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Molecular Typing of *Borrelia burgdorferi* from Lyme Disease Patients by PCR-Restriction Fragment Length Polymorphism Analysis

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Ninety-three *Borrelia burgdorferi* isolates obtained from erythema migrans lesions or blood of Lyme disease patients in Westchester County, N.Y., between 1991 and 1994 were characterized by PCR-restriction fragment length polymorphism (PCR-RFLP) analysis of the 16S-23S rRNA gene spacer. All isolates could be classified into three distinct RFLP types. Among the 82 skin biopsy isolates studied, 21 (25.6%) were type 1, 37 (45.1%) were type 2, and 21 (25.6%) were type 3. Three (3.7%) cultures contained a mixture of two isolates with distinct RFLP types. The 11 isolates cultured from blood showed a similar predominance of RFLP type 2 (6 of 11; 54.5%) relative to types 1 (2 of 11; 18.2%) and 3 (3 of 11; 27.3%). For one patient both skin and blood isolates were cultured, and RFLP analysis revealed that these isolates differed from one another. This study demonstrates that there is genotypic heterogeneity in *B. burgdorferi* strains infecting Lyme disease patients, and this typing approach may allow differentiation of isolates with various degrees of pathogenic potential.

Lyme disease, a multisystem disorder with potentially chronic neurological, cardiac, cutaneous, and arthritic manifestations (15), is caused by a genetically diverse group of spirochetes collectively referred to as *Borrelia burgdorferi* sensu lato. These spirochetes are transmitted to humans by the bite of an infected tick (3, 16). The application of several standard bacteriological methods to classification of *B. burgdorferi* sensu lato resulted in the subdivision of this group of closely related organisms into the four distinct species *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica* (2, 4, 8). These species vary in geographic distribution, and all but *B. japonica* have been associated with clinical illness in humans. Moreover, it has been suggested that different species may be responsible for distinctive disease manifestations (1, 4, 18, 20).

In North America, virtually all isolates of *B. burgdorferi* belong to the single species *B. burgdorferi* sensu stricto. This is distinct from the situation in Eurasia, where all four species have been isolated (2, 9, 12, 17). In a previous work, we reported the development of a PCR-restriction fragment length polymorphism (PCR-RFLP) typing method for identification of individual species of *B. burgdorferi* sensu lato (9). This technique combines PCR amplification of a portion of the 16S-23S rRNA gene spacer with RFLP analysis of the amplified product. The technique permits typing of both cultured isolates and uncultured specimens of *B. burgdorferi* sensu lato. Those experiments resulted in the subdivision of 17 individual *B. burgdorferi* sensu stricto isolates into two distinct PCR-RFLP types on the basis of *Hin*fI digestion patterns (9).

The objective of the present study was to assess further the genetic diversity among infectious clinical isolates of *B. burgdorferi* sensu stricto by typing a large number of *B. burgdorferi* isolates cultured between 1991 and 1994 from erythema migrans lesions or blood of Lyme disease patients residing in Westchester County, N.Y., or surrounding areas.

All *B. burgdorferi* clinical isolates were obtained from either skin biopsy specimens of erythema migrans lesions or blood of Lyme disease patients attending the Lyme Disease Diagnostic Center of the Westchester County Medical Center between 1991 and 1994. Biopsy of the lesions and growth and propagation of the cultures were as previously described (14).

Typing of *B. burgdorferi* clinical isolates. Samples for PCR amplification were processed as previously described (14), by using 0.5-ml aliquots of *B. burgdorferi* culture. In most instances analysis was performed on aliquots from the primary culture, and in all cases no analyzed sample was beyond the fourth in vitro passage. A 1,712-bp region of the 16S-23S rRNA gene spacer was amplified with primers P_A and P42 as previously reported (9). These fragments were subjected to RFLP analysis by digestion with either *Hin*fI or *Mse*I, and resolution of the resultant digestion products was achieved by electrophoresis on either a 1.5% (*Hin*fI) or a 2.4% (*Mse*I) agarose gel containing 0.5 μg of ethidium bromide per ml. Categorical data were analyzed by chi-square methods (Pearson product moment chi-square) with Fisher exact-type adjustments being made where appropriate (6).

