

Infection With Multiple Strains of *Borrelia burgdorferi* Sensu Stricto in Patients With Lyme Disease

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Objective: To assess human skin biopsy specimens from erythema migrans lesions for the presence of infection with multiple strains of the Lyme disease spirochete, *Borrelia burgdorferi*.

Design: Skin biopsy specimens were obtained prospectively from patients with erythema migrans. To determine allelic differences and strain identification of *B burgdorferi*, the biopsy specimens were analyzed by cold single-strand conformation polymorphism of an amplified fragment of the outer surface protein C (*ospC*) gene. Further single-strand conformation polymorphism patterns of amplified *ospC* genes from culture isolates were compared with polymerase chain reaction products obtained directly from erythema migrans biopsy specimens.

Setting: A private dermatology office and a university medical center outpatient department.

Patients: Sixteen patients presenting with erythema migrans.

Results: Two of the 16 patients in this cohort were infected with 2 *B burgdorferi* sensu stricto strains, as evidenced by 2 *ospC* alleles in their skin biopsy results.

Conclusion: This is the first documented description of the existence of more than a single strain of *B burgdorferi* sensu stricto in a human specimen.

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LYME DISEASE is an arthropod-borne infection that is caused by spirochetes of the *Borrelia burgdorferi* sensu lato group. Three genospecies have been identified as human pathogens. All 3 representatives of the *B burgdorferi* sensu lato group, *B burgdorferi* sensu stricto, *Borrelia garinii*, and *Borrelia afzelii*, have been reported in Europe,¹ but only *B burgdorferi* sensu stricto has been reported on the North American continent. However, the simple classification of *B burgdorferi* into 3 genospecies underestimates the genetic diversity

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of this species complex. Each of the 3 pathogenic species can be divided into subspecies, and additional nonpathogenic species of *B burgdorferi* have been isolated from ticks and other nonhuman sources in North America, Europe, and Japan.^{2,3} Immunological and genetic techniques used to elucidate genetic, phenotypic, and immunological heterogeneity in *B burgdorferi* have demonstrated a considerable degree of genetic variation^{4,5}; in the GenBank DNA Sequence Database (National Center for Biotechnical Information,

Bethesda, Md), more than 100 different strains from the United States and at least 300 worldwide are listed.

The outer surface proteins of *B burgdorferi* sensu stricto are heterogeneous, and multiple strains are often found in a single tick. The impact of this diversity in human infection has not been defined, but it appears that different strains may be associated with different clinical manifestations.⁶ We conducted a study to assess the rate of multiple infection with *B burgdorferi* in patients presenting with erythema migrans. To assess multiple infection in humans, skin biopsy specimens from erythema migrans lesions were prospectively obtained from 16 patients. Informed consent was obtained from all patients participating in this study. Cold single-strand conformation polymorphism (SSCP) analysis of amplified fragments of the outer surface protein C (*ospC*) gene was used to determine allelic differences and strain identification. This method has been shown to rapidly identify different alleles of this gene; different SSCP patterns define different alleles. Two representatives of each SSCP-defined allele were sequenced and found to be identical. Sequences of SSCP-defined alleles were similar, had variability of no more

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PATIENTS, MATERIALS, AND METHODS

