Outer Surface Protein C Peptide Derived from *Borrelia burgdorferi* Sensu Stricto as a Target for Serodiagnosis of Early Lyme Disease

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Current serodiagnostic assays for Lyme disease are inadequate at detecting early infection due to poor sensitivity and nonspecificity that arise from the use of whole bacteria or bacterial proteins as assay targets; both targets contain epitopes that are cross-reactive with epitopes found in antigens of other bacterial species. Tests utilizing peptides that contain individual epitopes highly specific for *Borrelia burgdorferi* as diagnostic targets are an attractive alternative to current assays. Using an overlapping peptide library, we mapped linear epitopes in OspC, a critical virulence factor of *B. burgdorferi* required for mammalian infection, and confirmed the results by enzyme-linked immunosorbent assay (ELISA). We identified a highly conserved 20-amino-acid peptide epitope, OspC1. Via ELISA, OspC1 detected specific IgM and/or IgG in 60 of 98 serum samples (62.1%) obtained from patients with erythema migrans (early Lyme disease) at the time of their initial presentation. By comparison, the commercially available OspC peptide PepC10 detected antibody in only 48 of 98 serum samples (49.0%). In addition, OspC1 generated fewer false-positive results among negative healthy and diseased (rheumatoid arthritis and positive Rapid Plasma Reagin [RPR+] test result) control populations than did PepC10. Both highly specific and more sensitive than currently available OspC peptides, OspC1 could have value as a component of a multipeptide Lyme disease serological assay with significantly improved capabilities for the diagnosis of early infection.

Lyme disease is the most common tick-transmitted disease in North America and Europe. The disease is caused by spirochetes of the genus *Borrelia* (including *B. burgdorferi, B. garinii*, and *B. afzelii*), which are transmitted to humans through the bite of infected *Ixodes* ticks (1, 2). Early disease is typified by the characteristic skin lesion erythema migrans (EM), which occurs in the majority of infected patients, as well as more-nonspecific symptoms that can include low-grade fever, headache, muscle and joint aches, and swollen regional lymph nodes. Though early disease is easily cured with an appropriate short course of antibiotics, if allowed to persist, disease progression can lead to permanent neurological and/or musculoskeletal damage (1–3). Early intervention is therefore critical to disease outcome. Unlike most bacterial diseases, in which the presence of the pathogen can be defined microbiologically by direct observation, culture, or PCR, Lyme disease is defined indirectly (9–12). EM is the classic marker of early infection and is considered pathognomonic in areas of endemcity (9). However, not all patients infected with *B. burgdorferi* develop EM (9), and even if present, it is fleeting and may be gone by the time the patient seeks medical attention. In the absence of EM, diagnosis of Lyme disease is based on the serological detection of antibodies against whole *Borrelia* and/or *Borrelia* proteins (10).

Current CDC guidelines for the serodiagnosis of Lyme disease mandate a two-tier analysis for improved accuracy, as current IgM and IgG serological assays lack sufficient specificity and/or are insensitive for the detection of antibody present at the time that many patients with early Lyme disease seek initial medical care (13–19). The first-tier assay is an enzyme-linked immunosorbent assay (ELISA), typically utilizing lysates of whole *Borrelia* as the target, which if positive or equivocal is followed by a Western blot assay containing several whole *Borrelia* proteins (9, 10, 20, 21). Some of the whole-protein antigens present in both whole-cell ELISAs and in Western blot assays contain epitopes that are cross-reactive with epitopes found in antigens of other bacteria (9). Because of the need to maintain a reasonable balance between specificity and sensitivity, current laboratory tests fail to serodiagnose early Lyme disease approximately 50% of the time (1, 4, 10, 20, 22). Clearly, new approaches are needed to develop better diagnostics. Peptides containing specific epitopes represent a logical alternative to whole-protein antigens as targets in diagnostics because this allows for the elimination of cross-reactive epitopes, retaining only those highly specific for *Borrelia*. An ELISA (IR6) using a peptide derived from the VlsE protein of *Borrelia* has demonstrated greater specificity in the detection of Lyme disease than whole-cell ELISAs and has been approved for use by the FDA (19, 23). However, C6, the peptide derived from VlsE, does not bind IgM particularly well, is derived from an antigen that is expressed only after infection is established (fewer than 1% of bacteria in the tick express VlsE, and transcription of the gene is suppressed prior to transmission of the bacteria), and the IR6 region of VlsE from which the peptide is derived has shown a greater degree of variability than originally thought (19, 23–26). Though the IR6 assay represents a significant improvement, in terms of specificity, compared to the whole-cell ELISA (27), these concerns have precluded the use of the IR6 assay as a stand-alone diagnostic test for early infection.