Ninety-three isolates obtained from 90 patients were analyzed. Of these, 82 were from skin biopsy specimens and 11 were from blood. Both skin and blood isolates were obtained for three patients. PCR amplification of the clinical isolates with the indicated primers resulted in a product of the expected size in all cases. In preliminary experiments, amplified products from a number of isolates were digested with either *Asn*I, *Dra*I, *Hin*dIII, *Hin*fI, *Mse*I, *Ssp*I, or *Taq*I. Some of these digestions resulted in patterns which provided little discrimination among isolates. On the basis of these experiments, *Hin*fI and *Mse*I were chosen for the subsequent studies.

RFLP results for representative isolates are presented in Fig. 1. All isolates tested had one of two *Hin*fI digestion patterns. The pattern designated H1 was identical to that found

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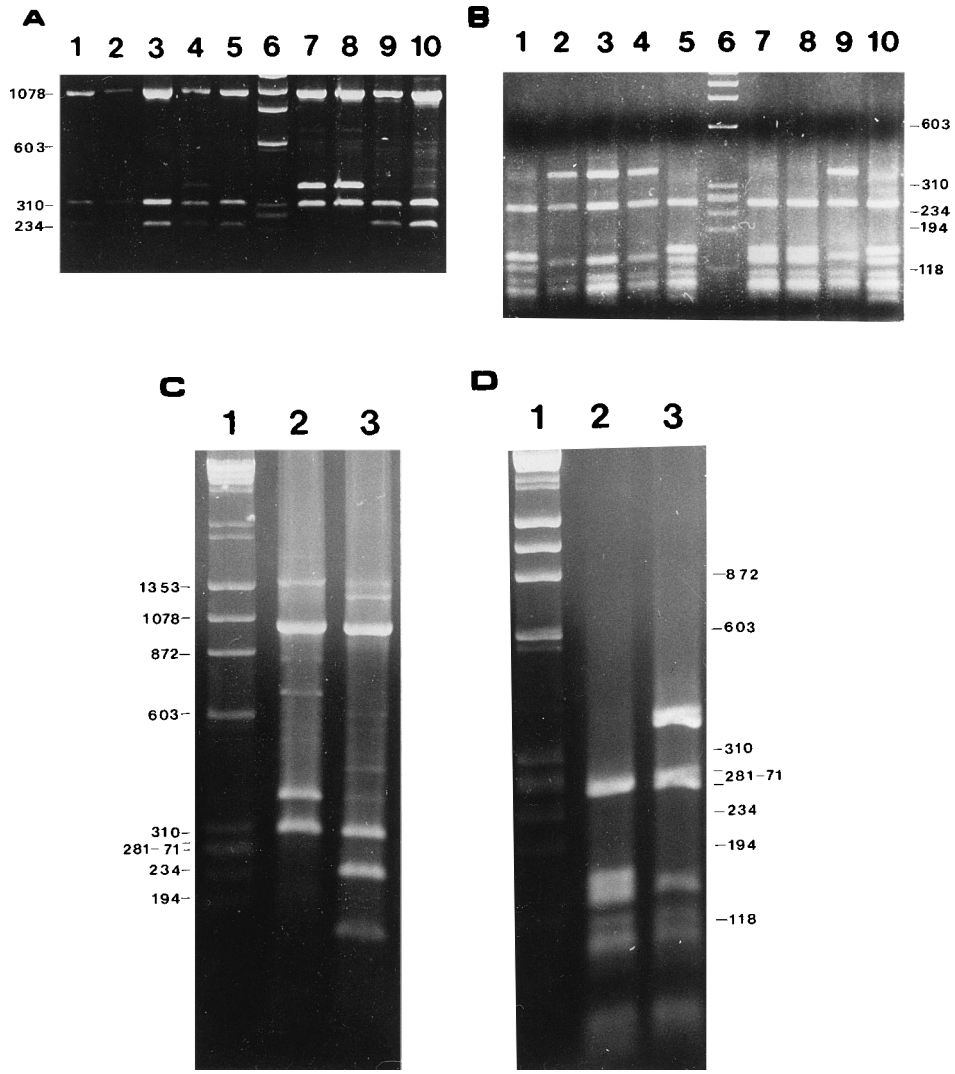

