Development of a multi-antigen panel for improved detection of *Borrelia burgdorferi* infection in early Lyme disease

Running Title: Ten-antigen panel for improved *Bb* detection

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ABSTRACT

The current standard for laboratory diagnosis of Lyme disease in the United States is serologic detection of antibodies against *Borrelia burgdorferi* (*Bb*). The Centers for Disease Control and Prevention recommends a two-tiered testing algorithm, however this scheme has limited sensitivity in detecting early Lyme disease. Thus, there is a need to improve diagnostics for Lyme disease at the early stage when antibiotic treatment is highly efficacious. We examined novel and established antigen markers to develop a multiplex panel that identifies early infection using the combined sensitivity of multiple markers, while simultaneously maintaining high specificity by requiring a positive test at two markers. Ten markers were selected from our initial analysis of 62 *Bb* surface proteins and synthetic peptides by assessing binding of IgG and IgM to each in a training set of Lyme disease patient samples and controls. In a validation set, this 10-antigen panel identified a higher proportion of early Lyme disease patients as positive at the baseline or post-treatment visit compared to two-tiered testing (87.5% and 67.5% respectively, \( P<0.05 \)). Equivalent specificities of 100% were observed in 26 healthy controls. Upon further analysis, positivity on the novel 10-antigen panel was associated with longer illness duration and multiple erythema migrans. The improved sensitivity and comparable specificity of our 10-antigen panel compared to two-tiered testing in detecting early *Bb* infection indicates that multiplex analysis, featuring the next generation of markers, could advance diagnostic technology to better aid clinicians in diagnosing and treating early Lyme disease.
INTRODUCTION

Lyme disease is the most prevalent vector-borne disease in the United States and is caused by the spirochete bacterium *Borrelia burgdorferi* sensu stricto (*Bb*) (1). Clinical diagnosis of early Lyme disease relies on identification of an active skin lesion termed erythema migrans (EM) and a history of exposure to disease-endemic areas (2-4). However, physicians face several challenges in diagnosing the early stage of disease: 20% of patients do not develop an EM, rashes may be atypical, and other presenting symptoms may be non-specific to *Bb* infection (5, 6). Antibiotic therapy (e.g., doxycycline) effectively treats early Lyme disease and prevents progression to disseminated stages involving neurologic, cardiac, and rheumatologic illness (1, 7-9). Therefore, proper diagnosis and treatment of Lyme disease in the early stage is imperative to limiting the number of patients who progress to later, more severe disease.

For a variety of reasons, neither direct laboratory detection of *Bb* nor laboratory culture from patient samples has been satisfactory (10, 11). The Centers for Disease Control and Prevention (CDC) currently recommends a two-tiered format for serologic detection of the patient’s antibody response to spirochete antigens (12). This strategy requires both a positive first tier enzyme immunoassay (EIA) and second tier Western blot, yielding overall specificity of 99.5% (13, 14). Two-tiered testing faces several limitations, namely it correctly identifies only 29-40% of patients presenting with an EM in the early stage of Lyme disease (10) and Western blot analysis is labor intensive, subjective, and can have issues with reproducibility (15-17).

Recent efforts to improve laboratory testing for Lyme disease have aimed to identify diagnostically superior individual antigen markers. Epitope mapping of *Bb* surface proteins has revealed novel peptides conserved across *Bb* genospecies that are not found in other bacterial species (18). Synthetic antigen peptides derived from these epitopes demonstrate equivalent sensitivity and decreased cross reactivity (19, 20).
Multiplex approaches have the potential to improve sensitivity and specificity of the current technology by assessing antibody responses to multiple \textit{Bb} proteins and these novel peptide markers simultaneously (21-25). Such platforms may detect cases where a patient is not responsive to the main immunodominant epitope, where the infecting subspecies of \textit{Bb} is polymorphic at the immunodominant epitope, or where temporal variations in antigen or antibody limit tests based on single antibody detection (26). Furthermore, multiplex systems may increase specificity by requiring two or more markers to be positive in a panel, thus minimizing false positive results due to cross-reactivity of any one single marker.

Herein, we sought to improve detection of anti-\textit{Bb} antibodies in early Lyme disease through the utilization of a multiplex antigen panel that combines the sensitivity of novel synthetic peptides with established markers. We demonstrate that a multiplex panel of 10 antigens with a two-positive rule improves upon the sensitivity of early \textit{Bb} detection compared to CDC two-tiered testing while maintaining equally high degrees of specificity. Positivity on the 10-antigen panel is associated with longer illness duration and the presence of multiple EM lesions. In total, these data support further development of multiplex technology utilizing the markers reported here as a method to better detect \textit{Bb} infection and aid diagnosis of early Lyme disease.

