Typing of Borrelia Relapsing Fever Group Strains

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Partial sequencing of the 16S-23S rDNA intergenic spacer showed two to four genotypes each for Borrelia hermsii and B. turicatae, both relapsing fever agents transmitted by argasid ticks, and for B. miyamotoi and B. lonestari, transmitted by ixodid ticks. Field surveys of Ixodes ticks in Connecticut and Sweden showed limited local diversity for B. miyamotoi.

The two major clades of species in the genus Borrelia are the Lyme borreliosis group and the relapsing fever group (1). The Lyme borreliosis group includes Borrelia burgdorferi, B. afzelii, B. garinii, and several other species not associated with human disease. The relapsing fever group includes several species, such as B. hermsii in the Nearctic ecologic region and B. persica in the Palearctic, that cause endemic relapsing fever in humans (2). The known relapsing fever agents are transmitted by soft (argasid) ticks, usually an Ornithodoros species. In 1995, B. miyamotoi was first isolated from Ixodes persulcatus hard (ixodid) ticks in Japan (3). Genomic DNA of the newly identified spirochete cross-hybridized to a greater extent with DNA of relapsing fever species than with DNA of Lyme borreliosis species. In 1996, B. lonestari was discovered in Amblyomma americanum, an ixodid tick of the southern and eastern United States (4,5). Although B. lonestari is associated with a Lyme borreliosis–like disorder in the southern United States (6), sequence analysis showed that B. lonestari, like B. miyamotoi, was in a clade with the relapsing fever group rather than the Lyme borreliosis group (4,5). More recently, spirochetes closely related to B. miyamotoi, and provisionally designated here as B. miyamotoi sensu lato (s.l.), were discovered in I. scapularis ticks in the United States (7) and I. ricinus ticks in Europe (8).

The Study

The public health importance of the newly discovered species remains to be determined. However, finding B. miyamotoi s.l. in I. scapularis, I. ricinus, and I. persulcatus, the predominant vectors of Lyme borreliosis in North America, Europe, and Asia, respectively, complicates interpreting epidemiologic studies of Lyme borreliosis and other ixodid-borne disorders. A method to identify and distinguish strains within species is needed to carry out studies of the population biology and of the possible etiologic roles of these organisms. Since most of these microorganisms are to date uncultivable or poorly cultivable, a method using DNA amplification by polymerase chain reaction (PCR) is preferable. On the basis of the findings of Liveris et al. (9), we further developed sequence analysis of the 16S-23S rDNA intergenic spacer (IGS) for strain typing and showed its advantages over other loci for the Lyme borreliosis agents B. burgdorferi and B. afzelii (10). For this study, we applied this approach to typing the new Borrelia spp. and included two relapsing fever agents, B. hermsii (endemic in the western and northwestern United States) and B. turicatae (endemic in the southwestern and south-central United States) (2).

Nine isolates of B. hermsii in our culture collection originated in New Mexico, Colorado, California, and Washington State and were either from Ornithodoros hermsii ticks, patients with relapsing fever, or, in one case, a bird (11). Two B. turicatae isolates were from O. turicata ticks from Texas and Kansas. B. miyamotoi strains HT24, HT31, and HK004 from I. persulcatus ticks and strains NB103-1 and FR64b from Apodemus spp. mice were from Hokkaido, Japan (3,12). Cultivable strains of these species were grown in Barbour-Stoenner-Kelly II medium. Uncultivated species were initially identified in total DNA extracts of ticks by using Borrelia genus–specific PCR, targeting flaB gene (5). Approximately 2% of A. americanum nymphs and adult females in collections from different areas of New Jersey, Illinois, and Missouri contained B. lonestari. B. miyamotoi s.l. spirochetes were identified in I. scapularis nymphs collected at a 7.2-ha field site in southern Connecticut and in I. ricinus nymphs collected at a 1.5-ha site in Blekinge County in Sweden (10). A Connecticut strain of B. miyamotoi s.l. strain was maintained in Mus musculus (7).

