Lyme Disease *Borrelia* Species in Northeastern China Resemble Those Isolated from Far Eastern Russia and Japan

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Fifty-nine *Borrelia burgdorferi* sensu lato culture isolates collected from northeastern China were characterized by 5S-23S rRNA intergenic spacer restriction fragment length polymorphism (RFLP) analysis and reactivity with monoclonal antibodies (MAbs). Among 59 culture isolates, 30 (50.8%) were *Borrelia garinii* and 17 (28.8%) were *Borrelia afzelii*. 2 were mixtures composed of *B. garinii* with RFLP pattern B and *B. garinii* with pattern C, and 9 were mixtures composed of *B. garinii* and *B. afzelii*. One isolate, ChY13p, produced a unique pattern and was identified as *B. garinii* based on analyses of 16S rRNA gene sequence, flagellin PCR-RFLP typing, and MAb reactivities. No *Borrelia burgdorferi sensu stricto* isolates were obtained from China. The results indicate that Lyme disease *Borrelia* species in northeastern China resemble those of *Borrelia* isolates from far eastern Russia and Japan.

Lyme disease is a multisystemic disorder caused by infection with *Borrelia burgdorferi* sensu lato, which is transmitted by ticks of the *Ixodes ricinus* complex (1, 15). Since the etiologic agent was first isolated from *Ixodes scapularis* in 1982 (6), a large number of *B. burgdorferi* sensu lato isolates have been obtained from patients, animal reservoirs, and vector ticks from various geographic areas of the world (2, 15, 26, 36). Genetically and immunologically, *B. burgdorferi* sensu lato, originally regarded as a single species (16), can be subdivided into nine species based on the reference methods for delineation of bacterial species (3, 7, 8, 10, 17, 19, 28, 34): *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, *B. japonica*, *B. andersonii*, *B. turdi*, *B. valaisiana*, and *B. lusitaniae*. The divergence within *B. burgdorferi* sensu lato may correlate with epidemiological and clinical features of Lyme disease (2, 31, 32). *B. burgdorferi* sensu stricto is present in North America and Europe but seems to be absent in Asia (22, 26, 30). Moreover, *B. burgdorferi* sensu stricto, found in the United States and Europe, is mainly associated with arthritic forms of Lyme disease. *B. garinii* and *B. afzelii* are present in Europe and Asia: the former is frequently associated with neurological manifestations, and the latter seems to be the exclusive agent of Lyme disease is also widespread in China, with endemic foci of the disease discovered and typical cases diagnosed in 31 provinces as well as the suburbs of Beijing (37). Many Lyme *Borrelia* species have been isolated in China, but few species determination studies have been published. We conducted a survey in northeastern China in May 1996. Fifty-nine *Borrelia* culture isolates were obtained from *Ixodes persulcatus* ticks and *A. penumis penumis* rodents. Here we report the genetic characteristics and species identification of these Chinese culture isolates by RFLP analysis and sequence analysis of 5S-23S rRNA intergenic spacer, 16S rRNA sequence analysis, flagellin molecular typing, and reactivity with monoclonal antibodies (MAbs).

One hundred twenty-seven *I. persulcatus* ticks were collected by beating vegetation and two *A. penumis* rodents were captured by snap traps in six different areas of Yakeshi in northeastern China from the end of May 1996 to the beginning of June 1996. The midgut of each tick and the earlobe of each rodent were inoculated into BSKII medium and cultured at 31°C for 4 weeks as previously described (4, 25). Fifty-seven *Borrelia* culture isolates obtained from the ticks were designated ChY01p to ChY57p, and two *Borrelia* culture isolates obtained from the rodents were designated ChYAE1 and ChYAE2. *B. burgdorferi* sensu stricto strain B31, the *B. garinii* strains 20047, ASF, and FujiP2, the *B. afzelii* strain VS461 and NT28, *B. japonica* HO14, and *B. henselii* HS1 were used as comparative reference strains.

The 5S-23S rRNA intergenic spacer was amplified by using primers RS1 (5’-TCAGGGAT TC CGGGAGA-3’) and RS2 (5’-TCTAGGCA TTCACCATGA-3’) (27), and RFLP analysis
was accomplished by digestion of the PCR products with MseI and DraI as described previously (22). The PCR product of Chinese culture isolate ChY13p was cloned into plasmid pGEM-5Zf by using a pGEM-T vector system kit (Promega Corporation, Madison, Wis.) and sequenced with a PRISM Ready Reaction DyeDeoxy Terminator Cycle Sequencing Kit (The Perkin-Elmer Corporation, Norwalk, Conn.). The 16S rRNA gene sequence of isolate ChY13p has been assigned accession no. AB003785. The accession numbers of reference strains used in this study are as follows: strain B31, accession no. L30127; 20047, L30119; ASF, D84403; VSY61, L30135; NT28, D84405; and H014, L30128.

