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Borrelia burgdorferi Genetic Markers and Disseminated Disease in Patients with Early Lyme Disease[▽]

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Three genetic markers of *Borrelia burgdorferi* have been associated with disseminated disease: the OspC type, the 16S-23S rRNA intergenic spacer type (RST), and *vlsE*. Here, we modified previous methods so as to identify the three markers by PCR and restriction fragment length polymorphism in parallel, analyzed *B. burgdorferi* isolates from erythema migrans (EM) skin lesions in 91 patients, and correlated the results with evidence of dissemination. OspC type A was found approximately twice as frequently in patients with disseminated disease, whereas type K was identified approximately twice as often in those without evidence of dissemination, but these trends were not statistically significant. The remaining seven types identified were found nearly equally in patients with or without evidence of dissemination. RST 1 strains were significantly associated with dissemination ($P = 0.03$), whereas RST 2 and RST 3 strains tended to have an inverse association with this outcome. The *vlsE* gene was identified in all 91 cases, using primer sets specific for an N-terminal sequence of *B. burgdorferi* strain B31 (*vlsE*^{B31}) or strain 297 (*vlsE*^{297}), but neither marker was associated with dissemination. Specific combinations of the three genetic markers usually occurred together. OspC type A was always found with RST 1 and *vlsE*^{B31}, type K was always identified with RST 2 and more often with *vlsE*^{297}, and types E and I were almost always found with RST 3 and equally often with *vlsE*^{B31} and *vlsE*^{297}. We conclude that *B. burgdorferi* strains vary in their capacity to disseminate, but almost all strains isolated from EM lesions sometimes caused disseminated disease.

Lyme disease, caused by the tick-borne spirochete *Borrelia burgdorferi*, typically begins with erythema migrans (EM) accompanied by flu-like symptoms (29). Weeks to months later, about 60% of untreated individuals have later manifestations of the infection, including neurological, heart, and joint abnormalities (31). The differences between patients with localized infection and those with disseminated disease are likely due to both spirochetal and host factors.

Several genetic markers of *B. burgdorferi* have been associated with disseminated infection in human Lyme disease (19, 27, 35, 37) or in murine model systems (7, 14, 15, 23, 24, 33, 34). In an initial study, Seinost et al. defined 21 outer surface protein C (OspC) types based on sequence heterogeneity of the protein (27). Of 118 EM skin biopsy samples in patients from Long Island, NY, 51 (43%) were type A, B, or I, 32 (27%) were type K, and 35 (30%) were other types, but hematogenous dissemination was associated only with types A, B, I, and K (27). In a later study of six *B. burgdorferi* blood isolates and one skin isolate from patients in Maryland and Pennsylvania, dissemination occurred with these and two other OspC types (H and N) (1). Recently, OspC was shown to bind a tick salivary gland protein, Salp15 (25, 26), which is important in establishing early infection in mice (10). However, the influence of OspC sequence variation on disease pathogenesis is not yet clear.

In a study of 104 skin biopsy samples from patients in Westchester County, NY, Wormser et al. reported that 28 (27%) had 16S-23S rRNA intergenic spacer (RS) type 1 (RST 1), 44 (42%) had RST 2, and 32 (31%) had RST 3 (37). RST 1 isolates were most often associated with a positive *B. burgdorferi* blood culture, multiple EM lesions, and more-severe symptoms, RST 2 strains had an intermediate association with these findings, and RST 3 isolates were least often associated with these outcomes (37). When mice were infected via needle inoculation, RST 1 strains reached greater densities in the blood, were present for longer durations, and caused greater organ system involvement than RST 3 strains (33, 34). When mice were infected by tick bite, both types caused similar organ system involvements (7). Since the 16S-23S rRNA intergenic spacer region is a noncoding sequence, it could not influence dissemination directly and is likely a marker for evolutionary relatedness among spirochetal strains.

In mice, linear plasmid 28-1 (lp28-1) has been associated with more-effective spirochetal dissemination (14, 15, 23, 24). In previous studies, spirochetes lacking lp28-1 were still able to disseminate, but they were cleared more rapidly and were detectable only in the joint or ear and not in the heart, bladder, or spleen (14, 15, 23). The variable major protein-like sequence expressed (VlsE) lipoprotein of the spirochete, which is encoded on lp28-1, undergoes frequent antigenic variation (38–40), suggesting that it plays a role in immune evasion (21, 38, 40). Thus, VlsE may contribute to dissemination by aiding in spirochetal survival during this vulnerable period (14, 23).

