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Borrelia burgdorferi ospC Heterogeneity among Human and Murine Isolates from a Defined Region of Northern Maryland and Southern Pennsylvania: Lack of Correlation with Invasive and Noninvasive Genotypes

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B. burgdorferi **invasiveness correlates with *ospC* genotype. To test this hypothesis and whether identical genotypes infect humans and small mammals in specific sites, *B. burgdorferi ospC* heterogeneity was tested among isolates from northern Maryland and southern Pennsylvania. Six culture-positive patients allowed collection of small animals from their properties, and spirochetes from animals trapped within 300 yards of each patient's home were isolated. 3′ *ospC* sequences were compared to reference sequences. Of the 7 human and 15 mouse DNA templates that produced reliable sequences, all clustered with references into only four and seven distinct clades, respectively. A human and a mouse isolate with the same *ospC* were seen in only one locality, and five of six sites contained two or more *B. burgdorferi ospC* clones. Four invasive patient isolates and six small mammal isolates clustered with "noninvasive" reference *ospC* genotypes. A high degree of *ospC* diversity exists among *B. burgdorferi* isolates in Maryland and Pennsylvania, even in narrowly defined geographic localities. Dissemination in mice and humans by noninvasive *ospC* types contradicts the *ospC* invasiveness hypothesis. Alternative genetic markers for *B. burgdorferi* disseminated disease should be investigated.**

Lyme borreliosis is an infectious disease caused by *Borrelia burgdorferi* sensu lato (4). These spirochetes are transmitted by *Ixodes* spp. ticks that maintain an infectious cycle by feeding on reservoir hosts such as *Peromyscus leucopus* and other small mammals; humans are accidental hosts (5, 9). Three *Borrelia* species that cause human disease include *B. burgdorferi* sensu stricto (North America), *B. afzelii*, and *B. garinii* (Europe and Asia) (2, 27, 33). In North America the geographic distribution is well documented, with *B. burgdorferi* and Lyme disease expanding and clearly endemic in the mid-Atlantic region including Maryland (1, 22).

B. burgdorferi plasmid-encoded outer surface protein C genes (*ospC*) are implicated in transmission and dissemination of *Borrelia* between ticks and mammals (12, 18, 20, 24, 32). Diversity of *ospC* has lead to the classification of *B. burgdorferi* clones into human invasive and "noninvasive" groups (14, 16, 25, 28), but these studies have not evaluated *ospC* as an invasive factor in small mammals. Because of *ospC* heterogeneity, it has been used as marker for clonality in epidemiological studies that suggest that human and small mammal isolates from the same region are restricted to only a single or a few clones (14, 16). Neither geographic nor *ospC* heterogeneity of *B. burgdorferi* infecting humans and mice has been extensively studied in the mid-Atlantic States. The present study was designed to test two hypotheses: (i) that only limited *ospC* diversity exists among *B. burgdorferi* infecting humans and mice from the same localities in northern Maryland, and (ii) that *ospC* genotypes associated with invasiveness in humans extends to Maryland and to small mammals.

MATERIALS AND METHODS

Patients and *Borrelia* isolates. From a study of clinical Lyme disease in Maryland during 2001 (J. S. Dumler, unpublished data), eight *Borrelia burgdorferi* isolates from six culture-confirmed patients were selected from those who also agreed to allow collection of small animals from their properties. Six isolates were from human plasma, and two were from erythema migrans lesions. Isolates were confirmed to be *B. burgdorferi* by PCR amplification of the *ospA* gene (data not shown), as previously described (7). Five patients resided in Baltimore County, Maryland, and one was from neighboring York County, Pennsylvania. All patients had early Lyme disease with erythema migrans lesions, and five had manifestations suggesting early disseminated disease, including one with multiple erythema migrans and four with influenza-like illnesses. Patients provided informed consent prior to the study as approved by the John Hopkins Medicine Institutional Review Board.

Growth of human *Borrelia* isolates. Low-passage (<2 passages) bacteria were propagated from dimethyl sulfoxide-preserved frozen patient isolates. Approximately 0.5 ml of each frozen isolate was transferred to 5 ml of modified BSK-II medium (31) and incubated at 34°C in 5% CO_2 until sufficient growth was confirmed by fluorescent microscopy using acridine-orange staining. Two control cultures were used: *B. burgdorferi* strain 297 (courtesy of Dennis Grab, the Johns Hopkins University School of Medicine) and a randomly selected human isolate previously prepared in the lab and used for internal control purposes (lab strain).

