OUTER SURFACE PROTEIN (OSP) F SUBFAMILY ANTIBODY RESPONSES DURING LYME DISEASE

A Manuscript Style Thesis Submitted in Partial Fulfillment of the Requirements for the Degree of Master of Science in Microbiology

Ashley Werner

College of Science and Health
Clinical Microbiology

December, 2018
OUTER SURFACE PROTEIN (OSP) F SUBFAMILY ANTIBODY RESPONSES
DURING LYME DISEASE

By Ashley Werner

We recommend acceptance of this thesis in partial fulfillment of the candidate's
requirements for the degree of Microbiology-Clinical Microbiology Concentration

The candidate has completed the oral defense of the thesis.

Steven Callister, Ph.D.
Theis Committee Chairperson

Date

Steven Lovrich, Ph.D.
Theis Committee Member

Date

Bernadette Taylor, Ph.D.
Theis Committee Member

Date

Peter Wilker, Ph.D.
Theis Committee Member

Date

Thesis accepted

Meredith Thomsen, Ph.D.
Graduate Studies Director

Date
Lyme disease is the most common tickborne illness in the United States. The CDC currently recommends a two-test system comprised of an enzyme linked immunosorbent assay followed by a Western blot for laboratory diagnosis. However, the approach is cumbersome and expensive, so identifying additional proteins that can be used to detect antibody responses associated exclusively with Lyme disease remains an important area of research. In this study, the genes encoding outer surface protein (Osp) F subfamily members OspF, OspEF-related protein G (ErpG), ErpK, and ErpL were cloned, and the resultant recombinant proteins were used to evaluate the antibody responses in well-characterized human Lyme disease sera. Although significant numbers of the Lyme disease sera contained antibodies that bound ErpG, ErpK, and/or ErpL, the responses were without an apparent pattern. In contrast, OspF antibodies were detected in a majority of patients with disseminated infection including 81% of patients with joint pain or swelling and each patient with cardiac abnormalities (3/3) or neuritis/neuropathy (2/2). Therefore, the findings failed to provide support for the diagnostic utility of the Erps, but additional studies to evaluate the utility of OspF for Lyme disease diagnostics are warranted.
AKNOWLEDGEMENTS

First, I would like to thank my major advisor, Dr. Steve Callister, for his support, advice with my research, and help with the writing process. I would also like to thank Dr. Steve Lovrich for his assistance with technical training and study design. Additionally, I would like to thank the other members of my committee, Dr. Peter Wilker and Dr. Bernadette Taylor, for their guidance throughout the course of my studies. Lastly, I would like to acknowledge the generous financial contribution of the Gundersen Medical Foundation.
TABLE OF CONTENTS

LIST OF FIGURES........................................................................................................vii

LIST OF TABLES........................................................................................................viii

INTRODUCTION............................................................................................................1

STUDY RATIONALE AND SPECIFIC OBJECTIVES.......................................................7

METHODS AND MATERIALS.......................................................................................10

Normal sera................................................................................................................10

Lyme disease sera......................................................................................................10

Chart review...............................................................................................................10

Western blotting........................................................................................................11

Selection, synthesis, and recovery of genes..............................................................11

Insertion into expression vector and propagation of plasmids.................................14

Transformation of BL21 Star cells.............................................................................16

Purification of proteins..............................................................................................16

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis....................................17

Enzyme linked immunosorbent assay......................................................................17

Statistics.....................................................................................................................19
# LIST OF FIGURES

<table>
<thead>
<tr>
<th>FIGURE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Schematic illustration and genetic sequence of pET100 vector. The insertion site is shaded.</td>
<td>15</td>
</tr>
<tr>
<td>2. Schematic illustration of cloning strategy</td>
<td>22</td>
</tr>
<tr>
<td>3. Detection of amplified genes by agarose gel electrophoresis. Lane 1 contains 50 bp-10 kbp Perfect DNA Marker, lane 2 contains <em>ospF</em> fragment, lane 3 contains <em>erpG</em> fragment, lane 4 contains <em>erpK</em> fragment, and lane 5 contains <em>erpL</em> fragment</td>
<td>23</td>
</tr>
<tr>
<td>4. Comparison of recovered <em>ospF</em> DNA sequence to the requested sequence. Vertical dashes indicate exact matches</td>
<td>24</td>
</tr>
<tr>
<td>5. Comparison of recovered <em>erpG</em> DNA sequence to the requested sequence. Vertical dashes indicate exact matches</td>
<td>25</td>
</tr>
<tr>
<td>6. Comparison of recovered <em>erpK</em> DNA sequence to the requested sequence. Vertical dashes indicate exact matches</td>
<td>26</td>
</tr>
<tr>
<td>7. Comparison of recovered <em>erpL</em> DNA sequence to the requested sequence. Vertical dashes indicate exact matches</td>
<td>27</td>
</tr>
<tr>
<td>8. Detection of recombinant proteins by SDS-PAGE. Lane 1 contains OspF, lane 2 contains ErpG, lane 3 contains ErpK, and lane 4 contains ErpL</td>
<td>28</td>
</tr>
</tbody>
</table>
### LIST OF TABLES

<table>
<thead>
<tr>
<th>TABLE</th>
<th>PAGE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Primer sequences and expected amplicon sizes</td>
<td>13</td>
</tr>
<tr>
<td>2. Significant optical density (OD) cut-off values for ErpK, ErpL, ErpG or OspF ELISA</td>
<td>20</td>
</tr>
<tr>
<td>3. Primary clinical abnormalities of patients with Lyme disease confirmed by CDC Western blot criteria</td>
<td>30</td>
</tr>
<tr>
<td>4. Detection of ErpG, ErpK, or ErpL antibodies in Lyme disease sera</td>
<td>32</td>
</tr>
<tr>
<td>5. Detection of OspF antibodies in Lyme disease sera</td>
<td>34</td>
</tr>
</tbody>
</table>
INTRODUCTION

Lyme disease was first described in 1975 after Dr. Allen Steere noted an unusual epidemic of juvenile rheumatoid arthritis in a group of children residing in Lyme, Connecticut (1). However, the affected children lived in wooded areas, had rashes, and reported tick bites, which raised suspicion of an infectious cause. After further study, a spirochete was recovered from the midguts of captured *Ixodes scapularis* ticks (2), and the sera from the patients contained antibodies that bound the spirochetes (3). The newly-described organism was subsequently named *Borrelia burgdorferi* and, following discovery in the United States (US), similar spirochetes were isolated from both *I. ricinus* ticks and patients in Europe with symptoms that mimicked the illness seen in the US patients.