In a previous study in which the OspC type and RST were identified in nine selected laboratory strains, OspC types A and B occurred with RST 1, OspC type K was found with RST 2, and OspC types D, E, I, M, N, and U occurred with RST 3 (5). Similarly, when the OspC types of five RST 1 and five RST 3 isolates were determined, all of the RST 1 isolates were OspC

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TABLE 1. PCR primers and conditions for amplification of *recA*, *ospC*, RST, and *vlsE*

Primer name (reference)	Target	Target location in *B. burgdorferi* strain 31 (base number)	Primer sequence	Amt of $MgCl_2$ (mM)[a]	T_m (°C)
nTM17.F (22)	*recA* F	194–223	GTGGATCTATTGTATTAGATGAGGCTCTCG	3.0	62[b]
nTM17.R (22)	*recA* R	415–386	GCCAAAGTTCTGCAACATTAACACCTAAAG	3.0	62[b]
ext (+) (35)	*ospC* F	6–29	AAAGAATACATTAAGTGCGATATT	2.5	45[c]
ext (−) (a) (35)	*ospC* R1[d]	605–584	GGCTTGTAAGCTCTTTAACTG	2.5	45[c]
P_A (18)	RST F1	1455–1471	GGTATGTTTAGTGAGGG	1.5	42[c]
P_{95} (18)	RST R1	3318–3301	GGTTAGAGCGCAGGTCTG	1.5	42[c]
P_B (18)	RST F2	1497–1516	CGTACTGGAAAGTGCGGCTG	1.5	45[c]
P_{97} (18)	RST R2	3285–3263	GATGTTCAACTCATCCTGGTCCC	1.5	45[c]
F-4340 (24)	B31-*vlsE* F	135–160	AGCCAAGTTGCTGATAAGGACGACCC	1.5	52[c]
R-4084 (24)	B31-*vlsE* R	418–389	ACGGCAGTTCCAACAGAACCTGTACTATCT	1.5	52[c]
F297C[e]	297-*vlsE* F	156–190[f]	AGCAATGTTGGTGAAGATAGTGGTAAGGATGATCC	3.0	57[c]
R297C[e]	297-*vlsE* R	461–429[f]	TACCCCATCAGCTTTACCAGCAGTCCCCTCTTC	3.0	57[c]

[a] A 50-μl reaction mixture included 5 μl of template, 1× QIAGEN PCR buffer, 0.2 mM deoxynucleoside triphosphates, 0.25 μM each of forward (F) and reverse (R) primers, and 1.25 U of QIAGEN HotStar *Taq* polymerase.
[b] Program 1 consisted of a polymerase activation step at 95°C for 15 min, followed by 39 cycles of denaturation at 92°C for 30 s, primer annealing at the indicated melting temperature (T_m) for 1 min, and extension at 72°C for 30 s. Final extension was at 72°C for 7 min.
[c] Program 2 consisted of a polymerase activation step at 95°C for 15 min, followed by 35 cycles of denaturation at 94°C for 30 s, primer annealing at the indicated T_m for 30 s, and extension at 72°C for 2 min. Final extension was at 72°C for 10 min.
[d] OspC R1 was modified by removing the first G nucleotide to lower the overall G+C content.
[e] Primers designed for this study.
[f] Location in *B. burgdorferi* strain 297 (GenBank accession number AB041949).

type A, and the RST 3 isolates were OspC type E or I (34). However, in both cases, the numbers of isolates tested were too small for statistical analysis to determine whether certain combinations of markers more often occurred together.

In this study, our goal was to identify the three genetic markers of spirochetal dissemination in *B. burgdorferi* isolates obtained in a large prospective study of patients with erythema migrans (30, 32) and to correlate these genetic markers with clinical and laboratory evidence of dissemination. For this purpose, we condensed and simplified previous methods so as to identify the three known genetic markers of spirochetal dissemination in parallel. We found that particular combinations of the genetic markers almost always occurred together. Although certain strains were more often associated with dissemination, almost all strains sometimes caused disseminated disease.

MATERIALS AND METHODS

Patients. During the summers of 1998 through 2001, 115 patients with physician-identified EM were recruited for the study at two field sites, one in Wakefield, RI (by N. Damle), and the other in East Lyme, CT (by V. K. Sikand). The study was approved by the Human Investigation committees at New England Medical Center (1998 to 2001) and at Massachusetts General Hospital (2002 to 2004). After informed consent was obtained, the physicians performed a clinical assessment of signs and symptoms of the infection, using a standardized questionnaire, and they obtained skin biopsy samples of EM lesions and blood samples. *B. burgdorferi* was recovered from the skin biopsy samples of 93 of the 115 patients (81%). Of the 93 isolates, two were contaminated and were discarded. The remaining 91 isolates were available for this study.

