JOURNAL OF CLINICAL MICROBIOLOGY, Mar. 1996, p. 652–656 Vol. 34, No. 3
0095-1137/96/$04.00+0

Multiple Infections of *Ixodes scapularis* Ticks by *Borrelia burgdorferi* as Revealed by Single-Strand Conformation Polymorphism Analysis†

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Received 10 July 1995/Returned for modification 14 September 1995/Accepted 7 December 1995

The genetic heterogeneity of the spirochete *Borrelia burgdorferi* within single adult black-legged ticks from Shelter Island, N.Y., was determined by cold, single-strand conformation polymorphism (SSCP) analysis. The central region of the *ospA* gene of *B. burgdorferi* from infected ticks was amplified by nested PCR. Amplified product of the correct size was obtained from 20 of 45 ticks (44%). This is the fraction of ticks that is expected to be infected with *B. burgdorferi*. Four variant classes were determined by SSCP analysis. Eight ticks were infected with a single variant, nine ticks were infected with two variants, two ticks were infected with three variants, and one tick was infected with all four variants. DNA from each variant was sequenced. Five different sequences were found. The sequence of each variant was different from that of another variant by a single base. SSCP analysis could distinguish three of the four single-base changes found in the region.

Lyme disease is a tick-borne illness caused by the spirochete *Borrelia burgdorferi* (4). Studies using various molecular and immunological typing systems have revealed a large amount of genetic variability within the species (3, 16). Consequently, the *B. burgdorferi* sensu lato species complex can be divided into a number of different genospecies, four of which have been formally designated: *B. burgdorferi* sensu stricto, *B. garinii*, *B. afzelii*, and *B. japonica* (3, 26). Additional strains which cannot be placed in one of these genospecies have been identified (21). The first three genospecies have been isolated from humans and are pathogenic. The others have been isolated from ticks of the genus *Ixodes* or mammals other than humans. Of the pathogenic genospecies, *B. burgdorferi* sensu stricto alone occurs in the United States, while in Europe all three occur.

The immune response to *B. burgdorferi* is represented by an early, prominent humoral response to the endoflagellar protein, p41, and a protein constituent of the protoplasmic cylinder, p93 (6). Both proteins are protected from the immune system by an outer membrane whose major components include OspA and OspB in the tick and OspC and OspD in mammals (22). When mice are immunized with a recombinant OspA, they are protected from challenge by the same strain of *B. burgdorferi* (11, 23) but not necessarily from challenge by heterologous strains of *B. burgdorferi* (12, 13).

A phylogenetic analysis was performed on 15 isolates of *B. burgdorferi* sensu lato by using DNA sequences obtained from the chromosomal genes *p41* and *p93* and the *ospA* gene, located on the 49-kb linear plasmid (9). Comparison of the resulting gene trees revealed that there was very little, if any, genetic exchange between *Borrelia* strains. This result showed that the genus *Borrelia* has a clonal population structure. The analysis also revealed a number of strains which were so highly divergent that vaccines developed against one are unlikely to confer resistance against others. Thus, determination of the genetic as well as the geographic variability of *B. burgdorferi* is vital for the development of efficient vaccines. Successful vaccines will also depend upon understanding how this variability influences cross-reactivity between the different *Borrelia* strains.

Lyme borreliosis presents itself in humans as a multisystemic disorder. Clinical symptoms can vary from an acute skin rash (erythema migrans) to severe dermatologic, arthritic, rheumatologic, cardiac, and neurologic manifestations (24, 25). Genetic variation within *B. burgdorferi* sensu lato has been shown to be responsible for some of the variable symptomatology of Lyme disease (1). Evidence suggests that infections caused by *B. burgdorferi* sensu stricto tend to lead to arthritic symptoms, while those caused by *B. garinii* and *B. afzelii* tend to lead to neurological complications (1, 2). On the basis of the previous findings of genetic and geographic variability in *Borrelia* genospecies and strains (9), it is logical to assume that the variable symptomatology of the disease could be due to variation in *B. burgdorferi* strains. Thus, we report a method that allows rapid screening for genetic variability within a natural population of *B. burgdorferi.*