The initial impression, therefore, was that the organisms affecting the European patients were also *B. burgdorferi*, but later studies showed that, in addition to illness from *B. burgdorferi*, Lyme disease in European and Asian patients could also be caused by transmission of genetically-distinct *B. afzelii* or *B. garinii*. Since then, researchers (4-6) have confirmed that multiple *Borrelia* spp. cause Lyme disease, and the spirochetes are now classified as members of the *B. burgdorferi* sensu lato complex until genetic characterization has been completed. Despite the great genetic diversity, however, the most common causative agent of Lyme disease in the US remains *B. burgdorferi*, which is now recognized officially as *B. burgdorferi* sensu stricto (ss). Since its discovery,
Lyme disease has become the most common tickborne infection in North America, and hyperendemic foci of *B. burgdorferi* ss-infected *I. scapularis* ticks are located throughout the midwestern and northeastern regions of the US.

The illness is categorized by severity of symptoms and duration of infection, and formally-recognized categories include early localized, early disseminated or late disseminated stages. Early localized infection manifests primarily as non-specific constitutional abnormalities such as fatigue, headache, fever, arthralgia, and myalgia. In addition, approximately 80% of patients develop a rash, termed erythema migrans (EM) (7), that is typically an expanding red ring with central clearing. In fact, the EM lesion is associated almost exclusively with early Lyme disease, so most clinicians consider a characteristic EM a pathognomonic sign of the illness (8).

If the early localized infection is not treated appropriately, the organisms disseminate to a variety of other locations. Therefore, the symptoms of early disseminated infection depend on where the spirochetes have colonized, but well-characterized complications include secondary EM lesions (9), facial palsy (10), or cardiac rhythm abnormalities (11). In addition to these possibilities, however, the most common complaint in US Lyme disease patients is likely arthralgia, presumably because *B. burgdorferi* ss organisms have predilection for joint tissue (12). If a disseminated infection remains untreated or is treated inappropriately, symptoms of late stage illness are more severe abnormalities that include heart blockage (13), a dementia-like syndrome (14) or, most commonly, recurrent joint swelling (8).
After the spirochetal microorganisms were identified as the causative agent of Lyme disease and researchers confirmed the organisms sequestered in multiple sites, fears that the infection caused an illness that mimicked syphilis caused by *Treponema pallidum* spirochetes emerged. This was especially worrisome, because the prevalence of syphilis continues to increase worldwide, and morbidity and mortality associated with the illness are significant. In addition, the syphilis spirochetes are difficult to eliminate by antibiotic therapy since the organisms readily cross the blood-brain barrier (15). Moreover, since ticks infected with *Borrelia* spp. are endemic throughout many regions of the US, the risk of acquiring Lyme disease would likely be much more significant.

Not surprisingly, therefore, the initial strategy for laboratory testing emphasized the need to detect the illness early after infection. However, directly confirming the infection by recovering the organisms remains difficult, because the culture medium is difficult to make, and appropriate commercial sources of prepared medium are not readily-available. In addition, directly detecting the *Borrelia* spp. DNA by polymerase chain reaction (PCR) technology was not yet developed and, even after development, the test suffers from the inability to obtain samples that reliably contain the spirochetes. Therefore, providing indirect evidence of infection remains the most viable option, and detecting antibodies formed against the spirochetes is the norm.

Primary among the antibody tests were enzyme linked immunosorbent assays (ELISA), indirect fluorescent antibody (IFA) tests, or Western blotting, but each suffered significantly from a gross lack of specificity (16). For example, antibodies produced in patients with rheumatoid arthritis (17), multiple sclerosis (MS) (17), mononucleosis (18), and multiple other infections or conditions often also bound *Borrelia* spp. proteins, and
the cross-reactivity caused considerable confusion. The result was individuals with neurologic illnesses like MS or amyotrophic lateral sclerosis (ALS) would obtain a positive result from a Lyme disease laboratory test and then use the result to lobby for treatment with antibiotics. The non-specific anti-inflammatory properties of some antibiotics (19) subsequently caused the patients to believe they were on the road to recovery, but the patients inevitably relapsed after the treatment was halted. This caused considerable confusion, especially concerning whether additional antibiotic therapy would be helpful. The confusion ultimately caused the Centers for Disease Control and Prevention (CDC) to mandate focused effort to improve the specificity of Lyme disease tests, and the result was adaptation of a two-test system.

The first test was a sensitive, but nonspecific *B. burgdorferi* ELISA that utilized sonicated *B. burgdorferi* organisms to screen for any antibodies that bound the antigens. A diagnosis of Lyme disease was then unlikely if the ELISA yielded a negative result. Lyme disease remained a possibility if a positive result was obtained, however, so the serum was then re-tested by using a Western blot and specific antibodies were required to confirm a diagnosis of the illness. An IgM Western blot was considered positive if antibodies bound at least two of the *B. burgdorferi ss* 24 kDa, 39 kDa, or the 41 kDa protein (20), and a positive IgG blot required antibodies to bind at least five of the 18 kDa, 21 kDa, 28 kDa, 30 kDa, 39 kDa, 41 kDa, 45 kDa, 58 kDa, 66 kDa, or 93 kDa proteins (21).

The two-test regimen resulted in more accurate confirmation of Lyme disease. However, the requirement is cumbersome and expensive, which caused many laboratories to resist implementation. Therefore, researchers have continued to evaluate
alternate diagnostic laboratory testing procedures. Most prominent among these are peptide ELISAs that offer higher specificity by detecting antibodies that bind specific regions of individual *B. burgdorferi* proteins. The most useful have been pepC10 or C6 ELISAs that detect antibodies that bind specific epitopes within OspC (22) or the variable surface expressed (VlsE) protein (23), respectively.