Definition of disseminated disease. In an early treatment study, carried out before practical laboratory markers of dissemination had been developed, disseminated disease was defined by a combination of clinical signs and symptoms (20). It was later shown that multiple symptoms, particularly the combination of headache, arthralgia, myalgia, and fever, were associated with PCR evidence of *B. burgdorferi* in blood (9). In this report, disseminated disease was defined by the laboratory marker of a positive PCR test for *B. burgdorferi* DNA in blood or by the clinical markers of multiple EM lesions or the combination of at least six of eight specific symptoms (headache, neck stiffness, fever, chills, myalgias, arthralgias, malaise, and fatigue), including the four that were previously shown to be associated with disseminated disease.

Samples. Two punch biopsies (1.5 mm) were performed at the leading edge of EM lesions, one for culture and one for PCR determinations. In addition, an EDTA-anticoagulated blood sample was obtained for PCR. The biopsy sample was placed immediately into modified Barbour-Stoenner-Kelly II medium (BSK-H; Sigma, St. Louis, MO), and all samples were shipped overnight at ambient temperature (32). Upon receipt in the laboratory, the isolates were grown to density (~10^8 organisms per ml) without being passaged and were frozen at −80°C. Blood and skin samples for PCR determinations were also stored at −80°C.

PCR for *B. burgdorferi* DNA in patient blood samples. DNA was isolated from 200 μl of the thawed blood samples, dissolved in 50 μl of buffer, and stored at −20°C, according to the protocol of a QIAGEN DNA miniprep kit for blood and body fluids (QIAGEN, Valencia, CA). PCR amplification of the *B. burgdorferi recA* gene was performed as previously described (22), with slight modifications (Table 1). The limit of detection for the PCR amplification was 10 spirochetes per 50-μl reaction mixture. Samples were visualized with ethidium bromide on 2% agarose gels (Invitrogen, Carlsbad, CA). The presence of a single 222-base-pair band indicated a positive result for spirochetal DNA in blood. Blood samples from normal individuals, which were included in each assay, always gave negative results.

DNA extraction of *B. burgdorferi* isolates. To recover *B. burgdorferi* DNA from the 91 skin isolates, spirochete cultures (200 μl) were centrifuged at maximum velocity (15,996 × *g*) for 5 min and washed with phosphate-buffered saline (pH 7.0) twice to remove culture medium. After resuspension of the pellet in 200 μl phosphate-buffered saline (pH 7.0), the DNA was extracted (QIAGEN DNA miniprep kit), resuspended in 50 μl of buffer, and stored at −20°C.

PCR amplification of the three genetic markers. PCR mixture and cycling protocols were modified from published protocols (17, 18, 24, 35) so that multiple targets could be amplified using consistent reaction mixtures and programs (Table 1). In preliminary experiments, the specificity of the cycling conditions for all markers described below was confirmed using *B. burgdorferi* laboratory strains N40 and 297, whose markers had previously been determined (35, 37).

The rRNA spacer sequence was amplified using a nested PCR (Table 1) (17, 18), which is predicted to amplify a 941-bp band in *B. burgdorferi* strain B31 (19). The *ospC* gene was amplified in parallel with the second round of the RS PCR in separate reaction mixtures, using the ext (+) primer and a slightly modified ext (−) (a) primer as described by Wang and colleagues (Table 1) (35), which are predicted to amplify a band of 600 bp from *B. burgdorferi* strain B31.

To confirm that the OspC type of the isolate was representative of the initial infection, the *ospC* gene was also amplified from DNA extracts of 74 available patient EM skin biopsy samples, as described for the *B. burgdorferi* isolates (Table 1), with PCR cycles increased from 35 to 42 to increase sensitivity. The resulting PCR products were purified into 50 μl of distilled water using a QIAquick PCR purification kit (QIAGEN) and then submitted for sequencing to

TABLE 2. Predicted restriction enzyme digestion fragments for *ospC* RFLP analysis