FIG. 1. PCR-RFLP analysis of selected *B. burgdorferi* clinical isolates. PCR amplification was carried out with primers P_A and P42, and amplified products were digested with either *Hin*fI (A) or *Mse*I (B). Lanes 1, B83; lanes 2, B48; lanes 3, BL55; lanes 4, B49; lanes 5, B65; lanes 6, *Hae*III-digested Φx174 DNA size markers; lanes 7, B31; lanes 8, B89; lanes 9, 297; lanes 10, B141. B31 is the *B. burgdorferi* type strain, and 297 is a cerebrospinal fluid isolate which has been previously described (2). All other isolates are from this study; all were cultured from skin biopsy specimens, except for BL55, which was cultured from blood. (C and D) PCR-RFLP analysis of skin and blood isolates obtained from the same patient. Amplified products were digested with either *Hin*fI (C) or *Mse*I (D). Lanes 1, *Hae*III-digested Φx174 DNA size markers; lanes 2, B14 (skin isolate); lanes 3, BL11 (blood isolate). The lengths (in base pairs) of the DNA size standards are given in the margins.

for *B. burgdorferi* type strain B31 (Fig. 1A, lane 7). The *Hin*fI pattern designated H2 resulted in two smaller fragments of 241 and 131 bp due to acquisition of an additional restriction site in the 372-bp fragment (Fig. 1A, lanes 1 to 3, 5, 9, and 10). There was a predominance (72%; 65 of 90) of the H2 RFLP in the clinical isolates studied ($P < 0.05$).

To obtain additional molecular typing information, PCR-amplified products were also digested with *Mse*I. On the basis of the known sequence of *B. burgdorferi* B31 (7), the amplified DNA should include 31 *Mse*I sites, yielding restriction fragments most of which are smaller than 30 bp. However, a distinct polymorphism among the largest fragments produced by digestion with this enzyme was observed. Profile M1 contains fragments of 102, 128, 136, 149, and 258 bp (Fig. 1B, lanes 1, 5, 7, 8, and 10). A second pattern (designated M2) results from the loss of two *Mse*I sites which link the 149- and 128-bp fragments with an intervening 87-bp fragment. As a consequence, the two former bands are lost and are replaced by a larger band of 364 bp (Fig. 1B, lanes 2, 3, and 9). The two *Mse*I RFLP patterns were relatively equally distributed among the 90 evaluable isolates (48 were M1, and 42 were M2).

By combination of the *Hin*fI and *Mse*I restriction patterns of PCR-amplified DNA, it was possible to classify all the patient isolates into three genetically distinct groups. These were designated RFLP type 1 (H1 and M1), type 2 (H2 and M2), and type 3 (H2 and M1). Interestingly, the fourth possible combination (H1 and M2) was not observed. A summary of the RFLP typing analysis results for these isolates is presented in Table 1. The vast majority of the clinical isolates available for analysis were from skin biopsy specimens of erythema migrans lesions. 47% (37 of 79) of these were RFLP type 2, with the remainder equally distributed between type 1 and type 3 (26.5%; 21 of 79 for each). A similar predominance of type 2 isolates was observed with the 11 isolates cultured from blood (55% [6 of 11] type 2, 27% [3 of 11] type 3, and 18% [2 of 11] type 1).

TABLE 1. PCR-RFLP typing of *B. burgdorferi* clinical isolates

Pattern or RFLP type	No. (%) of isolates from:	
	Skin	Blood
Digestion patterns[a]		
H1	23 (29.1)	2 (18.2)
H2	56 (70.9)[b]	9 (81.8)[b]
M1	43 (54.4)	5 (45.5)
M2	36 (45.6)	6 (54.5)
RFLP types[c]		
1 (H1, M1)	21 (26.5)	2 (18.2)
2 (H2, M2)	37 (47)[d]	6 (54.5)[d]
3 (H2, M1)	21 (26.5)	3 (27.3)

[a] Based on digestion pattern with either *Hin*fI or *Mse*I as defined in the text.
[b] By chi-square test of significance, $P < 0.05$ for comparison of H1 and H2.
[c] Based on RFLP patterns for both *Hin*fI and *Mse*I.
[d] By chi-square test of significance, $P < 0.05$ for comparison of RFLP type 2 and either type 1 or type 3; there was no significant difference between RFLP type 1 and type 3.