Part of the intergenic spacer was amplified by PCR with primers for the 3' end of the 16S rRNA gene and the ileT tRNA gene (10). As a comparison to the intergenic spacer locus and to assess linkage disequilibrium, we also partially sequenced the chromosomal gene for the P66 outer membrane protein (10,13) after amplification by PCR as described in the Table footnotes. The PCR products were either directly sequenced or first cloned into pCR2.1-TOPO vector (Invitrogen, Carlsbad, CA) before sequencing on a Beckman CEQ 8000.
Fullerton, CA) automated sequencer. The sequences were aligned automatically by using Clustal X software (http://www-igbmc.u-strasbg.fr/BioInfo/ClustalX) and then manually with MacCLADE version 4.05 (http://macclade.org/macclade.html) (10). The maximum lengths of the alignments (http://spiro.mmg.uci.edu/data) were set by the shortest available sequence. Accession numbers for the deposited sequences are given in the legend for the Figure and in a footnote for the Table.

The PCR products for the intergenic spacer locus varied in length between species and ranged from 388 bp for *B. miyamotoi* s.l. from Sweden to 685 bp for *B. turicatae*. The PCR product for the *p66* gene was 605–614 bp between species. The Table summarizes the statistics for the aligned intergenic spacer and *p66* sequences of the *B. miyamotoi* s.l., *B. lonestari*, and *B. hermsii*. The mean nucleotide diversity normalized for each aligned position was 38%–130% higher for the intergenic spacer locus than for the *p66* locus. At the same time, intragenic recombination was not detected at the intergenic spacer locus with Sawyer’s test (www.math.wustl.edu/~sawyer/mbprogs), which assesses the likelihood that polymorphisms in a sequence arose through recombination rather than mutation (data not shown). This result was consistent with the undetectable recombination at the intergenic spacer loci of *B. burgdorferi* (10).

The genetic diversity at the intergenic spacer and *p66* loci for the relapsing fever group species in a given geographic area was more limited than was the case for Lyme borreliosis species (10). This limitation was most apparent for the *B. burgdorferi* species. The phylogenetic relationships among the intergenic spacer genotypes were evaluated using the algorithm of Felsenstein (10). The maximum lengths of the alignments were set by the shortest available sequence. Accession numbers for the deposited sequences are given in the legend for the Figure and in a footnote for the Table.

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The genetic diversity at the intergenic spacer and *p66* loci for the relapsing fever group species in a given geographic area was more limited than was the case for Lyme borreliosis species (10). This limitation was most apparent with the *B. miyamotoi* s.l. sequences of 22 samples from Connecticut and 6 samples from Sweden. As shown by the phylogram (Figure), only one intergenic spacer genotype each was found for *B. miyamotoi* s.l. from the Connecticut site and from Sweden. In contrast, collections at the same sites and times, and from the same tick vectors, provided 8 intergenic spacer genotypes among 62 *B. burgdorferi* samples in *I. scapularis* and 9 intergenic spacer genotypes among 73 *B. afzelii* samples in *I. ricinus* (10). Accepting a type I error level of 0.05, we would expect to have detected a second genotype of *B. miyamotoi* s.l. in a sample size of 22 if its proportion was ≥14%. The findings at the *p66* locus for 10 Connecticut samples and for 4 samples from Sweden were similar: only one *p66* genotype was detected at each location.