OspC type	Fragment sizes (bp) after digestion with[a]:		GenBank accession no.
	AluI	HinfI	
A	258, 221, 75	381, 98	AF029860, U01894, L81131, AF416424, AJ749606, AJ749607, X84783, AJ749603, AJ749601, AJ749597, AJ749598, D49497, AF278579-AF78581, AF411451, AF41618-AF41622, AF467867, AF467868, U91792, L42887, U91797, U91798, U91801
B	208, 81, 76, 75	324, 129, 98	AF029861, X84765, X73625, X81522, AJ749604, AJ749605, AJ749602, U91795, L42868
C	218, 93, 81, 75	381, 98	AF029862
D	221, 127, 85, 75	198, 186, 98	AF029863, L25413
E	221, 93, 87, 78, 72	447, 98	AF029864, AF029866, U04240, L42894
F	221, 203, 103	259, 119, 98	AF029865, L42896, L81130, X83555
G	218, 99, 78, 73, 72	334, 116, 98	AF029867, AF223575
H	218, 174, 75, 73	381, 98	AF029868
I	336, 208	195, 186, 98	AF029869, U04281
J	221, 134, 75	453, 98	AF029870, U91802, AF097915
K	221, 213	454, 98	AF029871, AF029872, U08284, X84779, X84785, L42895
L	208, 126, 123, 87	381, 98	L42899, X81524, AF077661
M	225, 221, 71	387, 98	U01892
N	208, 134, 73, 72	453, 98	L42897
O	208, 153, 97, 72	378, 98	X84778
P	218, 209, 114	381, 98	U91796, X84769
Q	290, 218	332, 98, 90	U91790, AJ749600
R	306, 167	456, 98	U91791, U91800
S	290, 93, 81, 73	255, 192, 98	U91793
T	221, 119, 90	390, 98	AF065143
U	384, 221	459, 98	AF065144

[a] Fragments of <70 base pairs are not indicated here due to the difficulty of resolution on 2% agarose gels.

the Massachusetts General Hospital DNA Core Facility. Restriction fragment length polymorphism (RFLP) analysis could not be performed due to artifacts amplified from the human DNA in the skin samples.

The *vlsE* sequence from strain B31 (*vlsE*^{B31}), encoded on lp28-1, was amplified with previously described primers (Table 1) (24). This reaction has been shown to be specific for *vlsE* sequences on lp28-1 and does not amplify the *vlsE*-like sequence on lp38 (6, 8, 11, 12, 24). The *vlsE*^{B31} primers were predicted to amplify a 284-bp fragment. Since the *vlsE* gene sequence for *B. burgdorferi* strain 297 (*vlsE*^{297}) differed in the region where both B31 primers would anneal, a second set of primers was designed using the *vlsE*^{297} gene sequences (GenBank accession numbers AY052626 and AB041949). The *vlsE*^{297} primers were predicted to amplify a 305-bp fragment.

RFLP analysis of the three genetic markers. RFLP analysis was used to distinguish subtypes of the three markers from each other. Following enzyme digestion, 2% agarose gels (Invitrogen) with 0.5 μg/ml of ethidium bromide were used to visualize bands from all restriction digests for a given isolate together on the same gel. The RFLP patterns of the amplified 16S-23S rRNA spacer were determined as previously described (19). Ten microliters of the final PCR product was incubated overnight at 37°C with 4 μl of enzyme-specific buffer, 4 μl of 0.1 mg/ml bovine serum albumin (BSA), and 5 units of either HinfI (Fisher Scientific, Pittsburgh, PA) or MseI (New England Biolabs, Beverly, MA) in a total reaction mixture volume of 40 μl.

To determine the OspC type (A to U) of each sample, as defined by Seinost et al. and Wang et al. (27, 35), two restriction enzymes were selected based on their cutting of representative *ospC* sequences (NEBcutter V2.0 shareware program; http://tools.neb.com/NEBcutter2/index.php) (Table 2) (27). For these determinations, 10 μl of the final PCR product was incubated overnight at 37°C with 4 μl of enzyme-specific buffer, 4 μl of 0.1 mg/ml BSA, and 5 units of either AluI (Fisher Scientific) or HinfI (Fisher Scientific) restriction enzyme in a total reaction mixture volume of 40 μl (Fig. 1). When digested by HinfI, type K shows two possible fragment sizes. These two *ospC* mobility classes, OC12 and OC13, were combined in a single type, called K, according to current conventions (35). Bands with lengths differing by 18 bp on 2% agarose gels were distinct. Thus, with a few exceptions, the predicted AluI and HinfI fragments should comprise unique patterns for each OspC type. The RFLP patterns for OspC types J and N and for types L and T are predicted to be very similar, and for these pairs, direct sequencing of the *ospC* amplicons, followed by the nucleotide-nucleotide BLAST (2) of GenBank (3), would be necessary. The validity of our RFLP technique was confirmed by sequencing of *ospC* amplicons from representative samples of each OspC type at the Massachusetts General Hospital DNA Core Facility. Sequences were matched to those deposited in GenBank (3) by nucleotide-nucleotide BLAST (2).