\textbf{MATERIALS AND METHODS}

\textbf{Sample collection.} Serum samples were collected following informed consent under protocols approved by institutional review boards at the institutions outlined below.

Cohort 1 included 79 patients physician-diagnosed with early Lyme disease based on the presence of an EM (Supplemental Table 1), and five patients with untreated late Lyme disease presenting with Lyme arthritis, all collected through Johns Hopkins University in a Lyme-endemic region of Maryland (27, 28). In this prospective study, patients meeting all entrance
requirements were enrolled, prescribed a three-week course of doxycycline at the baseline visit, examined three weeks later upon completion of antibiotic treatment (post-treatment visit), and then followed for four additional visits (one month post-treatment, three months post-treatment, six months post-treatment, and one year post-treatment). Twenty-six healthy controls with no known history of Lyme disease were followed for three visits (baseline, six months, and one year).

Cohort 2 included 20 early Lyme disease patients with culture-confirmed EM, 107 late Lyme disease patients, and 30 healthy donors with no known history of Lyme disease, all provided by Massachusetts General Hospital (29-31) located in a Lyme-endemic region of Massachusetts. All late Lyme disease patients met the CDC criteria for Lyme arthritis, which included monoarticular or oligoarticular arthritis, accompanied by a positive two-tiered serologic test result.

Cohort 3 included 175 chronic fatigue syndrome (CFS) patients and 244 age- and sex-matched healthy control subjects collected by Stanford University, located in a non-endemic region of California, as part of the Myalgic Encephalomyelitis/CFS Initiative (32, 33). Additionally, serum samples from 200 healthy controls were acquired through blood banks, with 100 of these collected from Lyme-endemic regions of the United States.

Commercial two-tiered testing of cohort 1. Two-tiered serologic testing following CDC guidelines (12) was performed by Quest Diagnostics as previously described (28). Specifically, the first tier utilized the ZEUS ELISA *Borrelia* VlsE1/pepC10 IgG/IgM Test System (Alere, Waltham, Massachusetts). Second tier Western blots were performed using MarDx. *B. burgdorferi* Marblot Western blot systems (Trinity Biotech, Bray, Ireland), with IgG and IgM evaluated in patients with illness duration less than one month and only IgG evaluated if illness
duration was equal to or greater than one month. If a patient sample tested two-tier negative at
the baseline visit, a convalescent serum sample from the post-treatment visit was sent for two-
tiered testing.

Multiplex analysis of anti-\textit{Bb} antibody markers. We examined 62 candidate antigens,
including seven \textit{Bb} surface proteins and 55 synthetic peptides based on \textit{Bb} antigenic epitopes.
Several of the peptide antigens have been described previously (18-20, 34, 35). See
Supplemental Table 2 for the amino acid sequence of each synthetic peptide, vendor information
for each recombinant protein, and strain and annotated locus number for markers where
available. For peptide markers, the abbreviated protein name from which the amino acid
sequence was taken is followed by the specific residue numbers used in parentheses. A number
of markers consist of two distinct peptide epitopes, linked by triglycine. Markers containing
linked epitopes derived from two different parent proteins are named with the concatenated
protein name and residue numbers for each portion. A protein name followed with “combined”
indicates that the marker consists of two linear epitopes from a single parent protein which have
been linked with triglycine. Synthetic peptides which represent lipoprotein epitopes are in the
unprocessed form. Each antigen marker was conjugated to spectrally-distinct beads using
Luminex technology and established methods (23). The pooled antigen panel was incubated with
serum samples and binding was separately assayed using anti-human IgG conjugated to
phycoerythrin (PE) or anti-human IgM-PE. Beads were analyzed using a Luminex 200
instrument (Luminex Corporation, Austin, Texas) running Bio-Plex Manager Software v5.0
(Bio-Rad Laboratories, Hercules, California) and the median fluorescence intensity (MFI) of 200
beads events per analyte was quantified.
Training of markers in 10-antigen panel. Following multiplex screening of 124 markers, a positivity cutoff for each marker was defined as twice the 98th percentile MFI in a set of 222 healthy controls reserved at random from cohort 3 (Figure 1A). This definition of positivity was then used to select the two most sensitive markers at the baseline and post-treatments visits for a set of 39 early Lyme disease patients reserved at random from cohort 1. Eight supporting markers were chosen on the basis that they tested positive when the top two markers tested negative in the training set of early Lyme disease patients. To test positive, a sample was required to have two or more positive markers from this list of ten (Figure 1B), regardless of marker isotype.