Three skin isolates yielded composite RFLP patterns suggestive of a mixed culture (e.g., isolate B49; Fig. 1A, lane 4) and were not included in the data analysis. This could have been the result of laboratory contamination of the specimens or cultures or may have arisen by spontaneous mutation of the original isolate with subsequent expansion of the new genotype in culture. An argument against the latter possibility is that continuous passage of many isolates in culture in our laboratory has never revealed the conversion of an initially homogeneous culture (with regard to RFLP type) to a mixed culture. Simultaneous infection of *Ixodes ricinus* ticks with two distinct *B. burgdorferi* sensu lato species has recently been reported (13). Similarly, mixed *Borrelia* infection of reservoir mice in Japan has been described (11). It is, therefore, reasonable to expect that similar coinfection of *I. scapularis* with two or more genetically distinct *B. burgdorferi* strains occurs and that such ticks may transmit a mixed population of *B. burgdorferi* genotypes to humans on feeding. That this is also the situation in the current study site will be addressed by typing of uncultured tick and skin biopsy specimens. Regardless of the origin of the mixture, however, it is important to note that the technique has sufficient resolution to detect and type organisms from infections resulting from a heterogeneous inoculum of spirochetes, were this to occur.

Typing of skin and blood isolates from the same patients. Of particular interest are three pairs of isolates cultured from both skin and blood samples obtained at the same visit from an individual patient. For two of the pairs, both the skin and blood isolates were type 2. Interestingly, the third pair of isolates yielded two different RFLP patterns (Fig. 1C and D). The skin isolate (B14) displayed a type 1 RFLP pattern, whereas the blood isolate (BL11) was RFLP type 2. The patient from whom these isolates were obtained presented with a single erythema migrans lesion, no evidence of a tick bite, and no prior history of *B. burgdorferi* infection. This makes it unlikely that the presence of *B. burgdorferi* strains with different RFLP patterns in the two samples was the result of multiple tick bites or previous infection. The absence of a type 2 isolate in the skin biopsy culture of this patient could be due to variability in the organism load for each of the different *B. burgdorferi* RFLP types and/or type-specific differences in potential for dissemination. In any event, these results strongly suggest that an individual patient can be simultaneously infected with more than one *B. burgdorferi* genotype. Similar observations have been reported by others (5). Furthermore, this finding further supports the notion that two different spirochete genotypes can be transmitted to humans by the same tick bite. Since the blood isolate must have been disseminated from the initial erythema migrans lesion, this finding suggests that an individual tick may be simultaneously infected with *B. burgdorferi* strains of multiple genotypes and that this mixed infection can be transmitted to humans.

We reported previously the application of PCR-RFLP analysis for typing of *B. burgdorferi* sensu lato isolates from varied biological and geographic sources (9). The present study has focused on further refinement of this assay method in the evaluation of a large number of clinical isolates. All the isolates tested were obtained by culture of either the skin or blood of Lyme disease patients in Westchester County, N.Y., or surrounding areas during the period from 1991 to 1994. Despite a number of earlier reports indicating a high degree of homogeneity in North American isolates (19–21), it is clear from the present study that there is, in fact, significant genotypic heterogeneity in the clinical isolates obtained from even this restricted geographic region.

RFLP type 2 predominated among the clinical isolates tested, accounting for 47.8% (43 of 90) of evaluated specimens ($P < 0.05$). This pattern was even more striking when only the *Hin*fI-based RFLP was considered: 72.2% (65 of 90) of the isolates were type H2 ($P < 0.05$). The predominance of RFLP type 2 may be due to either a higher prevalence of this type in the local tick population, its enhanced ability to grow in culture, or greater human infectivity. The first possibility appears unlikely since our initial typing studies of *B. burgdorferi* in local ticks showed that all 18 strains analyzed so far were RFLP type 1 (10). Of course, the other RFLP types must be present in this tick population since they can be cultured from patients, and additional typing analysis will determine their distribution in nature. Similarly, typing analysis of uncultured spirochetes directly in skin biopsy specimens will allow us to distinguish between the other two possibilities.

The technique described here is a rapid and direct method for genotyping of *B. burgdorferi* clinical isolates. A major advantage of this approach is its applicability to uncultured specimens (9). Studies correlating the genotypes of cultured patient isolates with those obtained by direct PCR-RFLP analysis of skin and tick specimens are currently under way.

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