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TABLE 1. Accession numbers of the 21 major *ospC* group reference strains (from GenBank), used for alignment purposes, and their geographical origin/source

Major *ospC* group	GenBank accession no.	Geographical origin/source
A	AF029860	New York
B	AF029861	New York
C	AF029862	New York
D	AF029863	New York
E	AF029864	New York
F	AF029865	New York
G	AF029867	New York
H	AF029868	New York
I	AF029869	New York
J	AF029870	New York
K	AF029871	New York
L	L42899	United States
M	U01892	Connecticut
N	L42897	Connecticut
O	X84778	New York
P	U91796	France
Q	U91790	France
R	U91791	Spain
S	U91793	Germany
T	AF065143	New York
U	AF065144	New York

Mouse *B. burgdorferi* isolates. Mice were trapped using 60 Sherman live traps (H. B. Sherman, Tallahassee, FL) baited with bird seed and placed within 300 yards of each patient's home. After administration of a light anesthetic, a 2-mm-diameter ear tissue sample was taken using a biopsy tool (Accuderm Inc., Ft. Lauderdale, FL) and placed in BSK-II medium for transport. A blood sample was taken from each capture by retro-orbital sampling and placed directly on ice for transport. Captured rodents were released at the site of capture. Trapping and sample collection were performed according to Hofmeister et al. (9) as approved by the John Hopkins University Institutional Animal Care and Use Committee.

Mouse blood and skin cultures were incubated at 34°C in 5% CO_2 and examined weekly for up to 8 weeks. Acridine orange-positive samples were subcultured in BSK-II medium supplemented with 50 μg/ml of ciprofloxacin and 20 μg/ml of rifampin to reduce contamination with other bacterial species. When appropriate, 0.45-μm-pore-diameter syringe-driven filter units were used (11) to remove contaminating bacteria. After 2 to 3 weeks of log phase growth, aliquots of each culture were cryopreserved at −80°C. Despite the use of antimicrobials and filtering, contamination persisted for many cultures, resulting in poor *Borrelia* recovery in 11 field samples later excluded from further study. Despite all attempts, many isolates grew poorly in the medium preparations used.

DNA extraction. During log phase growth, 1.5 ml of each *Borrelia* culture was centrifuged at 13,800 × *g* for 25 min. The bacterial pellet was resuspended in 200 μl of phosphate-buffered saline, and DNA was prepared using a QIAamp DNA Mini kit (QIAGEN, Inc.) and the manufacturer's protocols. DNA preparations were stored at −20°C until analyzed.

PCR methods and conditions. The 3′ and 5′ ends of o*spC* were PCR amplified using conserved primers as described previously (25). PCR was performed using an eLONGase kit (Invitrogen Inc.) per manufacturer's instructions with the addition of 100 pmol of each primer and using 2 μl of *B. burgdorferi* isolate DNA that was not otherwise quantitated. The reaction was completed as follows: initial denaturation at 96°C for 2 min, followed by 40 cycles of denaturation at 95°C for 40 s, annealing at 54°C for 35 s, and extension at 72°C for 1 min. The 5′ *ospC* amplification did not produce a reliable product from all samples; therefore, all subsequent analyses were based on amplification of a ~314-bp fragment from the 3′ end. A negative control (deionized water) was included in each PCR. PCR amplification of a *B. burgdorferi*-specific fragment from the *flaB* gene was used to confirm that small mammal isolates from which *ospC* was amplified were *B. burgdorferi* (13).

Sequencing and phylogenetic analysis. *B. burgdorferi ospC* PCR products (25 μl) were electrophoresed in 2% agarose gels, stained with ethidium bromide, and excised for purification using a QIAquick gel extraction kit (QIAGEN Inc.). Sequencing was performed using the forward and reverse PCR primers (25) and a fluorescent automated method (ABI 3100 genetic analyzer). The resulting forward and reverse *ospC* sequences were initially assembled using the Contig Assembly Program in BioEdit (8) to generate consensus sequences. Discrepancies were resolved by inspection of the original chromatograms or by additional sequence analysis.