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Lyme disease. These limitations could be addressed by combining multiple peptide epitopes into a single assay (28). Thus, the identification of further epitopes is of paramount importance to the advancement of Lyme disease serodiagnostics.

OspC is a Borrelia surface protein required for transmission of the bacteria from the midgut of the tick into the human host (29). It is a protein of significant diagnostic value because it is required for entry into the mammalian host and therefore will always be present during infection and is expressed during the initiation of infection. However, the OspC protein is not highly conserved, containing numerous subtypes that have significant sequence variability. Furthermore, some previous studies using whole recombinant OspC as a serodiagnostic for Lyme disease demonstrated a high level of cross-reactivity within negative disease control samples (30, 31), though cross-reactivity was not often seen in normal control serum (30–34). In the present study, we mapped linear epitopes within the OspC protein to identify highly conserved regions lacking cross-reactivity with antigens from other bacteria. We have generated an antigenic peptide (OspC1) that binds to antibody from early Lyme disease patients with high specificity. OspC1 outperformed a previously identified peptide antigen from OspC (PepC10) in an ELISA and is a potential target for inclusion into a sensitive multipeptide serological assay for the diagnosis of early Lyme disease.

**MATERIALS AND METHODS**

**Serum samples.** Ninety-eight serum samples were obtained with consent, under institutional review board (IRB) approval, from patients with erythema migrans (early Lyme disease) at their initial presentation to the Lyme disease clinic at the Westchester Medical Center in Westchester, NY (n = 48), or the Gundersen-Lutheran Medical Center in La Crosse, WI (n = 50). Twenty serum samples were obtained from patients with Lyme arthritis (late Lyme disease) upon first clinical presentation at the Gundersen-Lutheran Medical Center in La Crosse, WI, with swollen joints (n = 20). Both areas are of high endemicity for Lyme disease. Forty-eight sera from healthy individuals residing in a region where Lyme disease is not endemic (New Mexico) were purchased from Creative Testing Solutions from healthy individuals residing in a region where Lyme disease is not endemic. Both areas are of high endemicity for Lyme disease. Eighty-seven disease control sera obtained from patients with results from negative controls via receiver operating characteristic (ROC) analysis using Prism 6.0 (Graphpad, La Jolla, CA). Cutoff values used for comparing sensitivity and specificity between the two peptides were 3 standard deviations (SD) from the mean of healthy controls (limit of detection). Statistical analysis of categorical data presented in the tables and the text was performed using a Fisher exact test with a two-tailed P value using Prism 6.0 (Graphpad).

**RESULTS**

Eight serum samples from Lyme disease patients were chosen for the initial epitope mapping based upon a high titer of anti-Borrelia antibodies as determined by the detection of 9 or 10 of 10 bands on Western blot strip test (5 of 10 bands is the minimum requirement for a sample to be considered positive). Epitope mapping was performed by ProImmune, Inc., using their proprietary ProArray Ultra peptide microarray technology. A partial sequence for OspC type K (B. burgdorferi OC12, accession no. AA886554) was expressed as an overlapping peptide library consisting of a total of 37 peptides (Table 1), each 15 aa in length, overlapping by 10 aa (offset by 5 aa). OspC type K was used for the epitope mapping because it has been associated with disseminated disease (37). In addition to lacking the first 10 aa, the partial sequence does not contain the final 10 aa, which correspond to the commercially available OspC peptide, PepC10. Of the 37 peptides assessed, only 3 were observed to bind more than 50% of the eight serum samples used in the epitope mapping. These were peptide 1, peptide 18, and peptide 30; in addition, 3 of 5 samples that bound peptide 1 also bound peptide 2, indicating that the epitope found in peptide 1 might extend into peptide 2. Here, these peptides are referred to as OspC1 (peptide 1 and 2), MTLFLFISCNNSKGDNNTSA, OspC18 (peptide 18, TLLAGAYTISKLITQ), and OspC30 (peptide 30, AK KAILITDAAKDKG). Serum binding was confirmed in subsequent ELISAs in which the 3 peptides were incubated with an additional 30 sera from high-titer Lyme disease patients (8 to 10 of 10 bands on Western blot strip assay) and 20 negative sera from...
TABLE 1 Borrelia burgdorferi OC12 OspC (type K) peptides

