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Evaluation of in vivo expressed *Borrelia burgdorferi* antigens for improved IgM serodiagnosis of early Lyme disease

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ABSTRACT

Improved serologic tests are needed for accurate diagnosis and proper treatment of early stage Lyme disease. We evaluated the 3 antigens currently used for 2-tiered IgM immunoblot testing (FlaB, OspC, and BmpA) in combination with 3 additional antigens (BBA65, BBA70, and BBA73) and measured the sensitivity and specificity against a serum repository of positive and negative controls. Using 3 statistical methods for positivity cutoff determinations and scoring criteria, we found increased sensitivities for early Lyme disease when 2 of 6 antigens were positive as compared with the 2 of 3 antigen IgM criteria currently used for second-tier immunoblot scoring. Specificities for negative controls were comparable or superior to using 2 of 3 antigens. These results indicate that IgM sensitivity and specificity of serological testing for Lyme disease in the early stages of illness can be improved by employing antigens that target the initial host antibody responses.

1. Introduction

Human infections caused by the tick-borne bacterial pathogen, *Borrelia burgdorferi*, the primary agent of Lyme borreliosis, are increasing in North America and are estimated to be ten-fold greater than officially reported cases (Hinckley et al., 2014; Nelson et al., 2015). More commonly known as Lyme disease, treatment with antibiotics is effective when infections are diagnosed early and accurately. However, accurate clinical diagnoses during early infection can be complicated as symptoms such as headaches, muscle aches, and fatigue mimic manifestations of other diseases. Moreover, the *Ixodes spp.* tick that harbors and transmits *B. burgdorferi* to humans via bloodfeeding is often not observed by its victim. The characteristic erythema migrans (EM) rash at the site of the tick bite within known regions of Lyme disease endemicity greatly aids diagnoses in the early stages of infection, however in some cases the EM may not appear or be apparent. Therefore, accurate diagnostic tests in the acute stage of infection are imperative for patient treatment or confirmation of infection.

The current test for Lyme disease is based on serological detection of host antibodies produced against *B. burgdorferi*. The standard is a 2-tiered test recommended by the Centers for Disease Control and Prevention whereby the first tier test is commonly an ELISA of either a total protein sonicate or a peptide of *B. burgdorferi* followed by an immunoblot of *B. burgdorferi* antigens as the second tier should the ELISA yield a positive or indeterminant result (Wormser et al., 2006). Several individual modified first- and second-tiered tests that use combinations of whole cell sonicate or recombinant antigens have been cleared by the U.S. Food and Drug Administration and are commercially available for Lyme disease testing (Molins et al., 2016).

Serologic testing detects antibodies in 97–100% of persons with Lyme arthritis (Bacon et al., 2003; Molins et al., 2016; Waddell et al., 2016). The test is more accurate at this stage of disease because the patient has had ample time to mount a robust antibody response against several *B. burgdorferi* antigens. However, serologic detection in patients with a duration of illness less than 30 days is much less sensitive, estimated at less than 50% (Aguero-Rosenfeld, 2008; Molins et al., 2016). The current 2-tiered recommendation is to test for both IgM and IgG antibodies for acute samples collected ≤30 days beyond duration of illness, with IgG testing only for suspected Lyme disease cases past 4 weeks. IgM testing beyond 30 days is not performed to limit potential false positive results.

Limitations of 2-tiered testing have been well documented particularly concerning the low sensitivity of detection in early acute infections with EM, estimated at 29–40% (Aguero-Rosenfeld et al., 2005), and the subjective nature of immunoblot interpretations in second tier testing (Aguero-Rosenfeld et al., 2005; Marques, 2015). Currently just 3 antigens are used for scoring IgM second-tier immunoblots; FlaB (flagellin), outer surface protein (Osp) C, and borrelial membrane protein (Bmp) A. These antigens were chosen because antibodies produced against them have been observed early following infection (Aguero-Rosenfeld et al., 1993). Despite limitations and concerns, IgM testing is still a part of

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the recommended 2-tier testing regimen; therefore, we sought to utilize the advantage of IgM antibody production within days of immune system recognition of infection to improve the sensitivity and specificity for serologic testing of early duration of illness Lyme disease patient samples.

A major drawback to using whole cell *B. burgdorferi* sonicate protein lysates in testing is that proteins synthesized when *B. burgdorferi* is grown in culture do not always correlate to those produced in a natural tick transmitted infection. Therefore, antibodies elicited against borrelial antigens expressed early after tick-borne infection would not be detected by assays that use cultured *B. burgdorferi* as antigens. We hypothesized that antibodies are elicited early in infection against *B. burgdorferi* antigens expressed in vivo that are not synthesized in culture lysates thereby representing novel targets for serologic testing. We also hypothesized that incorporating additional antigens into the IgM criteria algorithm would increase the sensitivity and specificity for detection of borrelial infection in the early stages of Lyme disease. In a preliminary study, we determined that the *B. burgdorferi* in vivo expressed proteins, BBA65, BBA70, and BBA73 reacted with early acute and early disseminated Lyme disease patient serum samples at levels comparable to antigens currently in IgM 2-tiered testing usage (Gilmore et al., 2007, 2008; Hughes et al., 2008; Weiner et al., 2015).

Here we evaluated the sensitivities and specificities using combinations of recombinant antigens FlaB, BmpA, OspC, BBA65, BBA70, and BBA73 in IgM serologic testing of early Lyme disease patient samples and negative controls.

