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Comprehensive seroprofiling of sixteen *B. burgdorferi* OspC: Implications for Lyme disease diagnostics design

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Abstract Early diagnosis of Lyme disease (LD) is critical to successful treatment. However, current serodiagnostic tests do not reliably detect antibodies during early infection. OspC induces a potent early immune response and is also one of the most diverse proteins in the *Borrelia* proteome. Yet, at least 70% of the amino acid sequence is conserved among all 21 known OspC types. We performed a series of comprehensive seroprofiling studies to select the OspC types that have the most cross-reactive immunodominant epitopes. We found that proteins belonging to seven OspC types detect antibodies from all three infected host species regardless of the OspC genotype of the infecting strain. Although no one OspC type identifies all seropositive human samples, combinations of as few as two OspC proteins identified all patients that had anti-OspC antibodies.

Introduction

Lyme disease (LD), caused by the spirochete *Borrelia burgdorferi*, is the most prevalent vector-borne disease in the northern hemisphere. Early diagnosis is critical to successful treatment and complete recovery [1,2]. However, clinical and serological diagnosis of Lyme disease is particularly difficult due to the phenotypic heterogeneity within and among species of the spirochete [3,1]. Even in regions where only one *B. burgdorferi* species is found, Lyme disease progresses very differently from one patient to another [4].

Current serodiagnostic tests for Lyme disease lack sensitivity and affinity for detection of anti-*B. burgdorferi* antibodies in the early stages of the disease. Sensitivity seldom exceeds 50% [5–8]. OspC was first identified as a seroreactive major outer surface protein in a subset of *B. burgdorferi* strains [9,10]. It is a virulence factor upregulated just prior transmission to the mammalian host and is indispensable for establishing infection [11–14]. Furthermore, OspC is the major protein expressed on the surface of *B. burgdorferi* during the first stages of infection [15] and induces a strong IgM immune response early on [16].

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Therefore, it is an essential antigen to include in serodiagnostic assays for early Lyme disease [17–23].

OspC is also one of the most diverse and heavily studied proteins in the *Borrelia* proteome. Distinct *ospC* genotypes are correlated with niche preference in natural reservoir species and invasiveness, pathogenesis and clinical manifestations in humans [24–31]. Twenty-one known OspC phyletic groups (referred to as OspC genotypes) classified by letters A to U [32–34] are distinguished by at least 8% amino acid sequence divergence. Given that there is at least 70% homology between all OspC genotypes [33], the presence of common epitopes that can be targeted for the development of new immunoprophylatic components has been explored [35]. We performed a series of comprehensive seroprofiling studies using serum panels from naturally infected white-footed mice, dogs and humans to screen for the OspC types that have common or cross-reactive immunodominant epitopes.

Materials and methods

B. burgdorferi strains

B. burgdorferi isolates were cultured from blood or *erythema migrans* skin biopsies of human patients seen at the Westchester Medical Center (kindly provided by Dr. Gary Wormser, New York Medical College (NYMC), Valhalla, NY). Fifteen OspC group-specific *B. burgdorferi* human isolates were typed for OspC phyletic group in Dr. Ira Schwartz laboratory (NYMC, Valhalla, NY) and were kindly provided to us for this study. Low passage *B. burgdorferi* were grown at 34 °C in Barbour-Stoenner-Kelly H (BSK-H) medium supplemented with antibiotic mixture for *Borrelia* (Sigma-Aldrich, St. Louis, MO). Total DNA was isolated from spirochetes using IsoQuik Nucleic Acid Extraction Kit (ORCA Research Inc., Bothell, WA). Patients provided informed consent and experimentation guidelines were followed as approved by the New York Medical College IRB.

Infection of mice with *B. burgdorferi*

Viability and number of spirochetes grown to mid- or late-log phase was done by dark field microscopy (Axio Imager, Zeiss, Germany). 10^5 bacteria were used to infect C3H-HeJ mice subcutaneously. Three weeks later mice were bled and the serum was tested for the presence of *B. burgdorferi* antibodies using the ViraBlot test (VIRAMED Biotech AG). Animal experimentation guidelines were approved by UTHSC's Animal Care and Use Committee.