| Peptide no. | Position in protein sequence | Peptide sequence |
|-------------|-----------------------------|-----------------|
| 1           | 11–25                       | MTLLFLFISCNNSGKD |
| 2           | 16–30                       | FISCNNSGKDGNTSA |
| 3           | 21–35                       | NSGKDGNSTANSADE |
| 4           | 26–40                       | GNTSANSDSVKGP   |
| 5           | 31–45                       | NSADESVKGNLITEI |
| 6           | 36–50                       | SVKGPNLTEISIKKT |
| 7           | 41–55                       | NLTIESSKITESNAV  |
| 8           | 46–60                       | SKKITESNAVLVAK  |
| 9           | 51–65                       | ESNAVLAKEIETL  |
| 10          | 56–70                       | VLAVKEIETLASID |
| 11          | 61–75                       | EIETLALSIDELATK|
| 12          | 66–80                       | LASIDELATRAIGK |
| 13          | 71–85                       | ELATAKGGKIQQNG |
| 14          | 76–90                       | AGKKIQQNGGLAVE |
| 15          | 81–95                       | IQQNGGVAEACHNG |
| 16          | 86–100                      | GLAVEAHGNTTLAG |
| 17          | 91–105                      | AGHNTGLAYATTIS |
| 18          | 96–110                      | TLLAGAYTISKLITQ |
| 19          | 101–115                     | AYTISKLITQKLDGL|
| 20          | 106–120                     | KLITQKLDGLKNEK |
| 21          | 111–125                     | KLDGLKNEKLEKIK |
| 22          | 116–130                     | KNEKLEKIKENAKK |
| 23          | 121–135                     | ILEEKENAKCKCEDF |
| 24          | 126–140                     | ENAKCKEDFTKKEI |
| 25          | 131–145                     | CSEDFTKKEGHEAQ |
| 26          | 136–150                     | TKKEGHAQLGIGEN |
| 27          | 141–155                     | GEHAQLGIEVTDEN |
| 28          | 146–160                     | LGIEVTDENAKAKI |
| 29          | 151–165                     | VTDENAKKAIITDA |
| 30          | 156–170                     | AKKAILITDADAKDG |
| 31          | 161–175                     | LITDAADKKGAELE |
| 32          | 166–180                     | AKDKGAELEKLFKA |
| 33          | 171–185                     | AALEKELFKAVENT |
| 34          | 176–190                     | KLFAVENLAKAAME |
| 35          | 181–195                     | VENLAKAAKEMLANS |
| 36          | 186–200                     | KAAKEMLANSKVETL |
| 37          | 190–204                     | EMLANSVICTLSPIV |

a Partial sequence; accession no. AAB86554.

healthy individuals (Fig. 1). Initially, 10 Lyme disease and 10 healthy control sera were titrated on peptide-coated plates to determine an optimal dilution for further study (Fig. 1, upper panels), and then peptide binding was confirmed at a single serum dilution of 1:100 (Fig. 1, lower panels) using an additional 20 sera from Lyme disease patients and 10 sera from healthy controls. OspC18 bound serum antibodies from Lyme patients and normal individuals equivalently (Fig. 1, upper and lower panels), indicating that the epitope contained in the peptide was cross-reactive and not specific for Borrelia. OspC18 was not used in further analyses. The mean absorbance of serum antibody binding to both OspC1 and OspC30 was significantly higher in Lyme disease patients than in normal individuals; however, OspC1 appeared to correctly identify more individual Lyme samples than did OspC30 (Fig. 1, lower panels).