Natural isolates of *B. burgdorferi* were sampled by collecting the black-legged or deer tick (*Ixodes scapularis*) from Shelter Island, N.Y. This tick is the primary vector for *B. burgdorferi*. A portion of the *ospA* gene of *B. burgdorferi* from infected ticks was amplified by PCR (10). Genetic variation was then surveyed at this locus by single-strand conformation polymorphism (SSCP) analysis, a technique which distinguishes short, fixed-length DNA fragments on the basis of nucleotide substitutions. Minor sequence differences will result in conformational folding differences when the DNA is single stranded. These conformational polymorphisms can then be discriminated by their electrophoretic mobilities on polyacrylamide gels (15). The region flanking the conserved tryptophan at residue 216 or 217 was determined to be hypervariable from a moving window population analysis of OspA from 15 European and North American isolates of *B. burgdorferi* (18). This region of *ospA* was used for determination of variability by SSCP analysis with DNA generated by a novel nested PCR amplification technique from single ticks.

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† This is contribution 946 from Graduate Studies in Ecology and Evolution, State University of New York at Stony Brook.

MATERIALS AND METHODS

Indirect immunofluorescence. Adult ticks were initially surface sterilized in 70% ethanol; this was followed by a 3% hydrogen peroxide wash and three rinses in sterile phosphate-buffered saline (PBS; pH 7.4). Midguts from individual ticks were dissected and triturated in PBS on 1-by-3-in (2.5-by-7.5-cm) microscope slides with Tuberculin syringes, allowed to air dry, and then fixed with acetone for 10 min. These preparations were overlaid with anti-*B. burgdorferi* fluorescein-labeled antibody (catalog no. 02-97-91; Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.), and then the mixtures were incubated at 37°C for 45 min. The antibody was diluted within the range of 1:500 to 1:1,000, depending on the optimum resolution on the test slides (slides with diluted, cultured *B. burgdorferi*), prepared fresh each day. The slides were rinsed three times in PBS and were viewed under a fluorescent microscope by using appropriate barrier filters to detect the spirochetes.

Extraction of DNA. Adult black-legged ticks (*I. scapularis*) were collected on Shelter Island, N.Y., and were stored at −20°C. Single ticks were transferred to individual 1.5-ml microcentrifuge tubes and were bisected with a sharpened laboratory spatula. All manipulations were performed with ethanol-flamed sterile instruments. A total of 500 μl of 5% Chelex (Bio-Rad) was added to each tube, and the tubes were rocked at 56°C overnight. After incubation, the tubes were vortexed at high speed for 15 s, heated at 95°C for 15 min, and vortexed for an additional 15 s. Finally, the tubes were centrifuged in a Beckman microcentrifuge for 5 min at maximum speed, and the supernatant (approximately 500 μl) was transferred to a fresh tube.

Nested PCRs. The strategy for the nested PCR amplifications was to use a very low stringency amplification on the first PCR round to generate a heterogeneous population of templates. This heterogeneous collection of templates would then be immediately subjected to a second round of PCR amplification with primers located internal to the first set. The second amplification would be initiated at very high stringency to preferentially amplify the gene of interest and reduce the amplification of spurious templates. A modified "touchdown" PCR (8) amplification strategy was used in later cycles to ensure that product yields were sufficiently high. The two rounds of amplification were performed in the same tube, with no cleaning or preparation of the first-round products before the addition of the second-round reagents.

ospA, encoding an outer surface lipoprotein in *B. burgdorferi*, was studied. The primers were external(+), 5′-AAA AAA TAT TTA TTG GGA ATA GG-3′, beginning at base pair position 4; external(−), 5′-GT TTT TTT GCT GTT TAC ACT AAT TGT TAA-3′, beginning at base position 695; internal(+), 5′-GGA GTA CTT GAA GGC G-3′, beginning at base position 220; and internal(−), 5′-GCT TAA AGT AAC AGT TCC-3′, beginning at base position 564. The *ospA* primer set was designed to amplify a 702-bp fragment in the first round. This fragment would then be used as a template by the two internal primers in the second round. The internal primers amplified a 345-bp fragment.