To validate the use of the 3′ *ospC* sequences for phylogenetic comparisons, 21 *B. burgdorferi* sequences corresponding to the major *ospC* groups (25, 30) were retrieved from GenBank (Table 1) and aligned using ClustalX (10). Primer sequences flanking the region of the alignment corresponding to the 3′ amplicon were removed, and the resulting sequences were gap stripped and realigned for phylogenetic analysis. Full-length and 3′ *ospC* trees were generated using the neighbor-joining algorithm, and topologies of the trees were compared. The consensus sequence assemblies of the study isolates were aligned with the reference 3′ *ospC* sequences. These combined sequences were then trimmed for optimal pairwise alignments and used to generate a neighbor-joining tree with bootstrapping 1,000 times. The tree was viewed with TreeView software (19).

RESULTS

Isolation and detection of *Borrelia*. Human and mouse specimens. With the broad assumption that patient exposure occurred on or around their properties, small mammals were collected from the properties of the six selected patients. From the six locations, a total of 61 small mammals (48 white-footed mice [*Peromyscus leucopus*], 9 chipmunks [*Tamias striatus*], 1 vole [*Microtus pennsylvanicus*], and 2 shrews [*Sorex* spp.]) were captured. Blood and ear biopsies for culture were obtained from each animal except for the shrews, from which blood was not obtained, and two chipmunks, from which ear biopsies were not obtained. From 118 small mammal tissue or blood cultures, spirochetes were observed in 71 (60.2%), including 27 blood and 44 ear biopsy cultures (Table 2). Contamination was a significant problem, as 54 (45.8%) cultures contained other bacteria. Contamination levels were minimal in all but 11 cultures. Bacterial contaminants could not be cleared from these 11 cultures, and they were subsequently excluded from further analysis. Growth of borreliae was also poor for most samples despite adequate growth of laboratory control cultures (*B. burgdorferi* strain 297).

***ospC* amplification.** An attempt to amplify the complete ~650-bp sequence of *ospC* was initiated, as previously described (25), but amplification was inconsistent. Therefore, the 3′ and 5′ ends of *ospC* were separately amplified. Although the 5′ *ospC* amplifications often failed, the 3′ reactions were more often successful and were used as the basis for phylogenetic analysis. To validate this approach, full-length sequences of 21

TABLE 2. Total number of animals and patient samples tested and number of acridine orange-positive and number of *ospC* PCR-positive samples

Specimen type	No. of samples	No. of AO-positive samples[a] (%)	No. of PCR-positive samples (%)
Patients			
Blood	6	6 (100)	6 (100)
Skin	2	2 (100)	1 (50)
Total (%)	8	8 (100)	7 (87.5)
Small mammals			
Blood	59	27 (45.8)	2 (7.4)
Ear	59	44 (74.6)	16 (36.4)
Total (%)	118	71 (60.2)	18 (25.4)

[a] Acridine orange (AO) staining on culture samples.