A limitation in the use of OspC in a diagnostic assay is the inherent variability found within the protein. Many OspC “types” (allelic variants) have been described in the literature (14, 35, 36), some having been associated with a greater propensity for dissemination of the bacteria from the site of initial infection following the tick bite. While the association of OspC type and disseminated disease is beyond the scope of this study, to be effective within the constraints of a diagnostic assay, an epitope must be highly conserved. Thus, we aligned the sequences of 15 different OspC types, assessing the amino acid variability in the epitopes OspC1 and OspC30 and comparing it to the degree of sequence variability found within the commercially available PepC10 sequence (PVVAESPKKP). Complete single sequences were not available for OspC types G, H, J, K, M, and U; when possible, multiple different sequences from the same OspC type were aligned to demonstrate the presence or absence of each epitope. As demonstrated in Fig. 2, the sequence for OspC1 is both present and highly conserved in all the OspC types analyzed, being identical to the consensus sequence generated by alignment of the different OspC proteins. This is similar to PepC10, which is also highly conserved and identical to the aligned consensus sequence, though this peptide does appear to be absent in OspC type U and type J. Complete sequences containing the C-terminal portion (where PepC10 is located) of OspC type C and type G were not available, so it is unclear if PepC10 is fully present in those types. On the other hand, OspC30 is poorly conserved among the different OspC types; the sequence identified in the epitope mapping was highly divergent from the consensus sequence generated by alignment of that position within the different OspC types. Indeed, a subsequent epitope mapping of OspC type A did not identify the analogous region for OspC30 as an epitope (data not shown). The high degree of conservation in both OspC1 and PepC10 may be due to their placement in the N-terminal and C-terminal portions, respectively, of the protein, as the highest degree of variation among the different OspC types falls in the middle of the protein (see Fig. S1 in the supplemental material). OspC30 was not used in further analyses.

To assess the potential of OspC1 in a diagnostic assay for early Lyme disease, we screened the peptide against a large panel of sera obtained from patients with EM at the time of their initial diagnosis and compared the results to those obtained with PepC10. As EM develops anywhere from 3 to 30 days after a tick bite (with the average being 7 days), the level of anti-Borrelia antibody within this patient population can vary greatly. Early disease is marked by elevated IgM antibodies against Borrelia. As the disease progresses and the immune response evolves, the IgM response diminishes and is replaced by the IgG response. Dependent upon when patients seek medical attention, they may present with IgM, IgG, or a mixture of both. As a result, we independently assayed for IgM and IgG against OspC1 and PepC10 (Fig. 3). Sera from healthy individuals collected in a region of nonendemicity for Lyme disease (the U.S. southwest) were used as a negative control and to set the limits of detection. In addition, sera from patients with RA or a positive RPR test were used as negative disease controls in this assay. RA is an autoimmune inflammatory disease marked by elevated serum antibody levels and joint destruction, which can also occur in Lyme disease. The RPR test is a first-tier screening test for syphilis, an infectious disease caused by the related spirochete Treponema pallidum, and was used as a negative control for cross-reactive antibody generated by infection with a related spirochete. Sera from 48 healthy donors were used to establish the limits of detection for the ELISA. The mean absorbances for OspC1 binding of IgM (0.316 ± 0.187) and IgG (0.139 ± 0.051) and for PepC10 binding of IgM (0.335 ± 0.187) and IgG (0.125 ± 0.101) were determined. A sample was considered positive if the mean
absorbance of three replicate wells was greater than 3 SD from the mean of the healthy controls, equivocal if it was between 2 SD and 3 SD from the mean of healthy controls, or negative if it was less than 2 SD from the mean of the healthy controls. IgG bound to PepC10 in 4 of 48 serum samples in the healthy control population with high absorbance levels. In two of these four samples, IgG binding occurred at absorbance values of >3 SD from the mean of the population. These two samples were treated as outliers, and while not removed from the analysis, they were not included in the calculation of cutoff values. All four high-binding samples were not positive for IgM binding to OspC10 or binding of either iso-type to OspC1 and were subsequently treated as bona fide false positives. All false-positive control samples were evaluated with commercially available Lyme disease diagnostic Western blot strip tests to determine if the serum was obtained from an individual with previously undiagnosed Lyme disease. All negative controls included in this evaluation were negative for Lyme disease via Western blot strips.

Positive binding of serum IgM to OspC1 was detected in almost one-half (47 of 97) of early Lyme disease patient sera (Fig. 3a; TABLE 2). IgG and IgM antibodies specific for OspC peptides in sera from patients with Lyme disease. Sera from patients with Lyme disease were confirmed to be positive for anti-"Borrelia" antibodies using commercially available Western blot strips prior to incubation with OspC peptides (10 μg/ml) in an ELISA. Antibody binding was detected using a polyclonal HRP-labeled goat anti-human IgG and IgM (γ- and μ-chain-specific) antibody. Upper panels show dose titration of Lyme disease patient sera (Lyme sera, n = 10) and healthy control sera (normal sera, n = 10) on OspC peptide-coated 96-well plates. Data are reported as mean absorbance ± SD. Lower panels depict binding of serum from Lyme disease patients and healthy controls (normal sera) at a single dilution of 1:100. Data are reported as absorbance at 450 nm; the solid lines represent means ± SD. Numbers of samples: for OspC1, Lyme disease, n = 20; normal, n = 10; for OspC18, Lyme disease, n = 20; normal, n = 10; for OspC30, Lyme disease, n = 17; normal, n = 10. Patient samples whose results are depicted in the upper panels are different from those used in the lower panels. *, P < 0.05 by the Mann-Whitney test.