First-round PCR amplifications used 10 μl of the extracted tick-*Borrelia* DNAs, deoxynucleoside triphosphates at 0.2 mM per nucleotide, Perkin-Elmer Cetus 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 2.5 U of native *Taq* polymerase (Perkin-Elmer Cetus), 0.5 μM (each) external primer, and sterile distilled water to a final volume of 50 μl. The reaction mixture was overlaid with approximately 100 μl of mineral oil. The temperature cycling profile was 2 min at 96°C for 1 cycle linked to 30 s at 94°C, 30 s at 37°C, and 2 min at 72°C for 20 cycles. At the end of these 20 cycles the second-round PCR reagents were added to the reaction vessel. The cocktail was composed of deoxynucleoside triphosphates at 0.2 mM per nucleotide, Perkin-Elmer Cetus 10× PCR buffer (500 mM KCl, 100 mM Tris-HCl [pH 8.3]), 2.5 mM $MgCl_2$, 1.25 U of native *Taq* polymerase (Perkin-Elmer Cetus), 1.0 μM (each) internal primer, and sterile distilled water to a final volume of 50 μl. The reaction tube was mixed and briefly centrifuged. The reaction profile for the second round of amplification was 2 min at 96°C for 1 cycle linked to 30 s at 94°C, 30 s at 60°C, and 1 min at 72°C for 10 cycles. The next 10 cycles used an annealing temperature of 55°C; this was followed by annealing at 50°C for 10 cycles and finally 45°C for 5 cycles.

Cold SSCP analysis. *Borrelia ospA* gene fragments, generated by the nested amplification protocol, were surveyed for point mutations by the cold SSCP protocol described by Hongyo et al. (15). A total of 14.6 μl of SSCP loading buffer (0.75% [wt/vol] Ficoll 400, 0.25% bromophenol blue, 0.25% xylene cyanol, 0.68× TBE [Tris-borate-EDTA] buffer) and 0.4 μl of 1 M methylmercury hydroxide (Alfa Aesaer) were added to 5 to 15 μl of the PCR product. The amount of PCR product used depended on the brightness of the band when the PCR product was run on an agarose gel. The mixture was heated at 95°C for 4 min and was then plunged into ice. A total of 15 μl of the reaction mixture was then loaded onto a precast 20% polyacrylamide–TBE gel (Novex), and the gel was electrophoresed in a Novex X-Cell Minicell electrophoresis unit with 1.5× TBE running buffer. The temperature of the lower buffer (which surrounds the gel plates) was maintained at 4 to 8°C by pumping it through a tubing coiled in a refrigerated circulating bath. To obtain the best strand separation, the gel was run for approximately 16 h at a constant 210 V. Shorter running times (4 h at 300 V) were also used, but they were found to give poorer strand separation. Precast 4 to 20% polyacrylamide–TBE gels were also found to give poorer strand separation. The gel was stained with ethidium bromide (5 μg/ml) in TBE for 20 min and was destained in deionized water for 30 min. The bands were visualized under UV light and photographed.

DNA sequencing. DNA sequencing of the PCR product was performed by following the protocol of Khorana et al. (17). The PCR products were electrophoresed in 1% low-melting-point agarose (BRL)–1× TAE (Tris-acetate-EDTA) buffer. The appropriate band was excised from the gel, and double-stranded DNA sequencing was performed directly on the DNA while it was still in the slurry agarose solution by using Sequenase II (U.S. Biochemical). The protocol was modified by the addition of 1 μl of Mn buffer.

Figures. The gels were scanned and analyzed by using Micrographix designer/photomagic 4.0.

RESULTS

The reliability of the nested PCR protocol for amplifying *B. burgdorferi ospA* from ticks was tested by using ticks from the zoonotic mouse colonies at the Centers for Disease Control and Prevention (CDC), Fort Collins, Colo. *B. burgdorferi* B31 is maintained in one colony, with transmission via *I. scapularis* ticks (14, 20). The B31 strain was passaged six times in medium before being inoculated into mice. This infected colony has been maintained for five cycles of infection, in which one cycle consists of passing the infection from mice to ticks and back to mice. Twenty-one ticks which had fed on mice from this colony and 10 ticks which had fed on mice from an uninfected colony were kindly provided by M. Dolan, J. Piesman, and W. Golde (CDC). The ticks from the infected colony are known to have an infection rate of 85 to 95%, while the ticks from the uninfected colony were completely free of *B. burgdorferi* (14). *B. burgdorferi* infection of the ticks was examined by the nested amplification protocol described above. The ticks were processed blind, with respect to their culture of origin, and all PCR reagents were pooled into a single cocktail before dividing them into aliquots and placing them into single reaction mixtures to provide additional controls. The cocktail reagents were added to the PCR tubes prior to the addition of template DNA to further remove any potential of cross-contamination. The results of the nested amplification showed that 0 of 10 ticks from the uninfected colony and 19 of 21 ticks from the infected colony tested positive (data not shown). The observed infection rate of 90.5% is consistent with the expected rate of infection in ticks fed on diseased mice.