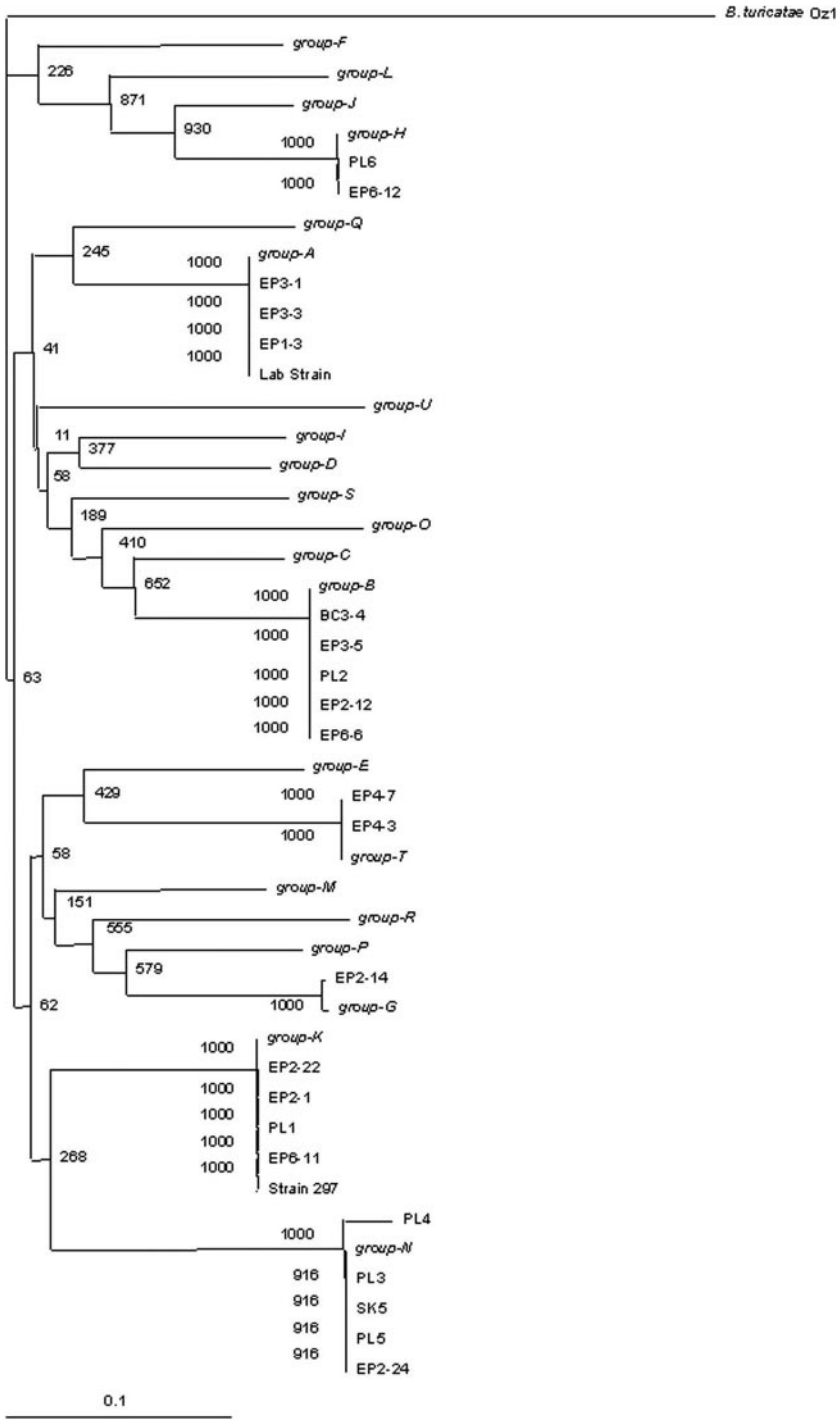

FIG. 1. Phylogenetic tree derived from the nucleotide sequences of *ospC* genes of humans (prefix PL or SK) and mouse isolates and the reference strains (italics) of *Borrelia burgdorferi*, using *Borrelia turicatae* (GenBank AF129434) *ospC*-like gene as outgroup. Lab strain and strain 297 were used as controls for amplification. The bar represents the number of nucleotide substitutions per 1,000 bp. Bootstrap values based on 1,000 replicates are shown for each node.

previously published *ospC* reference sequences and sequences corresponding to the predicted 3′ amplicons were separately aligned and resulted in similar tree topologies, for which each reference group identified a unique clade.

***B. burgdorferi ospC* PCR and sequencing.** All six *B. burgdorferi* DNA templates from human plasma cultures and one of the two human skin cultures produced an amplicon of approximately 314 bp with the 3′ *ospC* primers. Only 18 of the 71 *Borrelia* cultures derived from small animals amplified with the *ospC* primers. From the 18 PCR positives, 2 were from blood and 16 were from ear biopsy cultures. Both controls (strain 297 and lab strain) amplified in all PCRs; negative controls

TABLE 3. Site of the patients' specimens and small animal samples that tested *ospC* PCR positive

Site	Patient isolates (blood/skin lesion)	Small animal isolates[a]
1	PL1/none	EP1-3, BC1-10
2	PL2/none	EP2-1, EP2-12, EP2-14, EP2-22, EP2-24
3	PL3/none	EP3-1, EP3-3, EP3-5, BC3-4
4	PL4/none	EP4-3, EP4-7, EP4-6
5	PL5/SK5	None
6	PL6/none	EP6-6, EP6-9, EP6-11, EP6-12

[a] EP, ear biopsy; BC, blood culture.

(deionized water) did not produce detectable products. Unambiguous forward and reverse sequences were obtained from all except for three amplicons (one blood and two ear biopsies).