Specifically, the pepC10 ELISA detects antibodies that bind the carboxy terminus of the OspC protein and, since OspC is reliably expressed shortly after the spirochetes invade the mammalian host (24, 25), pepC10 antibodies are produced shortly after infection. A drawback, however, is lack of sensitivity during the earliest stage of Lyme disease because it typically takes several weeks of infection before antibodies are produced. For example, Mathiesen et al. (22) showed that pepC10 antibodies were produced by only approximately 40% of patients with EM. In addition, the antibody response does not persist after the spirochetes disseminate because OspC expression is downregulated as the spirochetes leave the skin (26), and the antigen appears to be processed independent of T-cells (27), which makes the response predominately IgM antibodies (28).

The C6 ELISA complements the pepC10 ELISA, because the test detects antibodies that bind a 26-amino acid peptide based on a conserved region of the VlsE protein, and the VlsE protein is expressed as the spirochetes disseminate from the skin to other tissues (23). This makes the test insensitive during early Lyme disease (29), but highly sensitive after the spirochetes have disseminated. For example, Liang et al. (30) showed that C6 antibodies were produced by 128 (93%) of 138 patients with clinical characteristics
consistent with disseminated Lyme disease. Additionally, although initially marketed separately, Zeus Scientific has recently combined the two assays into a single ELISA.

The combination of the pepC10 and C6 ELISAs has therefore greatly improved the efficiency of laboratory diagnostics, and the new tests offer the added benefit of objective evaluation versus the subjective analysis necessary when interpreting a Western blot. Despite the development of these ELISAs however, the CDC has not to date deemed the improvements sufficient to eliminate their recommendation for the two-test system. Therefore, studies to identify additional Lyme disease-specific antibody responses that may also be useful for diagnostic purposes remain important.
STUDY RATIONALE AND SPECIFIC OBJECTIVES

The identification of one or more additional proteins or individual epitopes within proteins that reliably induce highly specific antibodies during human Lyme disease remains an important research goal, especially if the antibody response complements the responses detected by using the pepC10 and C6 ELISAs. The OspEF-related (Erp) proteins (31) may contain good candidate antigens because many are expressed on the surface of the spirochetes during human illness (32). However, identifying appropriate candidates that warrant investigation within this family of proteins is complicated by the large number of possibilities. In fact, the family of Erp proteins is so large and diverse that investigators recently reclassified the proteins into subfamilies (33, 34) based on whether the proteins were similar to OspE (OspE-related), OspF (OspF-related), or the proteins have leader peptides that are a combination of both the OspE and OspF leader peptides (OspE/F-like leader peptides or Elps) (35, 36).

Despite the diversity, however, careful review of the scientific literature revealed some study results that suggested several proteins that warrant study for their diagnostic potential. For example, recent studies in mice suggested that antibodies specific for ErpG, ErpK, and ErpL could be used to reliably confirm infection with Lyme disease spirochetes. In addition, the antibody response may yield insight into the location of the infection, because the antibodies specific for each of these proteins appears to differ based on the duration and location of infection. In support, ErpL antibodies are detected
during the first few weeks of infection in mice and ErpK antibodies are not produced until after the spirochetes have disseminated (37, 38). Additionally, the temporal response may be due to predilection for certain tissues as other researchers (35, 39) have also showed that ErpG binds glial cells with high affinity and ErpK specifically binds respiratory epithelial cells. Moreover, the proteins apparently target heparan sulfate (40), a glycosaminoglycan (GAG) (35) that forms proteoglycans by linking with proteins on cell surfaces and also in the extracellular matrix. The structure or composition of the heparan sulfate may also be responsible for the tissue preference.

Another prominent candidate diagnostic antigen is OspF. In support, Magnarelli et al. (41) showed that 5 (25%) of 20 immune sera from patients with EM lesions contained OspF antibodies. More significantly, approximately 90% of patients with early Lyme disease were seropositive when the significant OspF antibody responses were combined with the responses detected by using the pepC10 and C6 ELISAs. In addition, Akin et al. (42) detected OspF antibodies in 80% of immune sera from patients with Lyme arthritis, which suggests the antibody response expands as the spirochetes disseminate. Despite these findings, however, other researchers reported less promising results. For example, Hefty et al. (43) detected OspF antibodies in only 39% of sera from patients with disseminated Lyme disease. In addition, significant sequence heterogeneity among ospF within B. burgdorferi ss isolates caused others (44, 45) to speculate that the genetic differences would be sufficient to limit its usefulness for diagnostics. The result of these contradictions was that few studies to evaluate the utility of OspF for diagnosing human Lyme disease have since been performed. However, Wagner et al. (46) recently showed that OspF antibodies were a reliable marker of Lyme disease in canines infected
throughout the US, which highlights the necessity for additional investigation into the utility of OspF as a diagnostic antigen for confirming human Lyme disease.

This study was therefore designed to characterize the ErpG, ErpK, ErpL and OspF antibody responses during human Lyme disease. This goal was accomplished by: 1) cloning erpG, erpK, erpL, and ospF into an expression vector and preparing ELISAs with each recombinant protein, 2) identifying an appropriate cohort of well-defined human Lyme disease serum samples, 3) reviewing the medical charts of the Lyme disease serum donors to determine the primary clinical abnormalities so that the findings could be used to predict the likely location of infection, and 4) characterizing the IgM and IgG antibody responses specific for each recombinant protein in the Lyme disease sera.
METHODS AND MATERIALS

Normal sera. Normal sera (n = 15) were obtained from healthy adult (>18 years of age) volunteers who resided in the region including and immediately surrounding La Crosse, WI. Each volunteer subject had no previous suspicion (chart review) of Lyme disease. The serum samples were each de-identified, assigned a unique number, and then frozen in 100 µl aliquots at -20°C until used.