Nested PCR amplification of the *ospA* locus was performed on 45 adult black-legged ticks collected from Shelter Island, N.Y. These ticks were collected as questing, unfed adults. The ticks consisted of approximately equal numbers of males and females. Twenty of these ticks gave strong positive results, indicating infection caused by *B. burgdorferi*. Figure 1 shows the results of the nested PCRs from 24 of the 45 Shelter Island ticks. The PCR products were electrophoresed in a 2% agarose gel with a ϕX174 *Hae*III standard. The 345-bp band is clearly visible for 12 of the 24 surveyed ticks. The observed infection rate of 44.4% for Shelter Island ticks is consistent with an infection rate of 40% determined by dissection. Of 76 ticks evaluated by indirect immunofluorescence, 30 were found visually to harbor a spirochete.

Cold SSCP analysis was run with the PCR product from the Shelter Island ticks which were positive for *B. burgdorferi* infection to determine the level of genetic heterogeneity within each tick. The SSCP gel is shown in Fig. 2. At least four strains of *B. burgdorferi* were present in the 20 Shelter Island ticks (Table 1). These strains have been arbitrarily named mobility class 1 (MC1) through MC4. These four mobility classes were found in 65, 40, 40, and 35% of the infected ticks, respectively. A total of 40% of the ticks were infected with a single mobility class of borrelia, while another 45% were infected with two mobility classes. Two ticks (10%) were infected with three mobility classes of borreliae, and one tick (5%) was infected

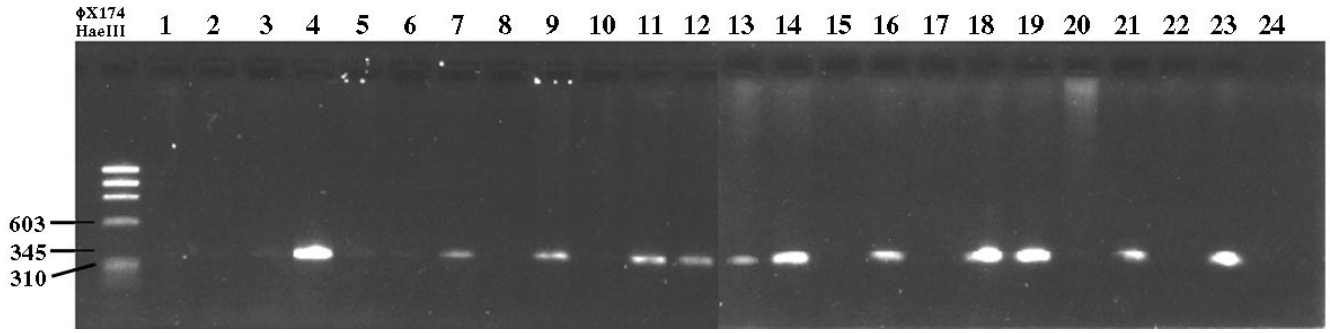

FIG. 1. Nested PCR amplification of a 345-bp fragment from the central region (base pairs 220 to 564) of the *B. burgdorferi ospA* gene from 12 of 24 Shelter Island ticks (lanes 1 to 24, respectively). The ticks in lanes 4, 7, 9, 11 to 14, 16, 18, 19, 21, and 23 are positive, showing the expected 345-bp amplified fragment. The standard (in base pairs) is a ϕX174 *Hae*III digest.

with all four mobility classes. No mobility class was found excessively alone or associated preferentially with other mobility classes. The amplified *ospA* gene segment of *B. burgdorferi* B31, which was originally collected from Shelter Island, was also subjected to SSCP analysis. Its mobility class was MC1.