Statistical analysis. Antibody Index (AI) was calculated as the ratio of the experimental MFI value to the positivity cutoff. Differences between proportions of test results were analyzed using a two-tailed Chi-square test. Illness duration groups were analyzed for differences using Kruskal-Wallis one-way analysis of variance followed by Dunn’s multiple comparison test. Early Lyme disease patients with multiple EM versus single EM were compared at each visit using Mann-Whitney U-test. Analyses were performed using GraphPad Prism software v5.01. P values less than 0.05 were considered statistically significant. Analytical precision of the multiplex antigen panel was calculated using the inter-assay coefficient of variance for three controls tested across 10 independent runs (Supplemental Table 3).

RESULTS

Generation of 10-antigen panel. To develop a multiplex panel with improved detection of anti-Bb antibodies in the early phase of infection, we screened a spectrum of novel synthetic peptides
and established proteins, and selected 10 antigens (Figure 1). Sensitivity and specificity of this
10-antigen panel were then examined for the training set and three validation sets (Table 1).

For early Lyme disease patients of cohort 1 at the baseline visit, sensitivities of the training
and validation sets were 56.4% and 55.0%, respectively. At the post-treatment visit, sensitivities
were 78.9% and 87.2%, respectively. All five late Lyme disease patients tested positive at each
visit (100% sensitivity) and none of the 26 healthy controls tested positive at any visit (100%
specificity).

Validation of the 10-antigen panel in cohort 2 demonstrated 100% sensitivity for 20 early
Lyme disease subjects with culture-confirmed *Bb* infection, 98.1% sensitivity for late Lyme
disease patients who were antibiotic-responsive (n=54) or antibiotic-refractory (n=53), and 100%
specificity within 30 healthy controls.

In cohort 3, two subjects tested positive of the 172 healthy controls from non-endemic
regions (98.3% specificity), while one subject of the 50 healthy controls from endemic regions
tested positive (98.0% specificity). Finally, two patients out of 175 CFS patients tested positive
with the 10-antigen panel (98.9% specificity). The specificity levels achieved by the 10-antigen
panel in cohort 3 are similar to those advertised by the manufacturer of the FDA-approved
enzyme-immunoassay (EIA) discussed below (98.5% specificity in 200 non-endemic controls)
(36).

Comparison of the 10-antigen panel, commercial EIA, and two-tiered algorithm. The 10-
antigen panel was compared to an FDA-approved EIA test and to two-tiered serologic testing
performed according to CDC guidelines in cohort 1 (Table 2). Whereas the 10-antigen panel
detected 55.0% of early Lyme disease patients at baseline in the validation set, the commercial
EIA detected 52.5% as positive or equivocal within the same patient set, and the two-tier algorithm was positive in 40% of these patients. By the post-treatment visit, detection using the 10-antigen panel had increased to 87.5%, the EIA had detected 87.5% as positive or equivocal and the CDC two-tiered algorithm had identified 67.5% as positive. Detection rates for the training set were comparable.

All five late Lyme disease patients in cohort 1 tested positive at the baseline visit by the 10-antigen panel, commercial EIA, and two-tiered serology. Of the 26 healthy controls followed in cohort 1, none tested positive at any visit by the 10-antigen panel or two-tiered serology (100% specificity). In contrast, the commercial EIA identified one of the 26 healthy controls as positive at the first visit (96.2% specificity) who later tested negative by the same EIA test at subsequent visits.

Thus, this novel 10-antigen panel detected *Bb* infection in a larger proportion of early Lyme disease patients at baseline or post-treatment visits than the CDC-recommended two-tiered algorithm (87.5% and 67.5%, *P*<0.05) while achieving equivalent specificity.

**Distribution of positive markers by disease type and time.** We examined the number of positive markers on the 10-antigen panel for subjects of each Lyme disease type over time. Early Lyme disease patients in cohort 1 who tested positive by the two-or-more criteria on the 10-antigen panel exhibited a dynamic distribution: there was a uniform distribution of positive markers at baseline that shifted to a normal distribution centered at six markers at post-treatment and five markers at the one month post-treatment visit (Figure 2A). Beginning at the three month post-treatment visit, some subjects who previously tested positive tested negative on the 10-antigen panel (Supplemental Figure 1). In contrast, the distribution of markers observed in the
five late Lyme disease patients was relatively static over time with three or four positive markers at every visit. Healthy controls from cohort 1 tested exclusively negative, with one or fewer markers positive.