The 16S rRNA gene of Borrelia isolate ChY13p was amplified by primers 5'-GTCTGCGGCACTGCGTCTTAAAGCATG-C-3' and 5'-GTGACGCGGCCTGTGTAACAGGCC-3' as described previously (12) and was sequenced as described above. Phylogenetic analyses of the 16S rRNA gene sequences were performed by the DNASTAR (Madison, Wis.) program with the CLUSTAL method (13). The 16S rRNA gene sequence of isolate ChY13p determined in this study has been assigned accession no. AB007450. The accession numbers of sequences used for phylogenetic analysis have been assigned as follows: strain B31, accession no. M88329; 20047, D67018; 9357, L39081; G1, M64311; G2, M69067; HT61, D67019; J1, L46697; Ip3, M75149; H014, L40597; IK2A, L40958; 20004, M64310; 1352, M66309; SH-2-82, M60969; and HS1, M60968. Flagellin PCR-RFLP analysis was carried out as described previously (11). The amplified DNAs were digested with HapII, HhaI, HincII (Tokara, Tokyo, Japan), CcII (Boehringer GmbH, Mannheim, Germany), and DdeI (Toyobo, Osaka, Japan). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described before (24). The monoclonal antibodies (MAbs) used were H9724, which is specific to the 12-kDa protein of Borrelia japonica; R091, M75149; HO14, L40597; IKA2, L40598; 20004, M64310; 1352, M66309; SH-2-82, M60969; and HS1, M60968. Flagellin PCR-RFLP analysis was carried out as described previously (11). The amplified DNAs were digested with HapII, HhaI, HincII (Tokara, Tokyo, Japan), CcII (Boehringer GmbH, Mannheim, Germany), and DdeI (Toyobo, Osaka, Japan). Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting were carried out as described before (24).

**TABLE 1.** SS-23S rRNA intergenic spacer RFLP patterns of Chinese Borrelia culture isolates

<table>
<thead>
<tr>
<th>Species</th>
<th>No. of culture isolates (% of total no.)</th>
<th>RFLP pattern* from digestion by:</th>
<th>No. (% of culture isolates reactive to MAb P3134 with OspA and OspB</th>
</tr>
</thead>
<tbody>
<tr>
<td>B. garinii</td>
<td>6 (10.2)</td>
<td>A (0.3)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>B. garinii (pattern B) + B. garinii (pattern C)</td>
<td>24 (40.7)</td>
<td>C’ (0.2) + C’’ (0.2)</td>
<td>1 (1.7)</td>
</tr>
<tr>
<td>B. afzelii</td>
<td>17 (28.8)</td>
<td>D’ (0.4) + D (0.4)</td>
<td>0</td>
</tr>
<tr>
<td>B. afzelii + B. afzelii</td>
<td>2 (3.4)</td>
<td>B’’ + D’’ + B + D</td>
<td>0</td>
</tr>
<tr>
<td>B. garinii (tentative)</td>
<td>7 (11.9)</td>
<td>C’ + D’’ + C’’ + D’’ + D’’ + C’’</td>
<td>3 (5.1)</td>
</tr>
</tbody>
</table>

* RFLP patterns are shown in Fig. 1, and their designations are given in the corresponding legend.

FIG. 1. Representative RFLP patterns of SS-23S rRNA intergenic spacer observed among Chinese Borrelia culture isolates. The PCR products were digested by HapII (A) or MseI (B). DNA was electrophoresed on a 10% polyacrylamide gel and stained with ethidium bromide. The molecular size standards are indicated on the left of the gel. Lane 1, pattern B (isolate ChY02p); lane 2, pattern C (ChY50p); lane 3, pattern D (ChY55p); lane 4, pattern P (ChY13p); lane 5, patterns B and C (ChY27p); lane 6, patterns B and D (ChY20p); lane 7, patterns C and D (ChY15p).
P62a, which is reactive to the 62-kDa heat shock protein of *B. burgdorferi* sensu lato but not *B. japonica*. The reference strains HS1 (*B. hermsii*) and HO14 (*B. japonica*) showed a negative reactivity with MAb P62a. Thus, there were no *B. japonica* isolates among these 59 Chinese cultures. We identified all culture isolates as *B. burgdorferi* sensu lato with genus-specific MAb G7 reactive to OspC. Five *Borrelia* culture isolates showed two OspC bands which might have resulted from mixtures of two isolates. These five culture isolates were also identified as mixtures by RFLP analysis. Twenty of 59 culture isolates showed cross-reactivity of both OspA and OspB to MAb P3134, including 15 *B. garinii* isolates with pattern C, 1 *B. garinii* isolate with pattern B, 1 isolate mixture of *B. garinii* with patterns B and C, and 3 isolate mixtures of *B. garinii* with pattern C and *B. afzelii* with pattern D (Table 1). Previous studies had reported that some *B. garinii* isolates from Japan and Russia showed cross-reactivity of both OspA and OspB with MAb P3134 (9, 17). Sequence analysis revealed that the ospA and ospB genes of these isolates share a conserved 282 bp sequence at their 3’ ends (35). To date, these isolates have been observed only in eastern Asia, not in North America or Europe.