Serum panels from naturally infected hosts

For the purpose of seroprofiling we used serologically characterized serum panels only. A panel, $n=43$, was obtained from the natural reservoir of *B. burgdorferi*, the white-footed mouse (*P. leucopus*) and was previously screened for *B. burgdorferi* infection by C6 ELISA (Immunetics, Boston, MA). A panel, $n=38$, was obtained from naturally infected dogs with Lyme disease previously tested for *B. burgdorferi* infection by whole cell sonicate ELISA. A panel, $n=25$, was obtained from naturally infected humans with Lyme disease from the United States. This panel was obtained from patients presenting with *erythema migrans* and was previously screened for *B. burgdorferi* infection by C6 ELISA (Immunetics, Boston, MA). The last panel, $n=40$, was obtained from naturally infected humans with Lyme disease from Europe. This panel comprises serum from 19 patients presenting with *erythema migrans* with IgM and IgG antibodies to *B. burgdorferi*; 11 patients with IgM and IgG antibodies to *B. burgdorferi* and 10 patients with IgM antibodies to *B. burgdorferi*. These 21 patients did not present with *erythema migrans*. Patients provided informed consent and experimentation guidelines were followed.

Cloning, expression and purification of recombinant OspC proteins

A 560 bp-fragment of each *B. burgdorferi* ospC type gene was amplified by PCR. A Nde I/BamH I fragment was cloned into pET9c (Novagen, Gibbstown, NJ). Plasmids were sequenced (GENEWIZ, Inc., South Plainfield, NJ) and the sequences of *ospC*-fragments were confirmed by ClustalW alignment with Genbank published sequences. Recombinant OspC proteins were expressed in *Escherichia coli* BL21 (DE3) and purified by ion exchange chromatography using Q-Sepharose Fast Flow (GE Healthcare, Sweden). Protein concentration was determined with the Bio-Rad Protein Assay Kit (Bio-Rad, Hercules, CA). OspC proteins were analyzed on a 15% SDS-PAGE Coomassie stained gel.

OspC seroprofiling

OspC-immunoarrays were done using ELISA. Purified recombinant OspC protein was used to coat Nunc MaxiSorp™ flat-bottom ELISA plates (eBioscience, San Diego, CA) and indirect ELISA was performed using serum (1:100) from C3H mice, *P. leucopus*, dog, or human. Species-specific IgG secondary antibody was used for mouse, *P. leucopus* and dog (1:50,000, Jackson ImmunoResearch, West Grove, PA). For human, anti-human IgM+IgG horseradish peroxidase-conjugated secondary antibody was used (1:50,000, Jackson ImmunoResearch, West Grove, PA).

Results

Cloning, expression and purification of group-specific OspC

Sixteen of the 17*ospC* genotypes endemic to the US were cloned. The *ospC* gene from 15 of the 17 genotypes were cloned from *B. burgdorferi* isolates cultured from blood or *erythema migrans* skin biopsies of human patients seen at the Westchester Medical Center (Valhalla, NY). These isolates were typed for OspC phyletic group by reverse line blotting in Dr. Ira Schwartz laboratory (NYMC) [36]. OspC genotype L was amplified from a plasmid constructed from *B. burgdorferi* DNA isolated from ticks. OspC genotype O is rare in the northeastern US and was not available. All *ospC* genes were cloned in an expression vector (pET9c) and sequences confirmed by ClustalW alignment against Genbank standards [33,26]. Each of the 16 recombinant OspC proteins (A–N, T and U) was expressed in *E. coli* BL21(DE3)pLys devoid