In preliminary studies, bands amplified from strain 297 DNA and from two *B. burgdorferi* isolates with *vlsE*^{297} were excised, gel purified using a QIAGEN kit,

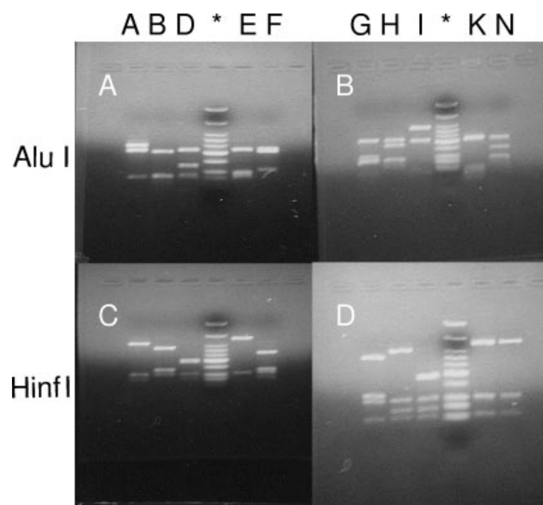

FIG. 1. RFLP patterns for the determination of OspC types. DNA was amplified, amplicons were cut with either AluI or HinfI, and the fragments were run on 2% agarose gels with ethidium bromide. The OspC type was determined based on digestion patterns predicted using NEBcutter V2.0 software. Panels A and B show AluI RFLP patterns for the 10 OspC types identified in our 91 isolates. Panels C and D show HinfI RFLP patterns for those same 10 OspC types. The ladder (*) used is a commercially available low-molecular-weight DNA ladder (New England Biolabs); 9 to 10 bands are visible on 2% gels, with a 766-bp band as the highest molecular weight marker, a 50-bp band as the lowest visible marker, and a 200-bp band with increased intensity as a reference point.

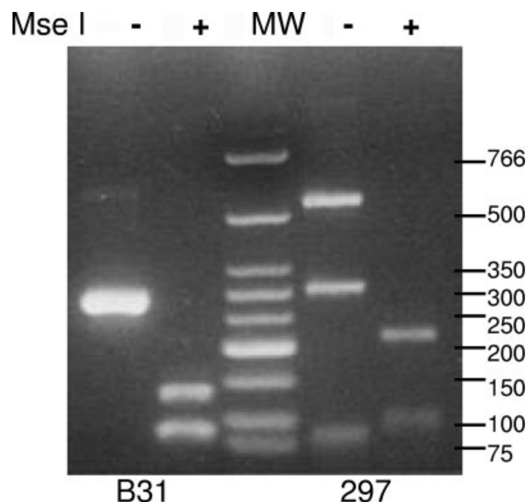

FIG. 2. RFLP patterns for the determination of *vlsE* types. PCR products amplified with either $vlsE^{B31}$ or $vlsE^{297}$ primer sets were cut with MseI, and the fragments were run on 2% agarose gels with ethidium bromide. Using NEBcutter V2.0 software and the GenBank-deposited *vlsE* sequences, the *vlsE* types are predicted to have the following patterns: $vlsE^{B31}$, 122 and 81 bp (doublet); and $vlsE^{297}$, 211 and 95 bp. Bands smaller than 70 bp resolve poorly on 2% gels and are not included. Digested and undigested PCR amplicons from a single isolate are shown here. MW, molecular weight marker.

and sequenced at the Massachusetts General Hospital DNA Core Facility. The sequences matched the GenBank-deposited strain 297 sequences by nucleotide-nucleotide BLAST. Later, amplicons from 9 of 52 isolates that had both $vlsE^{B31}$ and $vlsE^{297}$ were also sequenced, and *vlsE* amplicons from the remaining 43 isolates were confirmed by restriction digestion with MseI (New England Biolabs). For this analysis, 10 μl of the final PCR product was incubated overnight at 37°C with 4 μl of enzyme-specific buffer, 4 μl of 0.1 mg/ml BSA, and 5 units of MseI in a total reaction mixture volume of 40 μl (Fig. 2).

Statistical analysis. The identities of groups were compared by Fisher's exact test using SigmaStat 3.0.1 (SPSS). All *P* values are two-tailed. Because information has been published previously about OspC types or RSTs (27, 37), we did not make corrections here for multiple variables. For this study, *P* values of ≤0.05 were considered statistically significant.