Lyme disease sera. Archived serum samples from Gundersen Health system patients that tested positive for *B. burgdorferi* VlsE antibodies by a commercial chemiluminescent immunoassay (LIAISON, DiaSorin, Stillwater, MN) were targeted. Some had also already been tested by Western blotting and those that tested positive (n=61) by the IgM or IgG CDC criteria (47) were obtained. In addition, sera with high OD values (>3.5) from the chemiluminescent assay that had not been tested previously by Western blotting were tested and those that also contained sufficient levels of antibodies to satisfy the CDC requirement for Western blot positivity (n=50) were also included (47).

Chart review. The medical records of serum sample donors were accessed after approval by the Gundersen Health System institutional review board (IRB). Only the primary clinical abnormalities and previous laboratory test results associated with the Lyme disease diagnosis were retrieved.
**Western blotting.** Western blots were performed by using a commercially-available kit (Marblot® IgM and IgG Western Blot kit, Trinity Biotech, Jamestown, NY) according to the manufacturer’s instructions. A standardized procedure was used to detect antibodies that bound proteins within the provided nitrocellulose strips, and the band reactivities were scored by comparison to a provided serum band locator strip. A weakly reactive positive control was also included to prevent overdevelopment of the strips.

**Selection, synthesis, and recovery of genes.** Appropriate *ospF, erpG, erpK,* and *erpL* gene sequences were targeted by analyzing sequences available of the entire gene with a BLAST search. Requirements for selection included thorough characterization and recovery from *B. burgdorferi* 297. The latter requirement was important because the 297 isolate is a well-documented human pathogen (48) with a genetic sequence that closely matches Lyme disease spirochetes recovered in the La Crosse, WI region. Since the promoter sequences of the OspF-subfamily genes are highly similar, the individual gene sequences were chemically synthesized and incorporated into a pUC57 plasmid by a commercial company (Genscript, Piscataway, NJ).

The synthesized genes were then recovered from the pUC57 plasmid using a commercial kit (Phusion High Fidelity PCR Kit, New England Biolabs, Ipswich, MA) and inserted into the expression vector pET100. Briefly, a one µl amount of DNA from a solution of four µg of pUC57 resuspended in 50 µl of AE buffer was combined with 20.5 µl of master mix that consisted of 13.5 µl of PCR grade water, four µl of HF buffer, 0.5 µl of 10 mM dNTPs, one µl of forward primer (100 µM), one µl of reverse primer (100 µM), and 0.5 µl of DNA polymerase. To aid in the proper insertion into the pET100 cloning vector, each forward primer had a 5’ CACC overhang and the reverse primers
had an additional 3’ TAA stop codon (Table 1). Additionally, the primers were designed to create a truncated version of the whole protein (35), which aided in the efficiency of protein recovery by eliminating the N-terminal signal sequence (49). After mixing the suspension, the DNA was amplified by using an initial hold at 98°C for 5 minutes, 40 cycles of denaturation at 98°C for 15 seconds, annealing at 50°C for 30 seconds, elongation at 72°C for 45 seconds, and a final extension at 72°C for 7 minutes (Model 2720, Applied Biosystems, Foster City, CA).

The amplified DNA was separated by electrophoresis (Biorad) in a 2% 1X Tris-acetate-EDTA (TAE) agarose gel. Briefly, the poured gel was allowed to solidify by incubation at room temperature for approximately 30 minutes, 20 µl of amplified DNA combined with four µl of 6X gel loading buffer (Novagen, Temecula, CA) was loaded onto the gel, and the gel was electrophoresed at 100 V for 65 minutes. After electrophoresing, the gel was immersed in 100 ml of distilled water containing 0.7 µg/ml ethidium bromide for 15 minutes at room temperature with gentle rocking, and then evaluated by UV illumination. The size of the recovered DNA was approximated by comparison to perfect DNA marker (Novagen). The portions of the gel that contained the gene of interest were then excised, and the DNA was recovered from the excised portion of the gel by using a commercial kit (MinElute Gel Extraction Kit, Qiagen, Germantown, MD) that bound the DNA to a silica membrane followed by elution in low-salt buffer.
<table>
<thead>
<tr>
<th>Targeted gene</th>
<th>Primer Sequence</th>
<th>Expected Amplicon Size (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>ospF</td>
<td>aF: CACCGATTTAGAAGGGGCA R: TTATTATCTTTTTTGACTTCTCCATT</td>
<td>616</td>
</tr>
<tr>
<td>erpG</td>
<td>F: CACCGAAGATTTAAAACAAAATGTAA R: TTATTATTTTTATCTATATTTTGAGGCTC</td>
<td>520</td>
</tr>
<tr>
<td>erpK</td>
<td>F: CACCCTAAAAAAAGTTTAGAACAAGA R: TTACTATCTTTTTATTAGAATCTTTTAGATTCTT</td>
<td>688</td>
</tr>
<tr>
<td>erpL</td>
<td>F: CACCGTAAAAAATTTCAGAACAAAATC R: TTATTATCTTTTTATCTTTTCTATGCCTT</td>
<td>613</td>
</tr>
</tbody>
</table>

*a F= Forward primer; R= Reverse primer*
**Insertion into expression vector and propagation of plasmids.** The purified DNA was then inserted into a pET100 (Invitrogen, Carlsbad, CA) expression vector (Fig. 1). The DNA was initially diluted in elution buffer to a concentration of up to 5 ng/µl and a one µl volume was then combined with one µl of the supplied salt solution and 0.5 µl of pET100 vector. During a room temperature incubation, the DNA fragments were incorporated into the linearized plasmid by benefit of an attached topoisomerase.