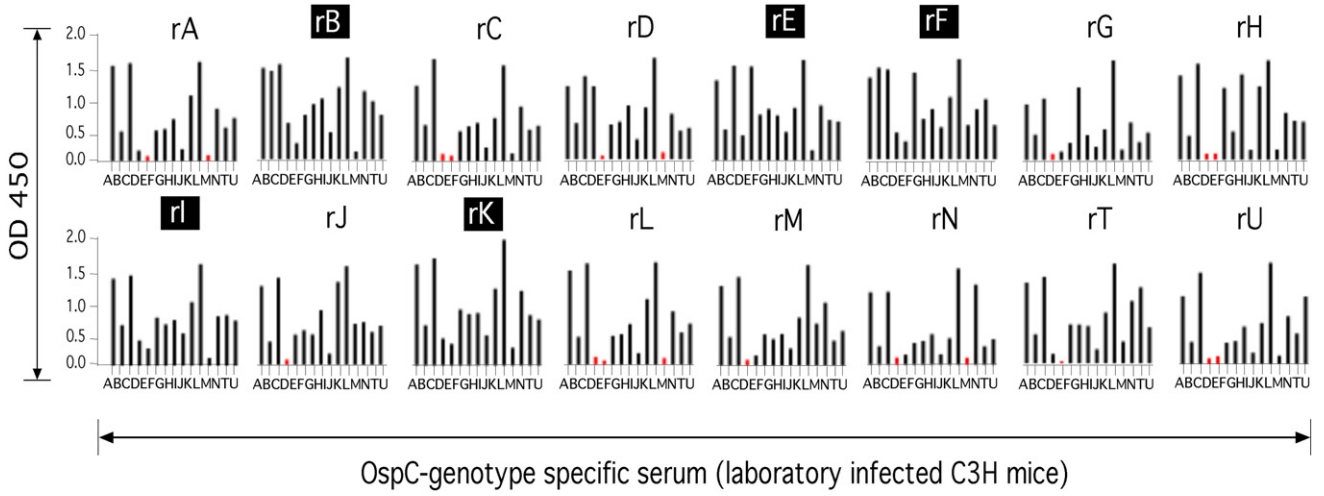

Figure 1 OspC seroprofiling of laboratory infected mice. ELISA immunoarrays of five rOspC proteins detect anti-OspC antibodies in all infected mice, regardless of the OspC-type of the *B. burgdorferi* with which the mouse was infected. All other rOspC proteins failed to detect anti-OspC antibodies from at least one *B. burgdorferi*-infected mouse (i.e. rOspC-type A did not detect mice infected with *B. burgdorferi* strains having either OspC-type D or M). Anti-mouse IgG HRP secondary antibody was used. ELISA readings below the detection cutoff (negative) have been highlighted in red.

of any markers or tags, purified under native conditions by ion exchange chromatography and protein purity was analyzed by Coomassie stained SDS-PAGE. All purified recombinant OspC proteins showed a single major band with an apparent molecular mass ranging between 20 and 25 kDa.

OspC screening for diagnostic design

Our main goal was to select proteins that detect *B. burgdorferi* anti-OspC antibodies induced by epitopes shared by all OspC types. To accomplish this we performed two comprehensive seroprofiling studies using 16 purified recombinant OspC types and serum panels from infected hosts that were pre-screened for *B. burgdorferi* infection by serological methods.

In the first trial, the level of OspC-type specific IgG antibody (OD_{450}) was determined in a serum panel from 15 C3H-HeJ mice infected in the laboratory with each strain of *B. burgdorferi* previously typed for its *ospC* phyletic group (Fig. 1). OspC type L-specific serum was not generated because this strain was not available. Positive reactions were determined using the OD_{450} from three serum samples from uninfected mice plus three standard deviations to calculate the cutoff. We observed that recombinant OspC proteins belonging to genotype L detected IgG antibodies induced by 80% of the OspC-typed *B. burgdorferi* strains; proteins belonging to genotypes A, C, D, H, N and U detected IgG antibodies induced by 87% of the OspC-typed strains; proteins belonging to genotypes G, J, M and T detected IgG antibodies induced by 93% of the OspC-typed strains; and proteins belonging to genotypes B, E, F, I and K detected group-specific IgG antibodies induced by 100% of the OspC-typed strains tested.

In the second trial, the diagnostic efficacy of all rOspC protein types was tested by evaluating the level of OspC-type specific antibody in serum obtained from naturally infected hosts: white-footed mouse (*Peromyscus leucopus*, $n=43$), dog (*Canis lupus familiaris*, $n=38$) and human (*Homo sapiens*, from the northeastern United States, $n=25$, and

Table 1 Percentage of naturally infected serum samples with anti-OspC antibody.