RESULTS

Characterization of OspC types. We determined the OspC type of *B. burgdorferi* isolates from EM skin lesions in 91 patients with early Lyme disease seen during a 4-year period at two locations along the coasts of Connecticut and Rhode Island. No significant differences were found between the two sites (data not shown); thus, the results were combined for presentation here. Of the 21 OspC types described by Seinost et al. (27), 10 were identified in the current isolates by RFLP (Fig. 1). The identities of 22 isolates, including at least 1 of each of the 10 RFLP-identified types, were confirmed by sequencing. The most common types were A and K, which accounted for 57% of the isolates. The least common were D and F, each of which was found in only one patient. All isolates contained a single OspC type, except for one isolate that had both types A and I.

To determine whether culture underestimated the genetic diversity of *B. burgdorferi* in the skin, *ospC* sequences were analyzed directly in the DNA extracts of all available EM skin biopsy samples from 74 patients. In 51 of the 74 samples, enough spirochetal DNA was present for typing, and in 50 cases, a single *ospC* sequence was found in the skin, which matched exactly that in the corresponding isolate. The sole disparity was a skin biopsy sample that yielded only a type I sequence when the matched isolate contained a mixture of types A and I. Thus, direct examination of the skin for the OspC locus of the spirochete did not suggest greater diversity than was found in the culture isolates.

Among the 91 patients, who were seen for a median of 4 days (range, 1 to 21 days) after the onset of EM, 58 (64%) had evidence of disseminated disease (Table 3). The distributions of OspC types were similar using clinical and laboratory evidences of dissemination (data not shown); thus, the results were combined for presentation here. Of the 10 OspC types identified, 9 were found among patients who did and did not have evidence of dissemination. OspC type A, the most common type, was identified approximately twice as frequently in patients with disseminated disease as in those who did not have evidence of dissemination (21 of 58 [36%] versus 6 of 33 [18%] patients; $P = 0.1$). Conversely, type K, the second most common type, was found approximately twice as frequently in patients who did not have evidence of dissemination as in those who did (13 of 33 [39%] versus 12 of 58 [21%] patients; $P = 0.09$). However, these trends were not statistically significant. Moreover, since type K was the second most common type, 20% of the 58 patients who had evidence of disseminated disease were infected with this type. Seven other less common

TABLE 3. Association between the genetic markers of *B. burgdorferi* and markers of disseminated disease

Genetic marker	No. (%) of patients with markers of dissemination[a]	
	Positive ($n = 58$)	Negative ($n = 33$)
OspC types		
A	21 (36)	6 (18)
B	8 (14)	3 (9)
D	1 (2)	0 (0)
E	1 (2)	2 (6)
F	0 (0)	1 (3)
G	1 (2)	1 (3)
H	3 (5)	1 (3)
I	4 (7)	3 (9)
K	12 (21)	13 (39)
N	6 (10)	3 (9)
A/I	1 (2)	0 (0)
RSTs		
1	29 (50)[b]	8 (24)
2	22 (38)	19 (56)
3	6 (10)	6 (18)
Mixed	1 (2)	0 (0)
vlsE types		
$vlsE^{B31}$[c]	50 (86)	30 (91)
$vlsE^{297}$[c]	36 (62)	27 (82)

[a] Twelve patients had at least six symptoms only, 5 had only multiple EM lesions, 2 had multiple EM lesions and at least six symptoms, 23 had only a positive PCR test for *B. burgdorferi* in blood, 2 had a positive PCR test and multiple EM lesions, 5 had a positive PCR test and at least six symptoms, and 9 had all three of these findings.

[b] $P = 0.03$ by Fisher's exact test.

[c] Fifty-two patients had both $vlsE^{B31}$ and $vlsE^{297}$ sequences, and they were included in both groups.

TABLE 4. Association between OspC type, RST, and *vlsE* type genetic markers

OspC type (no. of patients)[a]	No. of patients with indicated genetic markers				
	RST 1 (n = 37)	RST 2 (n = 41)	RST 3 (n = 12)	$vlsE^{B31}$ (n = 79)[b]	$vlsE^{297}$ (n = 62)[b]
A (27)	27[c]	0	0	27[c]	11
B (11)	10[c]	1[d]	0	11	4
D (1)	0	0	1	1	0
E (3)	0	0	3[e]	2	3
F (1)	0	1	0	1	1
G (2)	0	0	2	0	2
H (4)	0	4	0	3	4
I (7)	0	1	6[c]	6	6
K (25)	0	25[c]	0	20	25[c]
N (9)	0	9[c]	0	8	6