***B. burgdorferi ospC* heterogeneity among human and mouse isolates from the six properties.** A total of 45 sequences, including 21 references from GenBank, 2 laboratory controls, and 22 obtained from human and small mammal cultures, were aligned to generate a phylogenetic tree. Sequences derived from human isolates were found in four distinct clusters, while sequences originating from animal reservoirs were found in seven clusters (Fig. 1). Table 3 summarizes the findings and shows the 7 human and 15 small mammal isolates and the sites from which they were collected. Only at site 2 were *B. burgdorferi* isolates with the same *ospC* genotype observed in both human (isolate PL 2) and mouse (isolate EP2-12) isolates.

All *B. burgdorferi* sequences obtained from human isolates corresponded with *ospC* reference groups, including one each that clustered with groups K and B that are associated with invasive infections (Table 4) (25). The remaining blood isolates clustered with *ospC* groups not before identified to contain invasive strains, including PL6 in group H (Fig. 1) and PL3, PL4, and PL5 in group N (25). The one skin isolate (SK5) examined had an *ospC* sequence identical to that of a blood isolate from the same patient that clustered with group N as well.

From the 15 small mammal blood or ear biopsy isolate sequences, 10 clustered with invasive strains of major *ospC* groups (Fig. 1). Of those remaining, five clustered with noninvasive *ospC* groups (Fig. 1). Of those that matched invasive *ospC* groups, three each matched with group A (EP1-3 [site 1], EP3-1 [site 3], and EP3-3 [site 3]) and group K (EP2-1 [site 2], EP2-22 [site 2], and EP6-11 [site 6]). The remaining four matched with invasive *ospC* group B (EP2-12 [site 2], EP3-5 and BC3-4 [site 3], and EP6-6 [site 6]) (Table 4). The remaining four small-animal ear biopsy isolates EP2-14 (site 2; group G), EP4-3 and EP4-7 (site 4; group T), and EP6-12 (site 6; group H) clustered with *ospC* groups G, H, and T that are associated with noninvasive infections (Table 4) (25).

TABLE 4. Human and animal strains of Baltimore County, MD, and York County, PA, and the *ospC* groups with which each clustered

Source	Isolate name	Clustered with *ospC* group[a]								
		Invasive				Noninvasive				
		A	B	K	I	G	H	J	T	N
Small-animal ear biopsy or blood culture	EP1-3	×								
	EP2-1			×						
	EP2-12		×							
	EP2-14					×				
	EP2-22			×						
	EP2-24									×
	EP3-1	×								
	EP3-3	×								
	BC3-4		×							
	EP3-5		×							
	EP4-3								×	
	EP4-7								×	
	EP6-6		×							
	EP6-11			×						
	EP6-12						×			
Human blood	PL 1			×						
	PL 2		×							
	PL 3									×
	PL 4									×
	PL 5									×
	PL 6						×			
Human skin	SK 5									×
Control isolate	297			×						
	Lab strain	×								

[a] The major *ospC* groups were defined in references 25 and 30, and each group is represented by a letter. None of the listed isolates clustered with any *ospC* group associated with ticks (i.e., group D, E, F, or L). ×, isolate clustered with indicated group.

DISCUSSION

OspC of *B. burgdorferi* is a highly heterogeneous lipoprotein implicated as a potential virulence factor for tissue invasion and dissemination in humans (25). The diversity of *ospC* has been investigated extensively in tick and human isolates sampled from some regions (3, 9, 14, 23, 30) but has not been extensively explored among the isolates of small mammals that serve as critical reservoirs for maintaining the infectious cycle (6, 15). Moreover, few studies regarding the similarity of *Borrelia* isolates among small animals and humans from the same or related geographic regions have been conducted (16). The present study considers these factors in an examination of *B. burgdorferi ospC* heterogeneity in a Lyme disease-endemic region of northern Maryland and southern Pennsylvania.

PCR amplification of full-length *ospC* was unreliable for samples derived in this study; therefore, a modified method derived by comparing the sequences from ~240 bp of the 3′ end of *ospC* was used to yield a phylogenetic tree that allowed distinct identification of each reference group as demonstrated for the tree of the full-length gene (14). When applied to *B. burgdorferi* isolates from humans and mice collected within a limited geographic region in the Northern Maryland and Southern Pennsylvania, these data demonstrate that there is a high degree of *ospC* diversity. Matching human and animal isolates were only recovered from a single location/site, but this may be an artifact of small sample size. With the high degree of *ospC* polymorphism observed in this study, larger numbers of mammals may need to be captured to determine whether all or most mouse-infective clones are also infective for humans. Altogether, five of the six sites contained two or more *B. burgdorferi ospC* clones, including four at one site and three at another. Only a single clone was identified in site 5 (Pennsylvania). Unfortunately, *ospC* typing of animal isolates from that location was unsuccessful and would have likely revealed additional polymorphism.