Serum panel	% Positive															
	rA	rB	rC	rD	rE	rF	rG	rH	rI	rJ	rK	rL	rM	rN	rT	rU
NI *P. leucopus*	70	61	42	72	70	74	51	35	53	58	63	79	67	44	33	46
NI dog	68	82	61	32	66	74	53	24	66	13	74	66	58	55	26	63
NI human US	68	72	80	68	80	80	44	64	76	76	84	68	24	52	32	68
NI human EU	65	68	68	48	80	78	75	60	70	60	80	73	35	43	40	25
	A	B			E	F			I		K	L				

rA-rU represent purified recombinant OspC proteins; NI, naturally infected serum panels tested positive for *B. burgdorferi* infection by serological methods. NI *P. leucopus*, $n=43$, is serum panel from naturally infected white-footed mice; NI Dog, $n=38$, is serum panel from naturally infected dogs with Lyme disease; NI Human US, $n=25$, is serum panel from human North American patients with signs and symptoms of Lyme disease; NI Human EU, $n=40$, is serum panel from human European patients with signs and symptoms of Lyme disease; the serum panels included in this analysis tested positive for *B. burgdorferi* infection by serological methods; shaded gray, OspC proteins that detect the highest titer of antibodies.

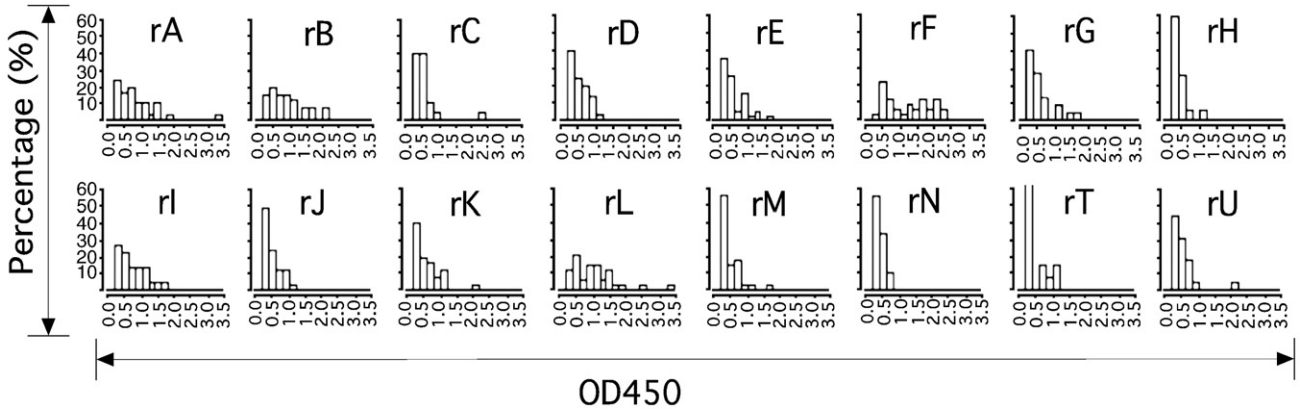

Figure 2 Variation among naturally-infected white-footed mice in the amount of antibodies detected by each rOspC protein. Each graph represents the frequency distribution of OD values obtained from the reaction of IgG in serum from naturally-infected white-footed mice (*P. leucopus*) to each type-specific-rOspC protein by ELISA. Serum panel tested positive for *B. burgdorferi* infection by the C6 ELISA assay.

from Europe, $n=40$). The four serum panels included in this analysis tested positive for *B. burgdorferi* infection by *B. burgdorferi* whole cell sonicate or C6 ELISA. Positive reactions were determined using the OD_{450} from three previously screened negative samples plus three standard deviations to calculate the cutoff. We detected substantial variation among individuals within a species in the proportion of positive reactions to each recombinant OspC protein (Table 1). Using serum from naturally infected white-footed mice (*P. leucopus*), IgG detection ranged between 33% (group T) to 79% (group L). Using serum from dogs with Lyme disease, IgG detection ranged between 13% (group J) to 82% (group B); using serum from human American Lyme disease, IgM+IgG detection ranged from 24% (group M) to 84% (group K) and using serum from human European Lyme disease, IgM+IgG detection ranged from 25% (group U) to 80% (groups E and K). No one rOspC type detected 100% of the *B. burgdorferi* infections in any of the species. However, rOspC types A, B, E, F, I, K and L detected infected hosts from all species (average 68.14%, sd=7.22).