PATIENT SAMPLES

Patients presenting with erythema migrans to the Lyme Disease Center at Stony Brook, NY, or the private practices of 2 collaborating physicians on eastern Long Island, New York, were asked to participate in this study. A total of 16 patients were enrolled. Thirty percent of the patients seen by the collaborating physicians during the summer of 1997 qualified for enrollment. For those giving informed consent, two 2-mm skin punch biopsy specimens were taken from the advancing border of the erythema migrans lesion. The first was placed in formalin for histopathologic examination and the second was used for culture and direct DNA extraction. We have found that 2-mm biopsies are less invasive and as efficient as 4-mm biopsies for yielding positive cultures, as reported by Wormser et al.⁸ All enrollees also consented to have blood drawn for serum antibody testing for Lyme disease. All patients met the Centers for Disease Control and Prevention surveillance definition of Lyme disease. Punch biopsy material was obtained using sterile technique. A small portion of the biopsy specimen (approximately one third) was ground in a disposable microtissue grinder (Kontes Pellet Pestle; Kontes Glass, Vineland, NJ), and DNA was extracted using a specially formulated Chelex resin (InstaGene Matrix; Bio-Rad Laboratories, Hercules, Calif) following the manufacturer's protocol. The rest of the biopsy specimen was immediately placed into a vial containing 4 mL of BSK-H culture media (Sigma Chemical Co, St Louis, Mo) with antibiotics. *Borrelia burgdorferi* was cultured by standard techniques. Two skin biopsy specimens from uninfected laboratory mice served as negative controls during culturing and DNA extracting procedures.

POLYMERASE CHAIN REACTION TESTING

An internal 340-bp fragment corresponding to the 5' region of *ospC* that was suitable in size to be studied by SSCP analysis was amplified using forward primer 5'-AAA GAA TAC ATT AAG TGC GAT ATT-3' and reverse primer 5'-CAA TCC ACT TAA TTT TTG TGT TAT TAT-3', as described previously.⁷ The 3' region of *ospC* (314 bp) was amplified using forward primer 5'-TTG TTA GCA GGA GCT TAT GCA ATA TC-3' and reverse primer 5'-GGG CTT GTA AGC TCT TTA ACT G-3'. Amplification was done in 50 μ L of a solution containing 10 \times polymerase chain reaction (PCR) buffer (100-mmol/L Tris hydrochloride [pH 8.3] and 500-mmol/L potassium chloride; Perkin-Elmer/Cetus, Norwalk, Conn), 2.5-mmol/L magnesium

chloride, deoxynucleoside triphosphates at 0.2 mmol/L per nucleotide, 2.5 U of *Taq* polymerase (Perkin-Elmer/Cetus), and each primer at 0.5 μ mol/L. The amplification reaction was carried out for 40 cycles in a DNA thermal-cycler (PTC-100; MJ Research Inc, Watertown, Mass), with an amplification profile of denaturation at 95°C for 40 seconds, annealing at 54°C for 35 seconds, and extension at 72°C for 1 minute, after an initial denaturation step at 96°C for 2 minutes. Negative controls were included in each experiment to control for contamination. The amount of DNA used for subsequent SSCP analysis was estimated after visualizing the PCR product on an agarose gel with ethidium bromide.

COLD SSCP ANALYSIS

Amplified *ospC* gene fragments were analyzed for genetic variations by the cold SSCP protocol described by Hongyo et al.⁹ This method allows the 2 strands to migrate separately in very specific, electrophoretic conditions. Migration patterns differ when there is sequence diversity between samples. By targeting variable regions of the *ospC* gene, genetic variability is quickly and easily detected. A volume of 0.5 to 15 μ L of the PCR product was added to a mixture containing 4 μ L of 5 \times Tris-boric acid-ethylenediaminetetraacetic acid (TBE) Ficol sample buffer (NOVEX, San Diego, Calif) and 0.4 μ L of 1-mmol/L methylmercury hydroxide (Alfa Aesaeer, Ward Hill, Mass). The final volume of 20 μ L was achieved by addition of the required amount of water. The sample mixture was heated to 95°C for 4 minutes, then plunged into ice prior to loading the entire 20 μ L into the gel sample well. The sharpest bands were observed when the sample was applied to a pre-cast 20% TBE gel (NOVEX). Electrophoresis was performed using a temperature-controlled electrophoresis system (ThermoFlow ETC Unit; NOVEX) with 1.25 \times TBE running buffer. Single-strand conformation polymorphism runs were conducted at a constant temperature of 8°C for 17 hours at 240 V in order to reveal discernible mobility shifts. Poor strand separation was achieved when shorter running times were used. Gels were stained with 0.5- μ g/mL ethidium bromide in 1 \times TBE buffer for 25 minutes and destained in distilled water for 30 minutes. Stained bands were viewed using a 340-nm UV staining box.