[a] Associations between markers were determined for the 90 isolates with a single OspC isotype.
[b] Fifty-two patients had both $vlsE^{B31}$ and $vlsE^{297}$ sequences, and they were included in both groups.
[c] $P < 0.001$ by Fisher's exact test.
[d] $P = 0.01$ by Fisher's exact test.
[e] $P = 0.002$ by Fisher's exact test.

types (B, D, E, G, H, I, and N) were identified nearly equally in patients with and without evidence of disseminated disease. Type F was found in only one patient who did not have evidence of dissemination. Thus, almost all of the OspC types identified sometimes caused disseminated disease.

Characterization of RSTs. Using PCR and RFLP analysis (19), 37 of the 91 isolates (41%) were 16S-23S RST 1, 41 (45%) were type 2, 12 (13%) were type 3, and 1 (1%) was a mixed type. RST 1 strains correlated significantly with disseminated disease ($P = 0.03$), whereas RST 2 and RST 3 isolates tended to have an inverse association with this outcome (Table 3). Nonetheless, about half of the patients with evidence of dissemination were infected with RST 2 or RST 3 strains.

Identification of *vlsE*. In addition to differences in the variable cassette regions of *vlsE* (38), *B. burgdorferi* strains also differ in their relatively conserved 350-bp N-terminal domains. We first used the published primers that detect this N-terminal sequence in *B. burgdorferi* strain B31 (24), but we found $vlsE^{B31}$ in only 88% of the isolates. Since the $vlsE^{B31}$ sequence is only 65% identical to that of *B. burgdorferi* strain 297 (13), we developed primers for this region of $vlsE^{297}$. With these two primer sets, *vlsE* sequences were found in 90 of the 91 isolates (Table 3), and in the remaining case, $vlsE^{297}$ was identified in the patient's EM biopsy sample. Isolates from 28 patients (31%) had only $vlsE^{B31}$, 11 (12%) had only $vlsE^{297}$, and 52 (57%) had both sequences. With these 52 isolates, sequencing or RFLP analysis confirmed that both sequences were present (Fig. 2). However, neither $vlsE^{B31}$ nor $vlsE^{297}$, alone or in combination, was significantly associated with disseminated disease (Table 3).

Associations among genetic markers of *B. burgdorferi*. Specific combinations of the three genetic markers were often found together in the 91 *B. burgdorferi* isolates (Table 4). OspC type A was always found with RST 1 and $vlsE^{B31}$ (for both comparisons, $P < 0.001$). In contrast, OspC type K was always identified with RST 2 and $vlsE^{297}$ (for both comparisons, $P < 0.001$). Finally, OspC types E and I were almost always found with RST 3 ($P = 0.002$ and $P < 0.001$, respectively) but equally often with $vlsE^{B31}$ and $vlsE^{297}$. Because particular OspC types and RSTs were always found together, the determination of both markers did not improve the statistical associations with dissemination compared with that of either marker alone.

DISCUSSION

In an effort to characterize *B. burgdorferi* strains associated with dissemination in a 4-year prospective study of patients with erythema migrans, we condensed and simplified previous methods so as to identify three known genetic markers of spirochetal dissemination in parallel. Previously, the cumbersome method of cold single-strand conformational polymorphism analysis was used to characterize OspC types (16, 27, 35). We found that most of the OspC types could be determined more easily by RFLP techniques, though four of the less common types still required sequencing for accurate identification. In an initial report, *vlsE* could not be detected by PCR in all strains using primers specific for an N-terminal sequence of *vlsE* strain B31, but probes used in Southern blot analyses hybridized to *vlsE* in PCR-negative strains (12). We found that two primer sets, one specific for the N-terminal sequence in strain B31 and the other based on the sequence in strain 297, identified *vlsE* in all of our patient isolates or skin samples. For the determination of RSTs, we followed previously published methods (17, 18, 35), but the number of PCR cycles and annealing temperatures were modified so as to analyze OspC, RS, and *vlsE* markers in parallel. Thus, we were able to analyze the isolates and skin samples for all three markers using standard PCR and RFLP techniques.