The distributions of positive markers for cohort 2 were similar to those observed in cohort 1 based on subject type. Early Lyme disease subjects in cohort 2 exhibited a broad distribution of positive markers and late Lyme disease patients exhibited a narrow distribution centered at three or four positive markers (Supplemental Figure 1). Additionally, healthy controls in cohort 2 tested exclusively negative, with one or fewer markers positive.

Ten-antigen panel positivity varies with illness duration and EM dissemination. To determine if detectable antibody diversity and titer increased with the length and extent of infection as noted in other reports (37), we analyzed the number of positive markers and the Antibody Index (AI) with respect to patient-reported illness duration and the presence of multiple EM.

Early Lyme disease patients in cohort 1 were grouped according to self-reported illness duration (Figure 2B). Those patients with reported illness duration of 14 or more days and late Lyme disease patients had significantly greater numbers of positive IgG markers on average than patients with illness duration of six or fewer days (2.69 vs 0.92, \(P<0.001\) and 3.2 vs 0.92, \(P<0.01\)) and significantly higher average IgG AI (18.3 vs 1.9, \(P<0.001\) and 28.2 vs 1.9, \(P<0.01\)). Among early Lyme disease patients with illness duration of 0-6 days, 7-13 days, 14 or more days, and late Lyme disease patients, there was a trend towards differential numbers of positive IgM markers and average IgM AI between the four groups that did not achieve statistical significance (\(P=0.0653\) and \(P=0.0736\), respectively).
Early Lyme disease patients who presented multiple EM lesions at the baseline visit were stratified from patients presenting a single EM (Figure 2C). The number of average positive markers in the multiple EM group was significantly elevated at baseline compared to the single EM group for IgG and IgM (2.5 vs 1.2, \( P<0.001 \) and 2.5 vs 0.9, \( P<0.01 \)). Furthermore, patients with multiple EM exhibited significant elevations in the average IgG AI compared to patients with a single EM at baseline, post-treatment, one month post-treatment, and three month post-treatment visits (\( P<0.001, P<0.05, P<0.05, \) and \( P<0.05 \)). Significant elevations in average IgM AI between the multiple EM group and the single EM group were only observed at the baseline visit (2.4 vs 1.0, \( P<0.01 \)).

**DISCUSSION**

We developed a novel multiplex bead array for detection of *Bb* infection on the premise that discrete assessment of multiple anti-*Bb* antibodies may improve sensitivity and specificity compared to existing testing formats. Indeed, our 10-antigen panel yielded more sensitive detection of early Lyme disease patients at either baseline or post-treatment visits than two-tiered testing in cohort 1. Importantly, the 10-antigen panel in combination with a two-positive rule achieved specificity comparable to two-tiered testing in the healthy controls in cohort 1. Our results provide a promising foundation for further development of these markers and the multiplex panel format that will improve laboratory diagnosis of Lyme disease.

Our most sensitive markers in early Lyme disease patients at the baseline visit were synthetic peptides targeted by IgG. Specifically, pErp59-mV (Erp51-65) IgG and pp35-mV (p35 101-115) IgG yielded the highest two sensitivities of 51% and 49%, respectively. Erp and p35 proteins, expressed by *Bb* in mammalian hosts, are early antigen targets of the mammalian immune response to *Bb* infection (38-40). The OspEF-related protein family, termed Erp, bind both...
complement inhibitor factor H and plasminogen allowing Bb to evade complement-mediated killing, bind host endothelium through plasminogen receptors, and gain surface protease activity (40, 41). The p35 protein binds fibronectin, enabling Bb to attach to the extracellular matrix of the host (42). This supports a hypothesis where peptides derived from early expressed virulence proteins have significant diagnostic utility.

Interestingly, a fraction of patients in cohort 1 clinically diagnosed by the presence of an EM exhibited no anti-Bb antibody response at any visit on both the 10-antigen panel and commercial EIA, including six of 39 patients in the training set and four of 40 patients in the validation set. Confirmatory culture of Bb from EM lesion biopsies was unfortunately not available in cohort 1. In these patients, one possibility is that other microbial pathogens are responsible for the clinical symptoms exhibited. Atypical EMs are a potential source of early clinical misdiagnosis, and Southern Tick Associated Rash Illness, for which the etiologic agent is unknown, can present an EM-like rash and has been reported in Maryland where cohort 1 was collected (43). Future studies testing serum from early Lyme disease cohorts from the upper Midwest in the U.S., where STARI is not reported, would serve as a means to remove this unknown factor in the evaluation of the 10-antigen panel for detection of Bb infection. Alternatively, these patients were infected with Bb and the laboratory detection of anti-Bb antibodies was unsuccessful using the 10-antigen assay and the commercial EIA because titers fell below detection cutoffs, responses were targeted against antigens not included in the assays, or antibody responses were potentially precluded by early treatment with antibiotics (37, 44).