DNA SEQUENCING

Double-stranded PCR fragments of each mobility class were purified by agarose gel electrophoresis and subjected to automated DNA sequencing using fluorescent dideoxy terminator chemistry and the forward and reverse primers originally used for PCR amplification, as described previously.⁷

than 2%, or had significantly different sequence divergence of at least 8%. Similar alleles include members of the same major group and, using these parameters, Wang et al⁷ defined 13 alleles representing 11 major groups. Using published sequences of *ospC* from GenBank, a total of 19 major groups were described. The hypothesis is that these major groups define *ospC* serotypes. Herein, we apply this technology to the analysis of isolates from a cohort of patients with human Lyme disease and show that SSCP patterns of amplified *ospC*

genes identify individuals infected with more than 1 strain of the spirochete.

RESULTS

CULTURE ISOLATION

Patients were all adults presenting with erythema chronica migrans. All had a single, primary lesion with 1 exception. Patient 97006 had 2 lesions associated with 2 sepa-

Characteristics of 16 Borrelial Strains Isolated From Erythema Migrans Biopsy Specimens*

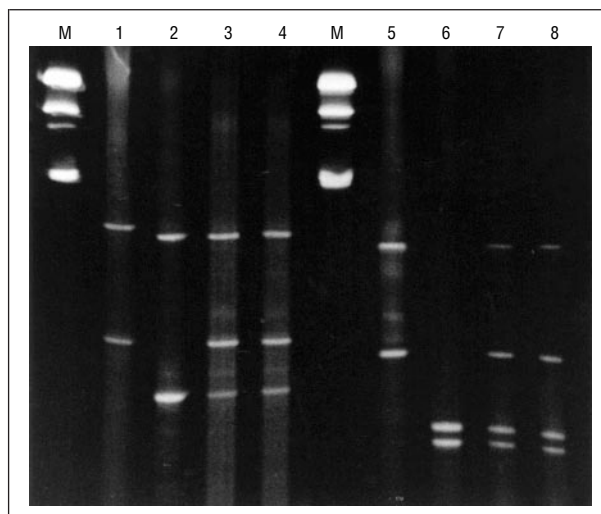
Patient No.	Culture	ospC MC	Template for PCR		ospC MC
			5' Region	3' Region	
97002	+	OC 12	ND	ND	OC 12
97003	+	OC 2	+	+	OC 2
97004	-	-	+	-	OC 10
97005	+	OC 15	+	+	OC 15
97006	-	-	+	+	OC 2 and OC 3
97007	+	OC 8	+	+	OC 8
97008	+	OC 2 and OC 3	+	+	OC 2
97009	+	OC 2	+	+	OC 2
97010	+	OC 12	+	+	OC 12
97011	+	OC 2	+	+	OC 2
97012	+	OC 15	+	+	OC 15
97013	+	OC 12	+	+	OC 12
97026	+	OC 15	+	+	OC 15
97051	-	-	+	-	OC 10
97061	+	OC 12	ND	ND	OC 12
97064	+	OC 1	+	+	OC 1

*DNA templates were used for polymerase chain reaction (PCR) testing, and outer surface protein C (ospC) gene mobility classes (MC) were identified by cold single-strand conformation polymorphism analysis. + Indicates positive culture; -, negative culture; and ND, no disease.

rate tick bites. A biopsy was performed on only 1 of the lesions in this case. All erythema migrans lesions were photographed before a skin biopsy specimen was obtained from the peripheral aspect of the primary lesion. Patients did not have secondary lesions and had not received antibiotic treatment for erythema migrans prior to the time biopsy samples were taken. *Borrelia burgdorferi* was successfully cultured from 13 of the 16 patients in the study. The **Table** shows strains that were isolated from the erythema migrans lesions.