The samples of the other relapsing fever group species were not prospectively acquired for population studies, and thus, the findings provide only a tentative view of population structure. Nevertheless, the results are consistent with an interpretation that the local strain diversity of the relapsing fever group species is more limited than that of Lyme borreliosis agents. The intergenic spacer sequences of five *B. miyamotoi* isolates from ticks or mice from Japan were identical, except for a single nucleotide in one isolate (Figure); the *p66* sequences were identical for each of the five isolates. Four intergenic spacer genotypes were detected from the nine isolates of *B. hermsii* from different regions of the western United States; the three intergenic spacer genotypes were examined each had a different *p66* allele. Two of the linked intergenic spacer and *p66* genotypes were unique to species from the Rocky Mountain region. The two strains of *B. turicatae* from Texas and Kansas differed in intergenic spacer genotype. *A. americanum* ticks collected in three states yielded three intergenic spacer genotypes from 20 samples positive for *B. lonestari* (Table and Figure). The three intergenic spacer genotypes were each linked to three unique *p66* alleles. Two of the linked genotypes were found at all three loca-
vertebrate reservoirs of should also be useful for identifying and characterizing the humans (14). Strain typing by PCR and sequence analysis are associated with certain virulence phenotypes in (1). Certain intergenic spacer genotypes of ered species. Sequencing this locus provides a means for further efficient for genotyping the relapsing fever group of demonstrates that, as for the Lyme borreliosis spirochetes conversion from horizontal gene transfer at this locus, pattern of relationship and the lack of evidence of gene spacer sequences, with or without the species with fewer polymorphisms (Table), the intergenic diversity at the intergenic spacer locus than did samples from other genomic groups (Figure). However, even for population structures remains to be determined. This variation may be the consequence of differences in pathogenesis between the organisms.

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tions; one was found in Missouri and New Jersey but not in Illinois.


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Conclusions

Samples of the B. miyamotoi s.l. showed greater genetic diversity at the intergenic spacer locus than did samples from other genomic groups (Figure). However, even for species with fewer polymorphisms (Table), the intergenic spacer sequences, with or without the p66 sequences, confirmed the monophyly of strains within each species. This pattern of relationship and the lack of evidence of gene conversion from horizontal gene transfer at this locus, demonstrates that, as for the Lyme borreliosis spirochetes (10), the intergenic spacer region is both sensitive and sufficient for genotyping the relapsing fever group of Borrelia species. Sequencing this locus provides a means for further epidemiologic and ecologic studies of the newly discovered Borrelia species of hard ticks, as well as of the relapsing fever agents that are reemerging as human pathogens (1). Certain intergenic spacer genotypes of B. burgdorferi are associated with certain virulence phenotypes in humans (14). Strain typing by PCR and sequence analysis should also be useful for identifying and characterizing the vetebrate reservoirs of B. lonestari and B. miyamotoi s.l.

The linkage disequilibrium between the intergenic spacer and p66 loci indicate that the relapsing fever group species, like Lyme borreliosis spirochetes (10,15), are highly clonal bacteria. Why these two groups of tick-borne spirochetes appear to have different population structures remains to be determined. This variation may be the consequence of differences in pathogenesis between the organisms.


table. Descriptive statistics for IGS* and p66 loci of three Borrelia species

| Species       | Locus | No. samples | No. variants | Base pairs | No. gapped | Polymorphisms (%) | π*       |
|---------------|-------|-------------|--------------|------------|------------|-------------------|----------|
| Borrelia miyamotoi s.l. | IGS   | 33          | 4            | 474        | 15         | 40 (8.4)          | 0.058    |
|               | p66†  | 19          | 3            | 617        | 9          | 38 (6.2)          | 0.042    |
| B. lonestari  | IGS   | 20          | 3            | 412        | 1          | 14 (3.4)          | 0.023    |
|               | p66†  | 7           | 3            | 346        | 0          | 5 (1.4)           | 0.010    |
| B. hermsii    | IGS   | 9           | 4            | 665        | 2          | 20 (3.0)          | 0.015    |
|               | p66†  | 5           | 3            | 516        | 3          | 8 (1.6)           | 0.010    |

*IGS, 16S-23S rRNA gene intergenic spacer region.
†π = mean nucleotide diversity at each aligned position.
‡Excludes an 81-bp indel.

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