The pET100 vector containing each DNA insert was transformed into TOP10 *E. coli* cells. Briefly, the TOP10 *E. coli* cells were thawed, pET100 vector was added to the cells, and the mixture was incubated on ice for 20 minutes. After incubation, cells were heat-shocked for 30 seconds at 42°C and immediately transferred back to the ice. The cells were then combined with 250 µl of Super Optimal Catabolite (SOC) medium (Invitrogen), followed by a one hour incubation at 37°C. Lastly, a 100 µl volume of the SOC medium containing the transformed TOP10 cells was spread onto 2x-YT plates containing 100 µg/ml of ampicillin, and the plate was incubated overnight at 37°C. Colonies of transformed TOP10 cells were then picked and cultured in 5ml of 2x-YT containing 100 µg/ml of ampicillin overnight at 37°C with shaking. The plasmids were finally recovered by using a commercially available kit (Qiagen) per the manufacturer’s instructions, forwarded for commercial sequencing using T7 promoter sequencing primers (Laragen, Culver City, CA), and evaluated by BLAST to confirm the integrity.
FIG 1 Schematic illustration and genetic sequence of the pET100 vector. The insertion site is shaded.
Transformation of BL21 Star cells. To transform the plasmids into BL21 Star (DE3) One Shot E. coli cells (Invitrogen), the cells were thawed, two µl of purified plasmid was added to the cells, and the suspension was incubated on ice for 30 minutes. Next, the cells were heat-shocked by incubation for 30 seconds at 42°C. The transformed cells were then combined with 250 µl of SOC medium, and the suspension was incubated for 30 minutes at 37°C with shaking. After incubation, the suspension was combined with 10 ml of 2x-YT containing 20 µl of ampicillin and 333 µl of 30% sterile glucose solution prior to overnight incubation at 37°C with shaking. Per the manufacturer’s recommendations, expression of each recombinant protein was then induced prior to freezing. Specifically, two ml of the culture was added to 200 ml of 2x-YT media containing 400 µl ampicillin and 7 ml of 30% sterile glucose solution, and the culture was incubated for two hours at 37°C with shaking. After incubation, 200 µl of 1M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added, and the culture was re-incubated for 24-36 hours at 26°C. After incubation, the cell suspension was divided into 50 ml fractions, the cells were pelleted by centrifugation, and the pelleted organisms were frozen at -80°C until used.

Purification of proteins. Recombinant proteins were recovered by using a commercial kit (Probond Purification System, Invitrogen). Briefly, the cell pellets were lysed by resuspension in 8 ml of guanidinium lysis buffer followed by rocking at room temperature for 10 minutes. Next, the cell lysate was sonicated (Branson Sonic Power W350, Danbury, CT), and the cellular debris was pelleted by centrifugation prior to transferring the supernatant to a fresh tube. The supernatant was then added to a column containing a nickel-charged affinity resin (Invitrogen) that bound the polyhistidine tag on
the N-terminus of the recombinant proteins. The bound proteins were then washed by passing two four-ml volumes of wash buffer (pH 6.0) over the columns followed by an additional two four-ml volumes of wash buffer (pH 5.3). An additional 6 one-ml volumes of wash buffer (pH 5.3) were then poured over the column prior to eluting the recombinant proteins by passing 5 separate one ml volumes of elution buffer (pH 4.0). The captured proteins were subsequently dialyzed and concentrated by using a centrifugal filter unit (Amicon®, Millipore Sigma, Burlington, MA).

**Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).**

SDS-PAGE was performed using standard techniques. Briefly, elution buffer that contained 11.5 µg of protein was combined with an equal volume of sample buffer, boiled in a water bath for 5 minutes, and allowed to cool to room temperature. A 30 µl amount was then loaded into an individual well of a 0.1% SDS-10-20% gradient polyacrylamide gel, and the proteins were separated by electrophoresing at 200 V for approximately one hour (Criterion, Biorad, Hercules, CA). Next, the gel was stained by immersing in buffer containing 0.125% Coomassie brilliant blue and rocking the solution at room temperature overnight. After staining, the gel was destained by using a buffer that contained methanol and glacial acetic acid, and the approximate sizes of proteins were determined using a standardized molecular weight standard (GE Healthcare, Pittsburgh, PA).

**Enzyme linked immunosorbent assay (ELISA).** Individual wells of microtiter plates (Immunolon 2 HB; Thermo Labsystems, Franklin, MA) were coated with purified recombinant protein diluted to one µg/ml in carbonate coating buffer (0.15 M sodium carbonate and 0.04 M sodium bicarbonate adjusted to pH 9.6) and incubated overnight at
4°C. After incubation, the plates were washed three times with phosphate buffered saline (pH 7.2) containing 0.05% tween (PBS-T), blocked by adding 200 µl PBS-T and 1% bovine serum albumin and incubating at room temperature for one hour. The plates were then washed with PBS-T, a 100 µl volume of immune serum diluted 1:400 with PBS-T was added to each well, and the plates were re-incubated at room temperature for one hour. After an additional wash, a 100 µl volume of horseradish peroxidase-conjugated anti-human IgM or IgG antibodies (SeraCare Life Sciences Inc., Milford, MA) diluted 1:15,000 or 1:20,000 with PBS-T, respectively, was added to each well, and the plates were incubated at room temperature for an additional hour. After incubation, the plates were washed and 100 µl of freshly prepared o-phenylenediamine dihydrochloride (OPD) substrate (Sigma-Aldrich, St. Louis, MO) was added to each well prior to incubation at room temperature for 30 minutes. The reaction was then stopped by adding 100 µl of 1N H₂SO₄, and the optical density (OD) value at 490 nm was measured by a spectrophotometer (VersaMax; Molecular Devices, Sunnyvale, CA).

Prior to using the ELISA, the optimum concentration of conjugated anti-IgM or anti-IgG antibodies was determined by performing checkerboard analyses with a known positive serum and a normal serum (50). The levels of IgM and IgG reactivity considered significant were determined by averaging the optical density (OD₄₉₀) value from 15 case-defined normal sera and then considering a significant reactivity as a reading greater than or equal to three standard deviations above the mean OD value. The result of the analyses were OD cut-off values that ranged from 0.46 to 1.05 (Table 2). A serially-diluted positive control was also included with each ELISA plate and interassay
variability was minimized by requiring the significant reactivity of the control serum to fall within one dilution during each successive run.

**Statistics.** The relatedness among the ELISAs was evaluated by using Cochran’s Q test to compare the positive and negative values. A p value $\leq 0.05$ was considered significant.
TABLE 2 Significant optical density (OD) cut-off values for ErpK, ErpL, ErpG or OspF ELISA.