One isolate, ChY13p, was observed to have an RFLP pattern never found before among *Borrelia* strains. To further confirm this characteristic of ChY13p, the 5S-23S rRNA intergenic spacer sequence was determined and compared with those of other representative strains (Fig. 3). ChY13p produced a 237-bp 5S-23S rRNA spacer amplicon that was similar in size to that of *B. japonica* (256 bp). Two fragments, 185 bp and 52 bp in size, were generated by digestion with DraI, and three fragments, of 105, 79, and 53 bp, were observed after digestion with MseI. Although the MseI pattern of ChY13p was almost identical to that of *B. japonica*, the DraI patterns of these strains were quite different (19). The sequence between nucleotide 73 and nucleotide 97 differed among the different *Borrelia* species. Compared with the sequences of *B. burgdorferi* sensu stricto and *B. garinii*, 10 nucleotides were missing from the sequences of *B. afzelii* VS461 and NT28 and 18 nucleotides were missing from those of ChY13 and *B. japonica*. Furthermore, ChY13p has the nucleotide sequence AAAACCA, was found specifically in the sequences of *B. afzelii* with RFLP pattern D and *B. japonica*. The sequence of ChY13p showed the highest similarity to those of *B. afzelii* with pattern D and *B. japonica*. To identify the species of isolate ChY13p, the 16S rRNA gene sequence of isolate ChY13p was determined to assess the phylogenetic divergence. About 90% of the whole 16S rRNA gene sequence was aligned and compared with pre-

![Fig. 2](link) Western blot analysis of Chinese *Borrelia* culture isolates with MAbs P62a, H9724, H5332, and G7. *B. burgdorferi* B31, *B. garinii* 20047, ASF, and FujiP2, *B. afzelii* VS461 and NT28, *B. japonica* HO14, and *B. hermsii* HS1 were used as comparative reference strains.

![Fig. 3](link) Nucleotide sequence alignment of 5S-23S rRNA intergenic spacer amplicons of Chinese isolate ChY13p and the reference *Borrelia* isolates. The 3' region of the 5S rRNA gene and the 5' region of the 23S rRNA gene are indicated in boldface type. The corresponding primers are underlined. The asterisks and boxes indicate identical nucleotides and the motif sequence, respectively, s. s., sensu stricto.
vously published sequences of *Borrelia* species. A neighbor-joining phylogenetic tree (29) was constructed on the basis of the sequence similarity matrix. Phylogenetic analysis placed the strains into a coherent cluster of the Lyme disease *Borrelia* and related genomic groups. According to this tree, ChY13p was clustered into the group of *B. garinii* strains (Fig. 4). The isolate ChY13p was further characterized by flagellin PCR-RFLP typing method. The following *Borrelia* strains have been previously determined as producing the flagellin RFLP types indicated: type I, *B. burgdorferi* sensu stricto; II, *B. garinii*; III, *B. afzelii*; IV, *B. turdus*; V, *B. turdus*; VI, *B. valaisiana*; VII, *B. japonica*; VIII, *B. lusitaniae*; IX, group DN127; and X, *B. andersonii* (11). ChY13p produced pattern II. Western blot analysis reveals that isolate ChY13p reacted with MAb D6, D and also to isolates were identified on the basis of 5S-23S rRNA intergenic spacer sequences of previously published sequences of *B. garinii* as determined by measuring the length of the horizontal lines connecting two isolates, s.s., sensu stricto.

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FIG. 4. Phylogenetic tree for the Lyme disease borreliae and their relatives constructed by using 16S rRNA gene sequences. Bar = 0.5% difference between sequences, as determined by measuring the length of the horizontal lines connecting two isolates, s.s., sensu stricto.