The amplified segments from ticks which were infected with a single mobility class were chosen for DNA sequencing. DNAs from two isolates of each mobility class were sequenced. The polymorphic sites are listed in Table 2, where the site numbering corresponds to the beginning of the coding sequence. DNA sequencing revealed that MC1 is heterogeneous. The sequences of two selected representatives of MC1 were different from the *B. burgdorferi* B31 sequence, with a T at position 348 instead of the expected C. Other ticks contained an MC1 strain similar to B31. Therefore, MC1 has been broken down into two cryptic subclasses, MC1a and MC1b. Strain B31 belongs to MC1a, while strain CA7-CA7 (5) belongs to MC1b. Strains N40 (9) and 42373-NY3 CA7 (5) were found to belong to MC3 by sequence comparison. No strains were recorded to have sequences that matched the sequences of MC2 and MC4. The sequence of strain ZS7 (9) is similar to that of MC2 except that ZS7 contains an additional change at position 490 and so could have been derived from MC2. The changes at positions 348, 465, and 511 are synonymous changes. Position 511 is a first-position synonymous change of leucine. The change of a G to an A at position 446 changes a glycine to a glutamate.

The amplified segments from ticks infected with multiple mobility classes were also sequenced. Figure 3 shows the double band at position 465 corresponding to the C/T polymorphism. This sequence is from tick 11 (Table 1), which contains mobility classes MC1, MC3, and MC4. There was no polymorphism at position 348, showing that this tick contained MC1a. There was a polymorphism at position 446, a distinguishing characteristic of MC1. From the DNA sequencing, there is no way to show that MC3 is in the sample when both MC1 and MC4 are present, as in this case.

DISCUSSION

The present study shows that SSCP analysis of PCR products encompassing a variable region of the *ospA* gene is a reliable method of rapidly screening for genetic variability within a natural population of *B. burgdorferi* sensu stricto. The basis of the analysis relies on the reliability of the PCR amplification of *B. burgdorferi* DNA from infected ticks. This was tested by the use of ticks from infected and uninfected colonies obtained from CDC. From a blind screening of the ticks, the amplification reactions were negative for all of the ticks from the uninfected colony. An infection rate of 90.5% was observed for ticks from infected colonies. These rates are in agreement with those previously determined by CDC, thus confirming the reliability of the PCR. In other words, the frequency of false-positive and false-negative results by this assay was shown to be insignificant. A further confirmation of the reliability of this assay is that the estimated infection rate of 44% by PCR agrees with the estimated infection rate of 40% by indirect immunofluorescence.

The SSCP analysis of the Shelter Island ticks positive for infection revealed four bands with different mobilities, referred to as MC1 through MC4 in Table 1. In this analysis, each band with a different mobility represents a different strain of *Borrelia* spirochete which has infected the tick under examination. Interestingly, 45% of the infected ticks tested contained two different strains of *Borrelia*, while another 15% contained three or more different strains. The ticks assayed were all questing adult ticks and had two blood meals. Since 15% of the adult ticks were infected with three or more different strains of spirochetes, more than one strain of *B. burgdorferi* must have

TABLE 1. Distribution of the mobility classes in infected ticks

Tick	Presence of the following mobility class:			
	MC1	MC2	MC3	MC4
1	+			+
2	+			
3	+			
4		+		
5			+	+
6	+	+		
7	+	+		
8[a]	+	+		+
9	+			
10[a]				+
11	+		+	+
12	+		+	
13	+			
14	+	+		
15		+	+	
16			+	+
17			+	
18	+	+		
19	+	+	+	+
20			+	
Total	13	8	8	7

[a] The SSCP pattern is not shown in Fig. 2.