PCR AMPLIFICATION

Fourteen skin biopsy samples were available for PCR analysis. We obtained PCR-amplified DNA from the 13 culture isolates using either the 5' or the 3' region of the *ospC* gene as a target. The 14 skin biopsy samples yielded PCR products corresponding to the 5' region of *ospC*, but only 12 PCR products were obtained from the 3' region of the gene (Table). The amplified 5' region is a 340-bp fragment corresponding to bp 6 to bp 346, based on the *ospC* sequence of strain B31 (GenBank accession number U01819, with the start codon as base 1). The amplified 3' region is a 314-bp segment of *ospC*. The fragments were visualized on a 1.6% agarose gel stained with ethidium bromide. The 3' region could not be amplified from 2 specimens, 97004 and 97051. Subsequent SSCP analysis of the 5' segment of *ospC* of these 2 specimens identified them as OC 10. The primer used in this reaction contains 8 mismatches with the OC 10 sequence. This explains the lack of product from these isolates. The skin biopsy sample from 1 individual (patient 97006) from which *B burgdorferi* was not isolated by culture showed multiple *ospC* alleles by SSCP analysis. All control specimens evaluated were culture negative. All of the specimens produced a *B burgdorferi*-



Identification of multiple clones of *Borrelia burgdorferi* in patients with erythema migrans. Included are single outer surface protein C (ospC) clones OC 2 and OC 3 in lanes 1 and 2 (front half of ospC), respectively, and lanes 5 and 6 (back half of ospC), respectively, along with multiple ospC alleles of samples 97006 (lane 3, front half; lane 7, back half) and 97008 (lane 4, front half; lane 8, back half). M indicates molecular weight marker I DNA/HindIII digest; it was only used to check running conditions and relative mobility, not to measure size.

feri-positive result by either culture or PCR amplification from tissue. None of the control specimens yielded a detectable band after the amplification procedure.

SSCP ANALYSIS

The SSCP patterns of all *ospC* amplification products were obtained. These products were run side-by-side in SSCP analysis with known examples of all of the SSCP classes in order to detect even slight shifts in electrophoretic mobility. We use DNA purified from representative strains of the bacterium of each of the *OspC* alleles. These PCR products of known *ospC* mobility class are the standards for analysis of new strains. Additionally, representatives of each *ospC* mobility class from the present study were sequenced in order to confirm SSCP analysis data.

The SSCP patterns of OC 1, 2, 3, 8, 10, and 12 from these patient samples were identical with the corresponding mobility classes of the 5' and 3' regions of *ospC* from strains isolated from ticks that were collected on the eastern end of Long Island.⁷ The analysis of isolates from patients 97005, 97012, and 97026 showed a new mobility class that had not been detected previously and was therefore named OC 15. Subsequent sequence analysis and homologous search demonstrated 100% homology with the *ospC* gene of strain 26815 (GenBank accession number L42897). This *B burgdorferi* strain was recently isolated from a chipmunk in Connecticut.¹⁰ Isolates from patients 97006 and 97008 were found to contain 2 mobility classes by SSCP analysis, OC 2 and 3 (**Figure**). Thus, 12% (2/16) of samples studied were found to contain multiple mobility classes by SSCP analysis. Results of SSCP analysis of DNA from culture isolates and skin biopsy samples were identical. Thus, we could confirm our method and rule out contamination with spurious am-

plicons. Contaminating *B burgdorferi* DNA would lead to a distinct *ospC* SSCP pattern within all samples.