FIG 1 IgG and IgM antibodies specific for OspC peptides in sera from patients with Lyme disease. Sera from patients with Lyme disease were confirmed to be positive for anti-"Borrelia" antibodies using commercially available Western blot strips prior to incubation with OspC peptides (10 μg/ml) in an ELISA. Antibody binding was detected using a polyclonal HRP-labeled goat anti-human IgG and IgM (γ- and μ-chain-specific) antibody. Upper panels show dose titration of Lyme disease patient sera (Lyme sera, n = 10) and healthy control sera (normal sera, n = 10) on OspC peptide-coated 96-well plates. Data are reported as mean absorbance ± SD. Lower panels depict binding of serum from Lyme disease patients and healthy controls (normal sera) at a single dilution of 1:100. Data are reported as absorbance at 450 nm; the solid lines represent means ± SD. Numbers of samples: for OspC1, Lyme disease, n = 20; normal, n = 10; for OspC18, Lyme disease, n = 20; normal, n = 10; for OspC30, Lyme disease, n = 17; normal, n = 10. Patient samples whose results are depicted in the upper panels are different from those used in the lower panels. *, P < 0.05 by the Mann-Whitney test.

FIG 2 Amino acid sequence alignment of different OspC types depicting the regions corresponding to OspC1, OspC30, and PepC10. Sequences were aligned using CLC Workbench and were trimmed to show only the regions corresponding to the peptides of interest. In several instances, complete sequences for the OspC types containing all three peptides were not available. When possible, multiple partial sequences for that OspC type were aligned, depicting the presence or absence of a particular peptide sequence. *, partial sequence; **, partial sequence used for epitope mapping. B. pacificus, B. burgdorferi strain isolated from Ixodes pacificus.
Late Lyme disease, Lyme arthritis.  

Total number of serum samples containing either IgM or IgG antibody binding to OspC1 or PepC10. Positive, more than 3 SD from mean of healthy controls; equivocal, between 2 and 3 SD from mean of healthy controls; negative, less than 2 SD from mean of healthy controls.

| Antibody | Diagnostic result | Lyme disease | Healthy | RA | RPR+ | Late Lyme |
|----------|------------------|--------------|---------|----|------|-----------|
|          |                  | OspC1 | PepC10 | OspC1 | PepC10 | OspC1 | PepC10 | OspC1 | PepC10 | OspC1 | PepC10 | OspC1 | PepC10 |
| IgM      | Positive         | 48.5  | 41.2  | 40.97 | 40.97 | 0     | 0     | 0     | 0     | 0     | 0     | 0     | 0     |
|          | Equivocal        | 8.2   | 13.4  | 13.97 | 13.97 | 2     | 2     | 2     | 2     | 2     | 2     | 2     | 2     |
|          | Negative         | 43.3  | 45.4  | 44.97 | 44.97 | 98    | 98    | 98    | 98    | 98    | 98    | 98    | 98    |
| IgG      | Positive         | 24.5  | 16.3  | 16.98 | 16.98 | 2     | 2     | 2     | 2     | 2     | 2     | 2     | 2     |
|          | Equivocal        | 16.3  | 11.2  | 11.98 | 11.98 | 4.2   | 4.2   | 4.2   | 4.2   | 4.2   | 4.2   | 4.2   | 4.2   |
|          | Negative         | 59.2  | 72.5  | 71.98 | 71.98 | 93.8  | 93.8  | 93.8  | 93.8  | 93.8  | 93.8  | 93.8  | 93.8  |
| Total    | Positive         | 62.1  | 49.0  | 48.98 | 48.98 | 2     | 2     | 2     | 2     | 2     | 2     | 2     | 2     |
|          | Equivocal        | 15.3  | 16.3  | 16.98 | 16.98 | 6.3   | 6.3   | 6.3   | 6.3   | 6.3   | 6.3   | 6.3   | 6.3   |
|          | Negative         | 23.5  | 34.7  | 34.98 | 34.98 | 92.7  | 92.7  | 92.7  | 92.7  | 92.7  | 92.7  | 92.7  | 92.7  |

a Positive, more than 3 SD from mean of healthy controls; equivocal, between 2 and 3 SD from mean of healthy controls; negative, less than 2 SD from mean of the healthy controls.

b Total number of serum samples containing either IgM or IgG antibody binding to OspC1 or PepC10.

c Late Lyme, Lyme arthritis.
bation with normal control serum resulted in the detection of fewer false positives in other negative-control populations.