<table>
<thead>
<tr>
<th>ELISA</th>
<th>Mean optical density (OD) values of normal sera (n=15)</th>
<th>Standard deviation</th>
<th>Significant cut-off value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
<td>IgM</td>
</tr>
<tr>
<td>ErpK</td>
<td>0.25</td>
<td>0.40</td>
<td>0.15</td>
</tr>
<tr>
<td>ErpL</td>
<td>0.26</td>
<td>0.56</td>
<td>0.11</td>
</tr>
<tr>
<td>ErpG</td>
<td>0.21</td>
<td>0.37</td>
<td>0.08</td>
</tr>
<tr>
<td>OspF</td>
<td>0.24</td>
<td>0.35</td>
<td>0.14</td>
</tr>
</tbody>
</table>

<sup>a</sup> Three standard deviations above the mean OD values of normal sera.
RESULTS

Recovery of recombinant (r) proteins. The cloning strategy was comprised of several distinct steps (Fig. 2), so it was necessary to ensure the validity of the recombinant proteins encoded by each construct by confirming the integrity of the products after each step. Amplification of the genes contained within pUC57 yielded appropriately-sized amplicons (Fig. 3), and the DNA sequences exactly matched the sequence deposited in GenBank after the DNA was purified, inserted into pET100, and transformed into TOP10 E. coli (Fig. 4-7). The molecular weight of each recombinant protein recovered after expressing each gene in BL21 Star E. coli was also appropriate (Fig. 8). In addition, the purified recombinants contained only small amounts of residual E. coli proteins.
FIG 2 Schematic illustration of cloning strategy.
FIG 3 Detection of amplified genes by agarose gel electrophoresis. Lane 1 contains 50 bp -10 kbp Perfect DNA Marker, lane 2 contains ospF fragment, lane 3 contains erpG fragment, lane 4 contains erpK fragment, and lane 5 contains erpL fragment.
FIG 4 Comparison of recovered *ospF* DNA sequence to the requested sequence. Vertical dashes indicate exact matches.
**FIG 5** Comparison of recovered *erpG* DNA sequence to the requested sequence. Vertical dashes indicate exact matches.
FIG 6 Comparison of recovered *erpK* DNA sequence to the requested sequence. Vertical dashes indicate exact matches.
FIG 7 Comparison of recovered *erpL* DNA sequence to the requested sequence. Vertical dashes indicate exact matches.
FIG 8 Detection of recombinant proteins (highlighted by ▶) by SDS-PAGE. Lane 1 contains OspF, lane 2 contains ErpG, lane 3 contains ErpK, and lane 4 contains ErpL. Other bands are residual *E. coli* proteins.
Characterization of Lyme disease sera. A total of 111 Lyme disease sera confirmed by Western blot were used. Of these, 25 (23%) contained *B. burgdorferi* specific IgM antibodies, 22 (20%) contained IgM and IgG antibodies, and 64 (58%) contained IgG antibodies (Table 3). In addition, the clinical complaints included non-specific (e.g. headache, fatigue) constitutional symptoms (n = 23), single or multiple skin lesions (n = 30), Bell’s palsy (n = 10), neuropathy/neuritis (n = 2), heart abnormalities (n = 3), arthralgia (n = 18) or arthritis (n = 25).
<table>
<thead>
<tr>
<th>Primary clinical complaint (no.)</th>
<th>No. (%) of sera confirmed by:</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgM and IgG</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>-----</td>
<td>-------------</td>
</tr>
<tr>
<td><strong>Constitutional</strong>&lt;sup&gt;b&lt;/sup&gt; (23)</td>
<td>4 (17)</td>
<td>6 (26)</td>
</tr>
<tr>
<td><strong>Erythema migrans</strong>&lt;sup&gt;c&lt;/sup&gt; (30)</td>
<td>13 (43)</td>
<td>4 (13)</td>
</tr>
<tr>
<td><strong>Arthralgia</strong> (18)</td>
<td>3 (17)</td>
<td>3 (17)</td>
</tr>
<tr>
<td><strong>Bell’s Palsy</strong> (10)</td>
<td>4 (40)</td>
<td>3 (30)</td>
</tr>
<tr>
<td><strong>Cardiac</strong>&lt;sup&gt;d&lt;/sup&gt; (3)</td>
<td>1 (33)</td>
<td>1 (33)</td>
</tr>
<tr>
<td><strong>Neuropathy/Neuritis</strong> (2)</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><strong>Arthritis</strong> (25)</td>
<td>0</td>
<td>5 (20)</td>
</tr>
</tbody>
</table>

<sup>a</sup> IgM= at least two of the 22kDa, 39 kDa, or the 41 kDa proteins
IgG= at least five of the 18 kDa, 21 kDa, 28 kDa, 30 kDa, 39 kDa, 41 kDa, 45 kDa, 58 kDa, 66 kDa, or 93 kDa proteins.

<sup>b</sup> Symptoms included fatigue, headache, fever, and myalgia.

<sup>c</sup> Includes patients with single (n=17) or multiple (n=13) EM lesion(s).

<sup>d</sup> Heart complications were bradycardia (n=2), heart block (n=2), asystole (n=1), or atrial fibrillation (n=1).
Detection of ErpG, ErpK, and ErpL antibodies. Significant levels of IgM and/or IgG antibodies that bound ErpG, ErpK, or ErpL were detected in the Lyme disease sera, but the response was without apparent pattern. For example, ErpG antibodies were detected in 16 (53%), ErpK was detected in 17 (57%), and ErpG was detected in 11 (37%) sera from patients with EM lesions (Table 4). However, the response failed to expand significantly after the spirochetes disseminated, since similar percentages of the sera from patients with arthralgia or arthritis contained antibodies. In addition, there were no readily-apparent differences when the positive and negative results were compared (p = 0.44).
**TABLE 4** Detection of ErpG, ErpK, or ErpL antibodies in Lyme disease sera.