COMMENT

In the present study, we have demonstrated the presence of infection with more than 1 strain of *B burgdorferi sensu stricto* in human patients. Using SSCP analysis, we were able to detect multiple infection with *B burgdorferi sensu stricto* in skin biopsy samples of patients suffering from early Lyme disease. Prior reports have demonstrated that ticks and reservoir mammals often harbor multiple *Borrelia* strains.¹⁰⁻¹² Different strains may be associated with different disease manifestations. A recent study in Europe underscored the relatively high prevalence of pluri-infection with different *B burgdorferi* genospecies in Lyme disease.¹³ In a group of patients with neuroborreliosis, 44% (8/18) were shown to be infected with at least 2 *B burgdorferi sensu lato* strains, as revealed by PCR testing and immunoblotting analysis. In addition, all 3 species of *B burgdorferi sensu lato* were identified among isolates from acrodermatitis chronica atrophicans lesions. That report also found that *B afzelii* is the predominant but not the exclusive etiologic agent of acrodermatitis chronica atrophicans.¹⁴ However, it remains to be established whether infection with multiple *B burgdorferi sensu lato* species influences the clinical course of patients with Lyme disease. Although *B burgdorferi sensu stricto* is the only pathogenic genospecies in North America, clones within this genospecies have different pathogenic potential. Specifically, *ospC* type correlates with secondary clinical manifestations in humans.¹⁵ Infection with multiple strains of the spirochete may augment pathogenicity in individuals in addition to increasing the potential of infection with a pathogenic strain.

The *OspC* protein has recently been the subject of a variety of investigations because of its potential as a highly immunogenic surface protein¹⁶ as well as a protective antigen in the mouse model.¹⁷ We used the *ospC* gene as a target for SSCP analysis because of the well-characterized heterogeneity of this gene, as described in earlier studies.⁷ The advantage of this approach is that a variety of strains can be identified by simply comparing different mobility shifts of single-stranded DNA with known standards. Diagnostic strain identification can then be achieved in less than 24 hours. Nevertheless, one needs to be aware of the fact that studying genetic polymorphism by SSCP analysis is sensitive to sequence variation within the gene but not necessarily sensitive to sequence variation at other loci in the genome, as described previously.¹⁷ In fact, SSCP analysis of *ospC* can detect single base pair changes with no determination of whether differences are silent within the DNA or affect a change in the amino acid sequence of the lipoprotein. Therefore, it is crucial to subsequently determine the gene sequences of the mobility classes that are detected by SSCP analysis.

Interestingly, when studying the heterogeneity of *ospC* in *B burgdorferi* that was obtained from ticks collected on Long Island, Wang et al⁷ recently identified 13 *ospC* alleles that were subsequently cloned and sequenced. These *ospC* genes served as standards for our study. The 5' and the 3' regions of the *ospC* gene served as templates for PCR testing, since it has been shown that

either the front or the back half can be used to study the heterogeneity of the *ospC* gene.⁷ Using these standards to compare the *ospC* patterns retrieved from human skin biopsy specimens with multiple infections, we were able to show the presence of a maximum of 2 alleles.

Thus, SSCP analysis was successful not only in identifying allelic differences in amplified fragments of *ospC*, but also in rapidly detecting infection with multiple strains of the spirochete. Interestingly, only 12% (2/16) of the human isolates we studied showed multiple infection. Wang et al analyzed allelic *ospC* differences in ticks collected on Long Island and found that every other tick (50%) was infested with at least 2 different strains of *B burgdorferi*. Given the high rate of multiple *B burgdorferi* strains in ticks, the unexpectedly low number of multiple infections (12%) in humans implies that not all strains are equally pathogenic. The lower rate of multiple infection cannot be explained by postulating that culturing techniques select for specific genotypes of *B burgdorferi*, as suggested by Norris et al.¹⁸ The *ospC* SSCP pattern of the cultured spirochetes was identical to the patterns found in the original punch biopsy specimens.

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Correction

Error in Byline. In the vignette titled "Two Familial Cases of Epidermolysis Bullosa Simplex Successfully Treated With Tetracycline," published in the August issue of the ARCHIVES (1999; 135:997-998), Carla Retief, MD, should have been listed as the senior author.