The effectiveness of each rOspC protein as a diagnostic tool is dependent on the probability of detecting anti-*Borrelia* OspC antibodies in infected hosts well above the limit of detection. Although low sensitivity rOspC proteins successfully identified anti-*Borrelia* antibodies in some infected animals, the majority of positive sera samples were very near the cutoff of detection C, D, H, J, M, N, T, U in *P. leucopus* (Fig. 2); C, D, G, H, J, N, T, U in dog (Fig. 3); H, M, T in human US (Fig. 4); and C, D, H, J, M, N, T, U in human EU (Fig. 5). In contrast, much of the positive sera that rOspC types A, B, E, F, I, K and L detected is far above the limit of detection, thus decreasing the risk of false negative assays. For example, rOspC type M detected anti-*B. burgdorferi* (OspC) antibodies in 67% of infected mice (Table 1), but nearly 60% of those were within 0.2 OD of the limit of detection (Fig. 2). rOspC type B also detected anti-*B. burgdorferi* (OspC) antibodies in 61% of infected mice (Table 1) and only 10% were within 0.2 OD of the limit of detection (Fig. 2). No single rOspC protein identified more than 84% (type K, Table 1) of infected individuals suggesting that a combination of rOspC

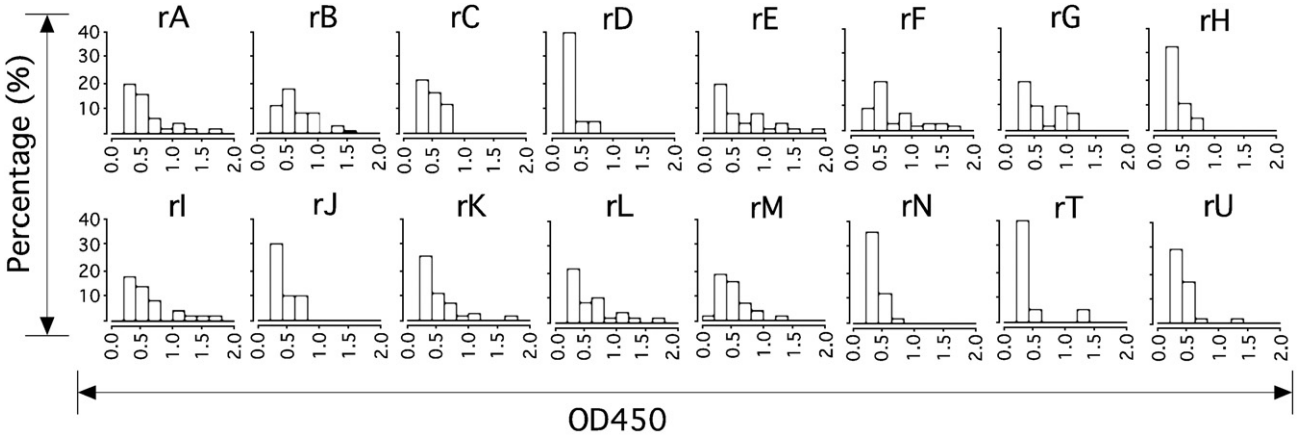

Figure 3 Variation among naturally-infected dogs in the amount of antibodies detected by each rOspC protein. Each graph represents the frequency distribution of OD values obtained from the reaction of IgG in serum from naturally-infected dogs (*Canis lupus familiaris*) to each type-specific-rOspC protein by ELISA. Serum panel tested positive for *B. burgdorferi* infection by the whole cell sonicate ELISA assay.