Late Lyme disease sera were obtained from patients with Lyme disease upon first presentation with swollen joints. OspC1 positively detected IgM in 3 of 20 (15%) and IgG in 5 of 20 (25%) patients with late Lyme disease. Unlike with early Lyme disease, all sera identified as unequivocally positive had only either IgM or IgG; thus, OspC1 detected late Lyme disease in a total of 8 of 20 samples (40%). On the other hand, PepC10 detected IgM in only 2 of 20 (10%) samples and IgG in only 2 of 20 (10%) samples for a total detection of Lyme disease in 4 of 20 (20%) samples.

The sensitivity and specificity of both peptides for identifying positive samples were determined by ROC analysis using 3 SD from the mean of healthy controls as a cutoff. When comparing detection of Lyme disease in patient sera to healthy controls, OspC1 demonstrated a specificity of 100.00% and sensitivity of 41.24% for IgM detection and a specificity of 97.92% and sensitivity of 24.92% for IgG detection. This is in contrast to PepC10, which demonstrated specificity and sensitivity of 100.00% and 29.90%, respectively, for IgM and of 91.67% and 17.35%, respectively, for IgG. Thus, both peptides were highly specific for the detection of Lyme disease, indicating that when a positive value is returned, it is highly likely that it is a true positive. When these values were recalculated comparing Lyme patient sera with all negative controls, OspC1 demonstrated specificity and sensitivity of 98.52% and 41.24%, respectively, for IgM and 92.59% and 24.49%, respectively, for IgG. In comparison, PepC10 demonstrated specificity and sensitivity of 99.26% and 29.90%, respectively, for IgM and 94.81% and 17.35%, respectively, for IgG. Thus, with respect to both healthy and disease controls, both peptides are highly specific. However, OspC1 had a marginally higher sensitivity than PepC10 (41.24% versus 29.90% for IgM and 24.49% versus 17.35% for IgG). Overall, the areas under the curve (AUC) for the two peptides were similar (OspC1 IgM AUC = 0.8047 versus PepC10 IgM AUC = 0.7406 [NS], and OspC1 1 IgG AUC = 0.7236 versus PepC10 IgG AUC = 0.7573 [NS]).

**DISCUSSION**

The inefficiency of current whole-cell- and whole-protein-based serological assays for Lyme disease diagnosis mandates the need for improved technologies. Though erythema migrans develops in a large majority of cases of Lyme disease, it does not develop in all of them and may be gone by the time a patient seeks medical care (9). Improved laboratory diagnostics are critical for the diagnosis of early Lyme disease in the absence of EM and other indeterminate cases. Peptide-based diagnostics, whereby cross-reactive epitopes can be excluded, are an attractive alternative to currently available assays. The IR6 assay, based on a peptide (C6) from the 6th conserved region of the VlsE protein of *B. burgdorferi*, demonstrates that a peptide target can significantly improve both sensitivity and specificity compared to whole-protein/cell-based assays. A recent report by Wormser et al. (27) provides evidence suggesting that the IR6 assay has both the specificity and sensitivity to function as a stand-alone diagnostic assay for Lyme disease. In that study, the authors compared the IR6 assay to a standard two-tier assay against a large panel of Lyme disease patient and negative-control sera and found that IR6 had a much higher sensitivity than the standard two-tier assay while demonstrating a nearly similar level of specificity. However, as the authors noted, the patient population was derived from a single area of hyperendemicity; therefore, it is unclear if similar results would be obtained using sera from different geographical locations. Furthermore, the individual second-tier Western blot assay utilized in the study has been demonstrated to be less sensitive than other, comparable kits, suggesting that the use of a more sensitive second-tier assay could alter the results. The use of a single epitope increases the likelihood of generating false negatives, as there is only a single antibody binding site and a relatively small variation in the amino acid sequence at that site would decrease the ability of such an assay to detect infection. C6, which is more variable than originally recognized, places the IR6 assay at risk for this limitation (19, 23, 24). Despite the recent results, it is unlikely that the current paradigm will be changed in favor of the single-peptide IR6 assay. However, these results strongly support the further development of peptide-based assays. The logical solution is to create a multipeptide assay, which would retain specificity while increasing sensitivity through the inclusion of multiple antibody binding sites (through multiple peptides), limiting the impact of sequence variability within any one epitope. An elegant analysis by Barbour et al. demonstrated that an effective diagnostic serological assay for the detection of early *Borrelia* infection would require a minimum of five distinct antigens to attain an appropriate level of both specificity and sensitivity that would be unaffected by antigenic variation (28).