The overall analysis is hampered by several potential biases. Although 60% of the 118 animal samples were culture positive, 18 were *ospC* PCR positive and only 15 were successfully sequenced despite repeated attempts. The inability to amplify *ospC* from field (mammal) isolates may be due to additional sequence variability at the priming sites resulting in failed PCR. Alternatively, the inability to amplify *ospC* and *fla* from small mammal blood cultures in which *Borrelia* were visualized could be the result of significant blood contamination and inhibition of PCR in spite of DNA purification methods. Another potential pitfall relates to the selection of *B. burgdorferi* clones in culture, possibly leading to a selection bias (17). For illustration, among the two human skin *B. burgdorferi* isolates examined, one failed to amplify with *ospC* primers, yet amplified with primers that target *ospA*, suggesting that even greater *ospC* heterogeneity exists among *B. burgdorferi* pathogenic for humans than is presently evaluated. Likewise, the inability to obtain unambiguous direct sequences from 17% of amplicons suggested that some cultures may be mixed. This proportion is similar to that described by Seinost et al. (26) and further illustrates the degree of potential heterogeneity in these regions.

Seinost et al. suggested that *ospC* typing could differentiate non-pathogenic from *B. burgdorferi* that are pathogenic for humans and may further differentiate strains that cause localized skin infection from those that cause systemic/invasive disease (25). However, four of the six human plasma isolates in the present study did not cluster with the major o*spC* groups previously implicated in invasion (25). In contrast, 10 of the 15 animal isolates clustered with the major *ospC* groups designated as invasive, potentially reflecting the innate ability of these groups to disseminate, even in the animal host. Unlike Seinost's observations, the major *ospC* groups A and K did not predominate in humans from this region (25, 29) but were found in 6 of 15 small mammal isolates (Table 4). Qiu et al. found group K to be one of the most common clones on the U.S. East Coast, including 14 of 22 *B. burgdorferi* identified in *I. scapularis* from Maryland (23), whereas it only accounted for 18% of our *ospC*-positive samples.

B. burgdorferi ospC groups G, H, J, N, and T are usually associated only with erythema migrans and not invasion in humans (25). However, in this study, their invasiveness in both animals and humans was demonstrated by identification in disseminated sites. Although not well investigated, animal invasiveness in groups G and T suggests the potential for invasion in humans.

The *ospC* groups D, E, F, and L, previously found only in ticks (25) were not identified in this study, confirming how infrequently they are observed in humans or reservoirs. Of more interest is the absence of these *ospC* groups in mice as well, despite their presence in ticks collected from this region (23). As *B. burgdorferi* exists in an obligate relationship that requires transmission between the tick and mammalian host (5), it is expected that these isolates should be identified in small animals. Alternatively, one may hypothesize that other mammalian hosts beside *P. leucopus* may harbor the *ospC* groups that have been characterized as predominantly tick associated. Another alternative could be that such *ospC* groups depend on transovarial transmission, a mechanism that is usually considered highly inefficient for *B. burgdorferi* (21). An expanded investigation may shed light on the extent of the host distribution of major *ospC* groups.

These Maryland and southern Pennsylvania studies confirm a high degree of *B. burgdorferi* genetic diversity and a lack of concordance between strains identified in animals and humans from the same locations. However, since 10 of the 15 animal isolates clustered with *ospC* types previously characterized as "invasive," it is reasonable to conclude that mouse invasiveness may be equivalent to human infectivity. Moreover, the identification of invasion in both mice and humans by noninvasive *ospC* types suggest that the grouping proposal of Seinost et al. (25) needs a more rigorous evaluation. Inclusion of additional isolates representing both a variety of geographic regions and infected human as well as reservoir hosts may help to determine whether all or some subpopulations of *B. burgdorferi* strains can cause disseminated human infection.

In accordance with other investigators using animal models (29), it is possible that *ospC* diversity is less important as a predictor of mammalian and human invasion than proposed previously (3, 25). Thus, given the extensive diversity of *B. burgdorferi ospC* types that may infect humans, alternative genetic markers should be investigated that may better correlate with and explain *B. burgdorferi* disseminated disease.

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