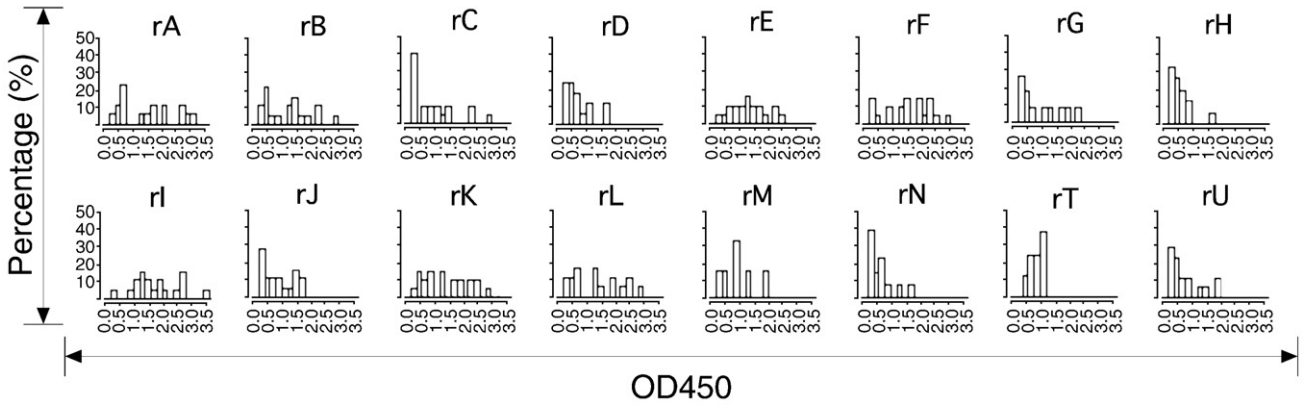

Figure 4 Variation among naturally-infected humans from North America in the amount of antibodies detected by each rOspC protein. Each graph represents the frequency distribution of OD values obtained from the reaction of IgG in serum from naturally-infected humans (*Homo sapiens*) to each type-specific-rOspC protein by ELISA. Serum panel tested positive for *B. burgdorferi* infection by the C6 ELISA assay.

components could be used to identify anti-*Borrelia* OspC antibodies.

In all four serum panels we observed that a number of individuals reacted to all 16 OspC types and that a number of samples did not have antibodies to any OspC. For naturally infected *P. leucopus*, $n=43$, 4 (9%) had IgG antibodies that bind to all OspC groups and 1 (2.3%) did not have antibodies to any OspC; for dogs with Lyme disease, $n=38$, none (0%) had IgG antibodies to all OspC groups and 2 (5.2%) did not have antibodies to OspC of any group; for humans in the Lyme disease American panel, $n=25$, 5 (20%) had IgM+IgG antibodies to all OspC and all samples had antibodies to all OspC groups; for humans in the Lyme disease European panel, $n=40$, 7 (18%) had IgM+IgG antibodies to all OspC groups and 5 (13%) did not have antibodies to OspC of any type. In humans, the low percentage of samples with antibodies to all OspC types (~19%) emphasizes the need for inclusion of OspC antigens from at least two groups in a diagnostic assay. The percentage of samples without antibodies to OspC of any type (0–13%) emphasizes the need for prudence when interpreting negative OspC results given that we only included serum panels that tested positive for *B. burgdorferi* infection.

In order to identify the most sensitive OspC types we analyzed the previously screened OspCs against OspC-positive serum (US and EU, Table 2).

The combination of rOspC types K and B identified 24 of the 25 (96%) North American human LD patients with confirmed antibodies to OspC. The combination of rOspC type K with either type E or type F detected all 35 (100%) European humans with confirmed antibodies to OspC (five European humans with confirmed LD did not have detectable OspC antibodies).

Discussion

The main objective of this study was to identify proteins that detect *B. burgdorferi* anti-OspC antibodies induced by epitopes shared by all OspC types, in order to identify the immunodominant OspC genotypes that are best suited to add to a multi-antigen diagnostic assay for early Lyme disease.