In the present study, we performed epitope mapping of OspC, a principal virulence factor for *B. burgdorferi* that is expressed at the initiation of infection, making it an ideal target for a diagnostic assay. We identified 3 potential epitopes for further analysis. Previous studies utilizing OspC as a serodiagnostic peptide for Lyme disease demonstrated substantial cross-reactivity with negative disease controls, indicating the presence of cross-reactive epitopes within the protein (30, 31). PepC10 is a commercially available, conserved peptide from the C-terminal region of OspC, which, when initially described, demonstrated a similar level of sensitivity and specificity with respect to healthy controls and a slightly reduced cross-reactivity to negative disease control sera similar to that of the whole OspC protein (30). Given the amount of previous data generated on the serodiagnostic capabilities and limitations of recombinant OspC protein (30–34), as well as the significant sequence variability associated with the different OspC types, we instead chose to draw comparisons in our study to the more efficacious peptide PepC10, rather than whole OspC protein. Of the three peptide epitopes identified, OspC1, a 20-aa peptide from the N-terminal region of OspC, was the only peptide that was both highly conserved among the different OspC types (Fig. 2) and capable of distinguishing between individual Lyme disease patient sera and healthy control sera (Fig. 1 and Table 2). OspC30 did bind to some Lyme patient serum samples during the initial screening process; however, as this sequence was found to be poorly conserved among the different OspC types, it was not included for further analysis because of the high likelihood that the peptide would be unable to consistently detect serum antibody in large patient populations from different geographical areas. In subsequent analyses, OspC1 was found to be highly effective at detecting the presence of IgM and/or IgG antibodies in sera obtained from patients diagnosed with early Lyme disease at the time of initial diagnosis, outperforming the commercially available peptide PepC10 (Fig. 3 and Table 2). OspC1 was also more effective at detecting late Lyme disease than PepC10 (Fig. 3 and Table 2). Much of the higher observed efficacy of OspC1 compared to

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that of PepC10 lay in reduced variability among negative healthy and diseased patient controls, resulting in lower cutoff values. Indeed, ROC analysis revealed that the specificity of both peptides for the positive detection of Lyme disease was very high, though ultimately both peptides functioned similarly in the assay (yielding comparable AUCs). As expected, however, the sensitivity of both peptides was lower than desirable, highlighting the need for multipeptide assay development (28). The observed sensitivity of OspC1 in binding IgM in our study mirrors the level of sensitivity demonstrated for full-length recombinant OspC in multiple previous studies, which was between 40 and 45% (30–33, 38). The initial study describing the efficacy of PepC10 also demonstrated that PepC10 performed at least as well as full-length recombinant OspC in the detection of Lyme antibodies (30). This indicates that OspC peptides have the capacity to function as well as full-length protein in serological assays, but with superior specificity. We assessed the ability of OspC1 and PepC10 to function in concert for the detection of Lyme disease (data not shown); however, the use of both peptides together resulted in an increased background staining in healthy control sera, which in turn led to an increased cutoff and did not enhance the ability of the assay to identify Lyme disease compared to that of assays using individual peptides.

As a target for a serological diagnostic assay, OspC1 has a number of desirable attributes: it is derived from a principal virulence factor that is required for mammalian infection, it is expressed very early in infection, increasing the likelihood of an immune response being mounted against it; it is highly conserved among different OspC genotypes, and it identified a significant majority of patients with early disease. Our data suggest that OspC1 should be considered as a viable candidate for testing in a multipeptide diagnostic assay. An assay containing 5 or more specific peptide antigens, derived from multiple B. burgdorferi proteins, would markedly improve upon currently available technologies in both specificity and sensitivity (28) and may represent a potentially viable stand-alone laboratory test for all phases of Lyme disease diagnosis, especially early disease.

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