If the high level of OspC diversity helps the borreliae escape host immune responses, the greater heterogeneity of OspC implies that a recombinant OspC vaccine preparation containing multiple antigens may be necessary.

The present study showed that RFLP of *ospC*-PCR products is a reliable epidemiological typing tool for *B. burgdorferi* sensu stricto. This method could also be used for rapid detection of mixed *Borrelia* cultures and multiple-species infections in mice and seems to be applicable to both ticks and clinical specimens.

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