<table>
<thead>
<tr>
<th>Primary clinical complaint (no.)</th>
<th>ErpG</th>
<th>ErpK</th>
<th>ErpL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>IgM</td>
<td>IgG</td>
<td>T&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Constitutional&lt;sup&gt;b&lt;/sup&gt; (23)</td>
<td>4 (17)</td>
<td>7 (30)</td>
<td>10 (43)</td>
</tr>
<tr>
<td>Erythema migrans&lt;sup&gt;c&lt;/sup&gt; (30)</td>
<td>13 (43)</td>
<td>7 (23)</td>
<td>16 (53)</td>
</tr>
<tr>
<td>Bell’s Palsy (10)</td>
<td>4 (40)</td>
<td>2 (20)</td>
<td>5 (50)</td>
</tr>
<tr>
<td>Neuropathy/Neuritis (2)</td>
<td>0</td>
<td>2 (100)</td>
<td>2 (100)</td>
</tr>
<tr>
<td>Cardiac&lt;sup&gt;d&lt;/sup&gt; (3)</td>
<td>0</td>
<td>2 (67)</td>
<td>2 (67)</td>
</tr>
<tr>
<td>Arthralgia (18)</td>
<td>4 (22)</td>
<td>8 (44)</td>
<td>10 (56)</td>
</tr>
<tr>
<td>Arthritis (25)</td>
<td>2 (8)</td>
<td>11 (44)</td>
<td>12 (48)</td>
</tr>
</tbody>
</table>

<sup>a</sup> T= Total no. (%) with IgM and/or IgG antibodies.

<sup>b</sup> Symptoms included fatigue, headache, fever, and myalgia.

<sup>c</sup> Includes patients with single (n=17) or multiple (n=13) EM lesion(s).

<sup>d</sup> Heart complications were bradycardia (n=2), heart block (n=2), asystole (n=1), or atrial fibrillation (n=1).
Detection of OspF antibodies. Similar to the findings with the Erp ELISAs, significant levels of IgM and/or IgG antibodies were detected in 13 (43%) of patients with likely localized Lyme disease manifesting as single or multiple EM lesions. However, in contrast to the Erp findings, the frequency of detection of OspF antibodies increased coincident with increased duration of infection and severity of symptoms (Table 5). Most notably, significant levels of OspF antibodies were detected in patients with likely joint infection manifesting as arthralgia (78%) or arthritis (84%), and the antibodies were detected in every patient with neuropathy/neuritis or heart complications. The collective findings therefore failed to provide compelling support for the diagnostic utility of the Erps, but confirmed that OspF antibodies were a more predictable immune response that was especially prevalent in patients with disseminated Lyme disease.
**TABLE 5** Detection of OspF antibodies in Lyme disease sera.

<table>
<thead>
<tr>
<th>Primary complaint (no.)</th>
<th>Likely site of infection</th>
<th>No. (%) positive sera</th>
<th>IgM</th>
<th>IgG</th>
<th>Total&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Constitutional&lt;sup&gt;b&lt;/sup&gt; (23)</td>
<td>Unknown</td>
<td>1 (4)</td>
<td>15 (65)</td>
<td>15 (65)</td>
<td></td>
</tr>
<tr>
<td>Erythema migrans&lt;sup&gt;c&lt;/sup&gt; (30)</td>
<td>Skin</td>
<td>4 (13)</td>
<td>9 (30)</td>
<td>11 (37)</td>
<td></td>
</tr>
<tr>
<td>Bell’s Palsy (10)</td>
<td>CNS</td>
<td>3 (30)</td>
<td>4 (40)</td>
<td>5 (50)</td>
<td></td>
</tr>
<tr>
<td>Neuropathy/Neuritis (2)</td>
<td></td>
<td>0</td>
<td>2 (100)</td>
<td>2 (100)</td>
<td></td>
</tr>
<tr>
<td>Cardiac&lt;sup&gt;d&lt;/sup&gt; (3)</td>
<td>Heart</td>
<td>0</td>
<td>3 (100)</td>
<td>3 (100)</td>
<td></td>
</tr>
<tr>
<td>Arthralgia (18)</td>
<td>Joints</td>
<td>5 (28)</td>
<td>14 (78)</td>
<td>14 (78)</td>
<td></td>
</tr>
<tr>
<td>Arthritis (25)</td>
<td></td>
<td>3 (12)</td>
<td>21 (84)</td>
<td>21 (84)</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> No. (%) with IgM and/or IgG antibodies detected.

<sup>b</sup> Symptoms included fatigue, headache, fever, and myalgia.

<sup>c</sup> Includes patients with single (n=17) or multiple (n=13) EM lesion(s).

<sup>d</sup> Heart complications were bradycardia (n=2), heart block (n=2), asystole (n=1), or atrial fibrillation (n=1).
DISCUSSION

Lyme disease is the most widespread tickborne illness in the US and confirming the illness by laboratory tests can be problematic. The most commonly used method is the two-test system comprised of a sensitive ELISA followed by a specific Western blot. However, performing the two-test system is cumbersome and expensive. In response, researchers developed C6 and pepC10 peptide ELISAs that improved the speed and affordability of testing, but the improvements were not sufficient to eliminate the CDC recommendation for the two-test system. Therefore, identifying additional antigens that could be useful for Lyme disease diagnostics remains an important goal. In this study, the OspF, ErpG, ErpK, and ErpL proteins were evaluated to determine whether patients with Lyme disease produced significant levels of antibodies specific for each protein and whether the antibody responses were sufficient to warrant additional studies that more completely evaluate this potential as an important diagnostic marker of infection.