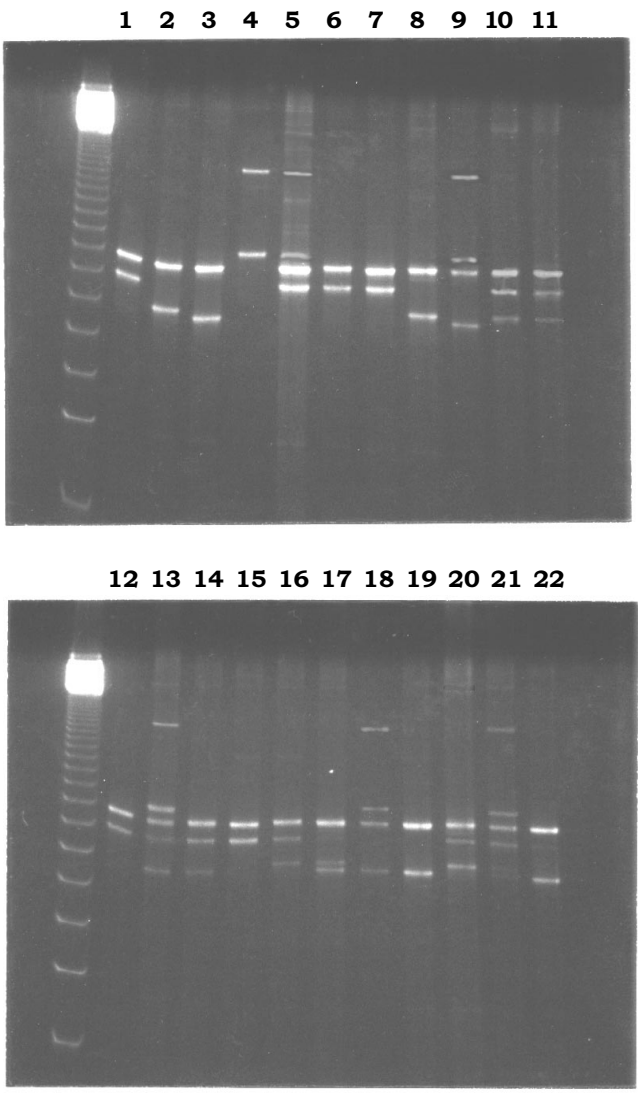

FIG. 2. SSCP gel of the *B. burgdorferi ospA* PCR product from the 20 infected ticks. Lanes 1, 2, 3, and 4, MC1, MC2, MC3, and MC4, respectively; lanes 5 through 11, ticks 1 through 7, respectively (Table 1); lane 12, tick 9; lanes 13 through 22, ticks 11 through 20, respectively. Ticks 8 and 10 (Table 1) do not appear in this figure. The size standards to the right of lanes 1 and 12 are a 123-bp ladder (catalog no. 15613; Gibco BRL). These were not used to measure size, since all of the amplified products are the same size, but were used to check running conditions and relative mobility.

been passed from a mammalian host to a tick during a single blood meal. This shows that the infection in ticks was established by a population of the spirochete rather than a single individual. Preliminary data (unpublished data) from a prospective study following the infection rate in nymphs and adults from the same cohort estimates the infection rate in nymphs to be 25%. If the probability of a tick being infected at each blood meal is the same and independent, then the infection rate in adults would be $1 - (1 - 0.25)^2 = 0.44$, and 6% of the ticks will have been infected twice. This compares with 26% of the ticks being infected with two or more strains.

In order to identify the polymorphisms responsible for the different migration patterns obtained by SSCP analysis, DNAs from representative bands of each mobility class were isolated and sequenced. The sequences from different ticks with the same mobility class were the same (except for the polymorphism found in MC1). This shows that these changes are not mutations arising in the sampled ticks but are polymorphisms in the population. The sequence shows that the amplified segments were from *B. burgdorferi* sensu stricto (9). Nucleotide substitutions (Table 2) were found to be the cause for the differences in mobility. The relationship between these strains is shown in Fig. 4. Each mobility class is different from another by a single mutation. Thus, there are no missing classes. Each mutational change alters a single base, and no class has been lost by genetic drift. This implies either that *B. burgdorferi* maintains a very large effective population size, something very rare in parasites, or that the classes have been maintained by frequency-dependent selection.