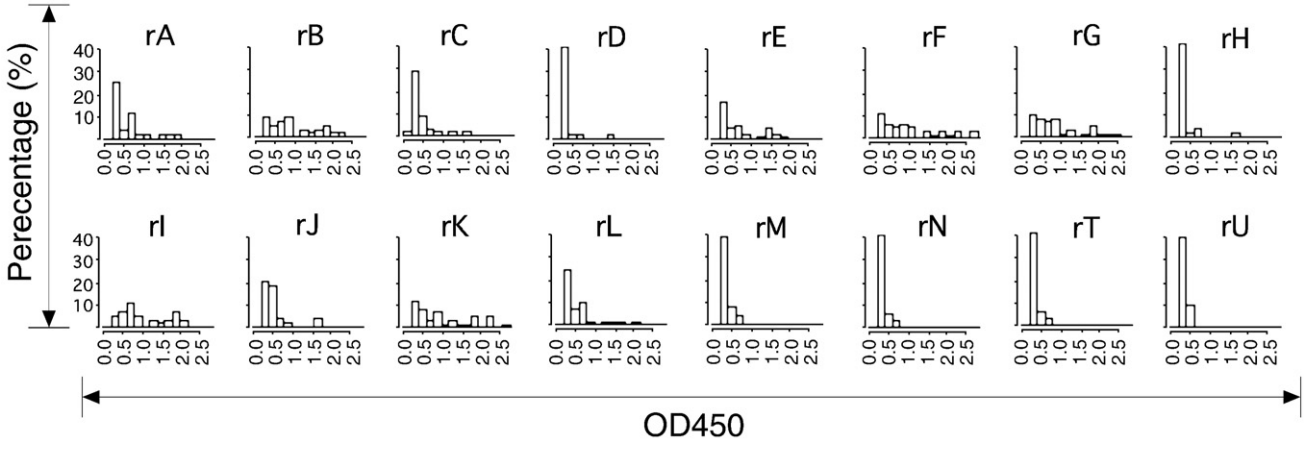

Figure 5 Variation among naturally-infected humans from Europe in the amount of antibodies detected by each rOspC protein. Each graph represents the frequency distribution of OD values obtained from the reaction of IgG in serum from naturally-infected humans (*Homo sapiens*) to each type-specific-rOspC protein by ELISA. Serum panel tested positive for *B. burgdorferi* infection by the whole cell sonicate ELISA assay.

Table 2 Percentage of seropositive Lyme disease samples correctly identified by OspC-pairs.

	% Positive US LD Panel							% Positive EU LD Panel						
	A	B	E	F	I	K	L	A	B	E	F	I	K	L
A	68	88	84	84	80	88	76	74	89	97	91	89	94	83
B		72	92	92	92	**96**	88		77	94	91	80	91	91
E			80	80	80	88	88			91	**100**	97	**100**	97
F				80	80	88	88				89	91	94	94
I					76	84	84					80	91	91
K						84	88						91	97
L							68							83

US LD, is serum panel from human North American patients with signs and symptoms of Lyme disease *and* IgM+IgG antibodies to OspC, $n=25$; EU LD, is serum panel from human European patients with signs and symptoms of Lyme disease *and* IgM+IgG antibodies to OspC, $n=35$.
Shaded grey, OspC protein combination that detect 98.3% of seropositive patients.

Data from our seroprofiling analysis indicate that seven rOspC proteins detected high anti-OspC antibody titers in infected hosts, regardless of species or the ospC genotype of the infecting *B. burgdorferi* strain. Although no one rOspC protein identified all humans with multiple signs and symptoms of LD, combinations of as few as two rOspC proteins identified all patients provided they had anti-OspC antibodies. Immuno-crossreactivity between distinct OspC type proteins, potentially due to antibodies targeting shared epitopes, along with the rapid and strong anti-OspC antibody response, makes these immunodominant rOspC proteins attractive for diagnostic tool development.

The polymorphism of the OspC gene, the immunoreactivity to the OspC protein and its implications for diagnostic design have been long investigated [17,37,32,33,34,26,29]. OspC alone is not sensitive enough to develop OspC-based assays for Lyme disease but it is an essential component of such diagnostic assays, especially if they are to be used to help identify early cases of the disease. In one study, when acute and convalescent-phase serum samples from patients with erythema migrans were tested for reactivity against rOspC by ELISA, the sensitivity of the IgM test was 73% and the specificity was 98% [19]. In another study, when serum samples from patients with EM and other symptoms of Lyme disease were tested against a synthetic peptide based in the C-terminal amino acids residues of OspC of *B. burgdorferi* by ELISA, the sensitivity of the IgM test was 36–45% while the IgG test was <8% [21].