Consistent with previous reports (27, 37), we identified 10 of the 21 OspC types and all three RSTs in our patient population, but the distribution of RSTs was slightly different. Compared with the experience in Westchester County, NY (37), we found a higher percentage of RST 1 strains (41% versus 27%) and a lower percentage of RST 3 strains (13% versus 31%). In addition, a previous report from Westchester County, NY, suggested that infection of the skin often results from multiple strains of *B. burgdorferi* (18). In that analysis, 14 of 31 patient skin specimens (45.2%) contained a mixture of two RSTs, as determined by PCR, whereas the matched low-passage skin isolate had only a single RST (18). In contrast, in an analysis of OspC types in skin biopsy samples and matched isolates from 16 patients from Long Island, NY, only 2 cases (12%) showed evidence of mixed infection (28). In our study of patients from Connecticut or Rhode Island, 50 had single, identical OspC types in both isolates and matched skin biopsy samples; only one culture (2%) showed mixed infection. Adjacent geographic regions may vary in the frequency of infection with multiple strains, but our study suggests that infection with multiple strains is rare in Connecticut and Rhode Island.

We then correlated the genetic markers of patient isolates with clinical and laboratory evidence of disseminated disease, defined as at least six clinical symptoms, multiple EM lesions, or PCR evidence of *B. burgdorferi* in blood. Similar results were obtained with clinical and laboratory criteria, and the 64% frequency of disseminated disease is consistent with previous reports (36). However, since spirochetal dissemination may occur in the absence of symptoms and since dissemination is intermittent (36), it is likely that some patients who were clas-

sified as not having evidence of disseminated disease did in fact have spirochetal dissemination.

As previously noted (27, 37), we found that some *B. burgdorferi* strains were more often associated with clinical or laboratory evidence of dissemination than others. For example, all studies show that RST 1 and OspC type A strains are more often associated with this outcome than other types (27, 37). However, in the only other large study (27), 15 OspC types were found in skin biopsy samples, but only OspC types A, B, I, and K were cultured from blood or cerebrospinal fluid specimens, suggesting that only certain strains disseminate. In our population of patients with erythema migrans, 10 OspC types were found in skin biopsy samples, but 9 of the types were sometimes associated with clinical or PCR evidence of dissemination. Since blood was not cultured in our patients, the OspC type of organisms in blood was not determined directly. However, since our patients rarely had mixed OspC types in skin biopsy samples, it seems unlikely that they would have had organisms with a different OspC type in skin than in blood. In the previous study (27), the number of blood isolates may have been too small to observe uncommon types, or spirochetes with uncommon types may be more difficult to culture from blood than organisms with common ones. Whatever the reason, our results suggest that most spirochetal strains that cause human infection may disseminate.

Although the three genetic markers seem to describe evolutionarily related strains that vary in their capacity to disseminate, none explains the mechanisms of dissemination. The RS, a noncoding region, is only a phylogenetic marker, which could not influence dissemination directly. OspC has been shown to bind a tick protein, Salp15 (25, 26), and expression of OspC is important in migration of the spirochete from the tick to the mammalian host (10) and in early immune evasion (25, 26). Brisson and Dykhuizen postulated that sequence variation in OspC, which is the basis of the OspC typing system, evolved to allow more-effective spirochetal transmission to a range of mammalian, avian, or reptilian hosts (4). However, it remains unclear whether OspC binds a mammalian ligand or has any critical function after initial infection. In addition to the OspC and RS markers, other genetic elements surely differ among these strains, including some that may be more directly involved in dissemination.

We did not find *vlsE* to be a helpful marker for spirochetal dissemination. The two markers that we used to identify *vlsE* often occurred together, and neither marker was associated with dissemination. The genetics of *vlsE* are complicated and not completely understood. This outer surface lipoprotein, which undergoes antigenic variation, is encoded by lp28-1. This plasmid varies in size (11, 13), it may be severely truncated (11, 12), and its gene organization may vary. Moreover, a *vlsE*-like sequence has been identified on another plasmid, lp38 (BBJ51-BBJ52), without *vls* recombination cassettes (6, 8). Since the strains in our study rarely had mixed OspC types or RSTs, the presence of two *vlsE* sequences raises the question of whether strains may sometimes have more than one *vlsE* gene.

Since the three known genetic markers of dissemination were determined in all patient isolates, we showed conclusively that specific combinations of the markers often occur together, suggesting that each marker identifies the same or closely related spirochetal strains. Therefore, is there a reason to use more than one method to type strains? For initial assessment, the RST system is technically simpler because there are only three RSTs. The determination of *vlsE*^{B31} and *vlsE*^{297} would appear to provide no additional benefit in the typing of strains. For clinical and epidemiologic analyses, OspC typing has the advantage of dividing the strains into a greater number of genetically distinct types. We believe that the PCR- and RFLP-based system described here for OspC typing will facilitate such analyses in large studies of human or animal isolates.

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