TABLE 2. Nucleotide changes defining the different mobility classes

Mobility class	Base at the following nucleotide sites:			
	348	446	465	511
MC1a	C	G	T	C
MC1b	T	G	T	C
MC2	C	A	T	T
MC3	C	A	T	C
MC4	C	A	C	C

The direction of change in Fig. 4 was determined by assuming that the base shared by *B. afzelii* and *B. garinii* is the original or primitive one and that the other one was derived recently by mutation. The T, A, C, and C residues are the primitive bases at positions 348, 446, 465, and 511, respectively. None of the mobility classes described in Table 2 contained only the primitive bases. MC4 contains three primitive bases, and all of the other mobility classes contain fewer. Thus, we have assumed

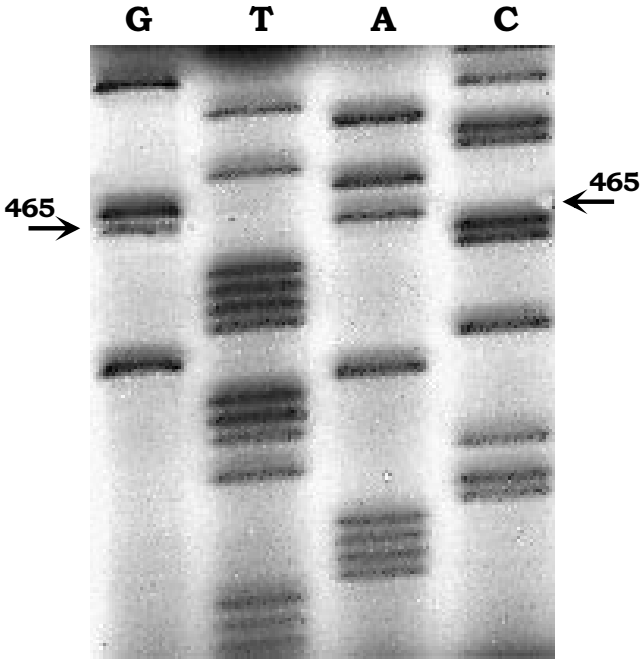

FIG. 3. Sample of the sequencing gel showing infection of a single tick by multiple strains of *B. burgdorferi*. The amplification product from tick 11 was sequenced with the internal(−) primer. A double band can be seen at position 465 corresponding to the T/C polymorphism in Table 2 which is consistent with infection by MC4 and another strain(s) (note that this sequence is off of the minus strand).

FIG. 4. A dendrogram of the relationships between the *B. burgdorferi* strains. The bars separating the mobility classes (strains) represent single nucleotide substitutions. The arrows point in the direction of the mutational or derived change.

that MC4 is the original type and that the others were derived from it by single mutational events. Thus, we are forced to assume that the T mutated to a C at position 348 in the lineage leading to *B. burgdorferi* sensu stricto and that the T in MC1b is a different mutation or that recombination has given rise to this inconsistency in the pattern of descent. In Europe, the larger amount of genetic variation found in *ospA* across the three species might prevent an accurate assessment of the genetic variation within a single tick, if there are polymorphisms in the regions of primer binding causing differential amplification.

Since the ticks acquire multiple spirochetal strains from a host during a single bite, a high degree of variability in *Borrelia* strains must be maintained in the host population. Mixed infections of different *Borrelia* species have been found in mice in Japan (19) and in humans (7). This aspect of the population biology of *Borrelia* species has not been thoroughly examined to date, but infection by multiple strains could be responsible for the variable symptomatology experienced by patients resulting from a single tick bite. As stated earlier, evidence suggests that the infection caused by different species of *B. burgdorferi* sensu lato leads to different symptomatologies in humans (1). This pattern may be repeated on a more local scale, with variable symptomatology being the result of different strains of *B. burgdorferi* sensu stricto from a single natural population. Therefore, identification of the population of strains of *Borrelia* which exist both in the natural rodent reservoir and in individual ticks would be relevant for the understanding of the variable clinical manifestations of Lyme disease and for the evaluation and development of vaccines against Lyme Disease.

ACKNOWLEDGMENTS

We thank Marc Dolan, Joseph Piesman, and William Golde for providing infected and uninfected adult ticks from the zoonotic colonies maintained at the Fort Collins facility of CDC. We thank John Dunn for discussions.

This study was supported by a grant from the National Institute of Allergy and Infectious Diseases (RO1AI33454) and by cooperative agreement number U50/CCU206608 from CDC.

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