Previous reports demonstrating that OspC immunization is protective against only *B. burgdorferi* expressing the same OspC type [38,39] raised the question of OspC-type specificity. OspC-type specificity was further supported by a study of seven recombinant OspC types that found that despite strong sequence conservation in the N- and C-terminus of OspC, the antibody responses to this protein were type specific. That is, serum from mice infected with type A or D strains was immunoreactive in a type-specific manner and there was little or no cross-reactivity with other OspC types [29]. In sharp contradiction, we find that all 16 rOspC proteins in our library cross-react with a minimum of 12 other OspC proteins. Further, five *B. burgdorferi* genotypes (B, E, F, I and K) induce OspC antibodies that react to all 16 rOspC-types. Three *B. burgdorferi* genotypes (D, E and M) induced the most type-specific OspC antibodies, but still cross-react with 8, 9, and 12 rOspC types, respectively. The difference in conclusion between the former [29] and the current study is likely due to methodology; ELISAs are far more sensitive and quantitative than are immunoblots. Our conclusions are also supported by the observation that most patients infected with *B. burgdorferi* (regardless of strain type) develop anti-OspC antibodies that bind to OspC belonging to genotype A used in commercial serodiagnostic assays in both ELISA and immunoblot formats. Our results suggest major cross-reactivity between OspC antibodies. Although the protective OspC epitopes are genotype-specific, shared OspC epitopes elicit detectable antibody responses for use in diagnostic applications.

A combination of only two rOspC proteins identified 59 of the 60 human LD patients that had positive anti-OspC serology from Europe and North America. However, the best combination of rOspC proteins for LD diagnosis differed on the two continents. rOspC types B and K identified 96% of the US LD patients, with one patient's serum reacting only to type J. Over 76% of North American patients reacted positively with type J, suggesting that a diagnostic assay based in these three proteins may decrease false negative results. Combinations of rOspC types E and K as well as F and K identified all European patients that had anti-OspC antibodies. These data are not an indication of overall diagnostic efficacy of an OspC only-based assay but rather suggest that the OspC types identified are the best candidates to include in a multi-antigen assay for the diagnosis of Lyme disease. The winning of different rOspC combinations on each continent may correlate with differences in the composition of *B. burgdorferi* genotypes to which European and North American humans are exposed. *B. burgdorferi* genotypes A–O, T and U are endemic in the United States, genotypes A, B and J are endemic in both continents, while genotypes P, Q, R and S appear to be restricted to Europe [26,40].

No anti-OspC antibodies were detected in five of the forty (13%) *B. burgdorferi* seropositive European patients. By contrast, all 25 North American patients tested positive to at least one rOspC type. This discrepancy could be explained by the prevalence of multiple genospecies of *B. burgdorferi sensu lato* in Europe. However, it could also be due to the absence of some of the European OspC types in our rOspC library.

OspC genotypes correlate with human invasiveness. It has been suggested that only ospC genotypes A, B, I and K caused systemic disease in humans [26] and that these four ospC types comprise more than 80% of the cases of culture-confirmed early Lyme disease associated with spirochetal dissemination [41]. Additional genotypes have been found in disseminated sites, albeit rarely (genotypes C, D, N, F, H, E, G and M) [29,42]. Further, other studies suggest that OspC typing does not necessarily correlate with *Borrelia* invasiveness [43]. However, the two OspC types (H and N) identified in this study in human blood have since been included in the group of rare disseminators [42]. Interestingly, our best OspC candidates (B, E, F and K) detect anti-OspC antibodies present in serum samples from 59 of 60 seropositive patients infected with several types of *B. burgdorferi*. Only three *B. burgdorferi* genotypes (D, E and M) have low cross-reactivity with recombinant OspCs B, E, F and K, indicating they may be more difficult to detect. However, genotypes D, E and M appear to be rarely found in disseminated sites and are less

likely to cause LD. Two recombinant OspC types, B and K, that also belong to the four types found in 80% of disseminated infections, appear to be the best pair combination to add to a Lyme disease assay.

Although it has been determined that the polymorphism of OspC is due to positive selection favoring diversity at the amino acid level in the variable region [37] and that the immunodominant epitopes of OspC reside in the variable domains of the protein [29] it would appear that common epitopes present in OspC types B, E, F and K detect most anti-OspC antibodies present in serum samples from seropositive patients infected with *B. burgdorferi*. Contrary to the dogma, our results indicate that OspC proteins belonging to these four genotypes may be among the best candidates to develop additional diagnostic tools for early Lyme disease. As with all serodiagnostic assays, caution should be used given that up to 13% of samples, with proven anti-*B. burgdorferi* antibodies from three different hosts, did not react to any of the 16 OspC tested. This highlights a source of false-negative results that could indirectly lead to the increase in the incidence of late Lyme disease.

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