Significant numbers of the Lyme disease sera contained antibodies that bound ErpG, ErpK, and/or ErpL, but the responses were without apparent pattern. In addition, none of the antibody responses were particularly predominant in any stage of infection. For example, approximately 50% of the sera from patients with single or multiple EM lesions contained antibodies against one or more of the Erps, but the response failed to expand significantly thereafter. In fact, only 49-67% of patients with arthralgia or
arthritis produced the antibody response. In addition, there were no significant (p = 0.44) differences among the responses detected by using either of the ELISAs, which contradicted previous findings where antibody responses against the Erps varied with duration of infection (37, 38). Furthermore, the findings provided scant support for previous reports (35, 39) that ErpG and ErpK could be differentiated by affinity for glial or epithelial cells, respectively. For example, ErpG antibodies were detected in serum samples from the Lyme disease patients with neuritis/neuropathy, but also detected commonly in patients with skin (EM) or joint (arthritis/arthritis) infection. Similarly, ErpK antibodies were detected in the sera from patients with a probable infection of the nervous system manifesting as Bell’s palsy. These contradictory results are likely due to early literature combining results from different OspF-related proteins and a lack of consistent nomenclature. The collective findings therefore failed to provide much support for the diagnostic utility of the Erps.

In contrast, however, the OspF antibody response clearly expanded coincident with the increasing duration of infection and severity of the clinical abnormalities. For example, while relatively few sera from the patients with EM lesions (37%) contained OspF antibodies, the antibody response clearly predominated as the spirochetes disseminated. Most notably, significant levels of OspF antibodies were detected in most (81%) patients with joint pain or swelling and each patient with cardiac abnormality or neuritis/neuropathy. The results therefore contradicted a previous finding (43) that OspF antibodies were only rarely produced by patients with disseminated Lyme disease and instead confirmed the results from another study (42) that showed that OspF antibodies were reliably (80%) produced by patients with Lyme arthritis. More significantly, the
results also refuted previous studies (44, 45) that suggested the heterogeneous antibody responses due to significant genetic differences within \textit{ospF} would hinder reliable detection of the antibody response, since the seropositive patients resided in diverse locations throughout the 3-state geographic region served by the Gundersen Health System.

Therefore, the findings suggest that the predominant OspF antibody response is targeting a conserved region within OspF and additional studies to discriminate the epitope(s) would be useful. Moreover, the results provide strong evidence that, in contrast to the findings using the Erps, OspF may be a valuable serodiagnostic marker that is especially useful after the Lyme disease spirochetes have disseminated. In addition, even though the antibody response was less frequently detected in the patients with early Lyme disease, a test that detects OspF antibodies may still be valuable for these patients if the response occurs independent of the pepC10 and C6 antibody responses. Curiously, Magnarelli et al. (41) showed in 2002 that almost 90\% of early Lyme disease sera were seropositive when the positive OspF antibody responses were combined with those obtained using a pepC10 and C6 ELISA, but the possibility was not formally pursued thereafter. The findings in this study, however, provide added impetus for additional effort to also more completely evaluate this possibility.

Several strengths and a weakness of this study should also be highlighted. As strengths, the intent to recover four recombinant proteins at sufficient volumes to perform ELISAs could have been daunting, but the universal cloning strategy was efficient, and the experience gained with each clone streamlined subsequent efforts. In addition, recovering the recombinant proteins by utilization of a His-tag yielded recombinant
proteins with little contamination from *E. coli* proteins. Moreover, the legitimacy of the immune sera was irrefutable, since antibodies against other proteins were sufficient to satisfy the CDC Lyme disease surveillance criteria. A significant shortcoming, however, was that the recombinant proteins still contained a small amount of *E. coli* proteins that could have confounded precise evaluation of protein concentrations and, in turn, affected the accuracy of the ELISA OD values considered significant. In addition, the specificity of each antibody response was not evaluated by also testing sera from patients with unrelated illnesses that may have potentially contained cross-reactive antibodies. In summary, however, identifying additional *B. burgdorferi* ss proteins that reliably induce antibodies in Lyme disease patients remains an important goal, especially if the response can ultimately be used to eliminate the necessity for confirming the illness by using the cumbersome two-test system. While the results in this study provided little evidence of the utility of the Erps for serodiagnosis, the OspF antibody response was reliably produced, especially in patients with disseminated infection with *B. burgdorferi* ss. Therefore, additional studies that more critically evaluate the utility of detection of OspF antibodies for laboratory confirmation of Lyme disease are especially warranted.
REFERENCES


disease with carditis complicated by posttreatment Lyme disease syndrome. Case
Rep Infect Dis 2017:5847156.

burgdorferi sensu lato spirochetes is largely complement independent. PLoS One
9:e108013.

carditis: an interesting trip to third-degree heart block and back. Case Reports
Cardiol 2016:1–3.

14. Kristoferitsch W, Aboulenein-Djamshidian F, Jecel J, Rauschka H, Rainer M,


16. Lane RS, Lennette ET, Madigan JE. 1990. Interlaboratory and intralaboratory
comparisons of indirect immunofluorescence assays for serodiagnosis of Lyme

Molecular and Clinical Laboratory Immunology, 7th Edition. ASM Press,

common clinical pitfalls, and future directions for laboratory diagnosis of Lyme

pleiotropic family of compounds with promising therapeutic properties. Review


APPENDIX A
TRUNCATED SEQUENCES OF OSPF, ERPG, ERPK, AND ERPL
OspF: Genbank # HM756745.1

ATGCGGGGTTCTCATCACAATGGGCTGAGATGATGAACGCCAACATCTCACATG
CTGGTGACAGCAATGGGTGCTGAGATGACGATAAGGATCATCCCTTACC

ErpG: Genbank # AE001576.1

ATGCGGGGTTCTCATCACAATGGGCTGAGATGATGAACGCCAACATCTCACATG
CTGGTGACAGCAATGGGTGCTGAGATGACGATAAGGATCATCCCTTACC

ErpK: Genbank # AE001578.1

ATGCGGGGTTCTCATCACAATGGGCTGAGATGATGAACGCCAACATCTCACATG
CTGGTGACAGCAATGGGTGCTGAGATGACGATAAGGATCATCCCTTACC

46
ErpL:

ATGCGGGGTTCATCATCATCATCATCATCATGTATGGCTAGCATGACTGGGTGG
ACAGCAATGGGTGCAGGATCTGTCGACGATGACGGATTGATCCCTGC

Green = Vector sequence
Blue = Overhang added for insertion into vector
Red = Repeated stop codon
APPENDIX B
VECTOR pET100 /D-TOPO SEQUENCE DATA