The 10-antigen assay described in this report may be improved through further marker development and panel selection. As the known repertoire of Bb surface and secreted proteins is expanded over time, additional markers can be tested and included in future iterations of this
panel. Furthermore, investigation into the three-dimensional conformation of highly
immunogenic antigens may reveal previously-unappreciated tertiary epitopes that are targeted by
the immune system. The inclusion of markers featuring stabilized three-dimensional epitopes
may detect antibodies beyond those recognized by the linear peptides in this panel.

In summary, we evaluated the utility of novel diagnostic markers on a multiplex antigen
panel in improving serologic detection of anti-\textit{Bb} antibodies in early Lyme disease patients in the
United States. While these findings must be validated in larger cohorts and in patients infected
with \textit{Borrelia} genospecies from Europe and Asia, within our sampling the 10-antigen panel
achieved more sensitive detection of the first stage of Lyme disease than the current two-tiered
scheme and achieved comparable specificity. These data provide a foundation for development
of a new generation of highly accurate and robust diagnostics that will better aid the clinician in
diagnosing and treating early Lyme disease.
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REFERENCES


Diagnostic challenges of early Lyme disease: lessons from a community case series.

BMC Infect Dis 9:79.
Table 1. Performance of 10-antigen panel

<table>
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Table 2. Comparison of 10-antigen panel versus commercial two-tiered testing

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<td>Seropositive at baseline</td>
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Note. EIA, enzyme immunoassay

a EIA seropositive includes both positive and equivocal test results
b One subject out of 20 tested equivocal
c One subject out of 21 tested equivocal
FIGURE LEGENDS

Figure 1. Training and validation of the multi-antigen panel. A, Using Luminex technology, 62 candidate antigen markers were assessed separately for IgG and IgM reactivity, for a total of 124 markers. A positivity cutoff for each marker was defined as twice the 98th percentile MFI observed in 222 healthy controls. The sensitivity of each marker was evaluated in a training set of 39 early Lyme disease patients at baseline and post-treatment visits. The two most sensitive markers and eight supporting markers were selected for inclusion into a 10-antigen panel. Samples with two or more positive markers were defined to test positive on the 10-antigen panel, and the performance of this panel was validated in three independent cohorts. B, List of the markers in the 10-antigen panel.

Figure 2. Distribution of antigen markers detected on the 10-antigen panel and marker positivity as a function of illness duration and dissemination of infection. A, The number of subjects testing positive for a given number of markers on the 10-antigen panel was plotted as frequency distributions for cohort 1. Subjects in the gray shaded region are negative based on the test criteria. B, For each antibody isotype (IgG or IgM), the number of positive 10-antigen targets and AI for Lyme disease patients in cohort 1 were stratified by patient-reported illness duration at baseline. Bars represent the mean ± SEM. C, Similarly, the number of positive targets and AI were stratified by the presence of multiple erythema migrans (EM) lesions at the baseline visit. Late Lyme disease and healthy control values were displayed for reference. Symbols represent the mean ± SEM. Abbreviation: PT, post-treatment. For all panels, *P<0.05, **P<0.01, ***P<0.001.
Cohort 2
20 Early Lyme
107 Late Lyme
30 Healthy controls

Cohort 3
175 Chronic fatigue syndrome
222 Healthy controls

**A**

**Marker Screening**
62 IgG + 62 IgM

**Training**

- **Define Positivity Cutoff**
  2x98th percentile of 222 healthy controls
- **Select Markers**
  Training set of 39 early Lyme disease patients
- **10 markers**
  ≥2 positive markers for test positive

**Validation**

- **Cohort 1 (longitudinal)**
  40 Early Lyme
  5 Late Lyme
  26 Healthy controls

- **Cohort 2**
  20 Early Lyme
  107 Late Lyme
  30 Healthy controls

- **Cohort 3**
  175 Chronic fatigue syndrome
  222 Healthy controls

**B**

**10-antigen panel**

- pErp59-mV (Erp51-65) IgG
- pp35-mV (p35 101-115) IgG
- pOspF-mV (OspF 86-105) IgG
- OspC B. burgdorferi IgM
- pFlaB-mV IgM
- DbpB B. burgdorferi IgM
- p100 B. burgdorferi IgM
- pOspC type K combined IgM
- pLA-7 (91-110) IgM
- pOspC type K combined IgG