2. Materials and Methods

2.1. Recombinant protein expression and purification

Recombinant proteins were generated and purified in *Escherichia coli* using the pETite N-His vector in accordance with the T7 Expresso system instructions (Lucigen, Middleton, WI). Truncated (i.e., lacking signal sequence and lipidation motif) genes encoding BBA65, BBA70, BBA73, and OspC were amplified by PCR from *B. burgdorferi* strain B31 genomic DNA using primers described previously (Weiner et al., 2015). Truncated BmpA was cloned for expression using the primers: forward 5′ CATCATCACCACCATCACAGTGGTAAAGGTAGTCTTGGG 3′, and reverse 5′ GTGGCGGCCGCTCTATTAAATAAATTCTTTAAGAAACTT 3′. Since FlaB does not possess a signal sequence, the entire coding sequence was cloned using primers: forward 5′ CATCATCACCACCATCA CATTATCAATCATAATACATCA 3′, and reverse 5′ GTGGCGGCCGCTCTA TTATCTAAGCAATGACAAAACATA 3′.

Cloned genes in expression plasmids were transformed into *E. coli* 10G (Lucigen) and selected for growth on Luria-Bertani (LB) medium plates supplemented with 50 μg/ml kanamycin. Plasmid DNA from transformant colonies was purified by miniprep (Qiagen, Valencia, CA) and was sequenced for insert confirmation. Recombinant plasmids with the correct gene inserts were transformed into *E. coli* BL21(DE3) (Lucigen). Following transformant screening for the appropriate clones, colonies were grown in LB-kanamycin (50 μg/ml) broth, and recombinant protein expression was induced by the addition of isopropyl-D-thiogalactopyranoside (IPTG; 1 mM). Cells were harvested at late-log-phase growth, and recombinant protein was purified under nondenaturing conditions using a nickel-nitrilotriacetic acid (Ni-NTA) Fast Start His tag affinity purification kit (Qiagen). The FlaB protein was purified following manufacturer's instructions for preparation of insoluble protein. Proteins were dialyzed into PBS (pH 7.4) and quantified by bicinchoninic acid (BCA) assay (Thermo-Fisher Scientific, Rockford, IL) before use.

2.2. ELISA

Recombinant antigens were diluted with carbonate buffer (90 mM $NaHCO_3$, 60 mM Na_2CO_3; pH 9.6) and bound to 96-well Immulon 2HB format plates overnight at 4 °C (Thermo Scientific, Rockford, IL) at a final concentration of 200 ng/well. The plate wells were subjected to 5 washes with Tris-buffered saline–Tween 20 (TBS-T; 20 mM Tris, 140 mM NaCl, 2.7 mM KCl, 0.05% Tween 20 [pH 7.4]) using a BioTek 405 Select plate washer (BioTek, Winooski, VT), followed by addition of blocking buffer (TBS-T with 3% fetal bovine serum) for 45 min at room temperature. Serum samples were diluted to 1:100 in blocking buffer, then added to the wells coated with each of the 6 antigens, and the plates were incubated for 60 min with moderate agitation at room temperature followed by 5 washes with TBS-T. Alkaline phosphatase-conjugated goat anti-human IgM (KPL, Gaithersburg, MD) was added at 1:2500 in TBS, and plates were incubated for 45 min with agitation at room temperature followed by the wash step. For development, 100 μL of para-nitrophenyl phosphate (PNPP) substrate (Thermo-Fisher Scientific) was added to each well, followed by incubation with agitation at room temperature for 20 min. The reaction was stopped by adding 50 μL of 2 N NaOH to wells. Plates were read at an optical density at 405 nm (OD405) using an ELx808IU Ultra microplate reader (BioTek). Each serum sample was assayed in duplicate, and was performed by 2 different laboratory personnel. Optimal serum and conjugate dilutions were determined prior to the assays as described previously (Weiner et al., 2015).

2.3. Serum samples

The Lyme Serum Repository (LSR) was the source of human serum panels used in this study, and samples were collected by the Division of Vector Borne Diseases, Bacterial Diseases Branch, Centers for Disease Control and Prevention. A detailed description of the LSR, which is composed of serum obtained from well-characterized Lyme disease patients, control serum from healthy individuals, and serum from patients with other diseases, has been published (Molins et al., 2014). Lyme disease patient samples were subdivided into groups as follows: early Lyme disease with EM, which consisted of paired patient serum samples taken at the acute and convalescent phases of disease (stage 1; $n = 78$); early Lyme neuroborreliosis (stage 2; $n = 9$); and early Lyme carditis (stage 2; $n = 7$). Informed consent and Institutional Review Board approval was granted for the testing of these samples.

2.4. Statistical analyses and cutoff calculations

To normalize for anticipated daily variation of the assay measurements, duplicate positive control wells employing a reactive serum control against rOspC were included on each plate**.** Optical density (OD) values were normalized by dividing all OD values on the plate by the positive plate controls' average ODs. Natural logarithms (ln) of the normalized values were computed for use as the primary measure in analyses. A preliminary analysis showed relatively little variance attributable to user or date replications, so sample replicates were averaged over these before being analyzed further.

We used 3 different methods to calculate cutoffs to declare samples positive for *B. burgdorferi* infection. Averages and standard deviations (SD) of the normalized ln(OD) values were computed for the healthy non-endemic samples ($n = 102$) for each antigen, and the first cutoffs were then set at the resulting average + 2 SD for each antigen. These cutoffs were applied separately to the original 3 antigens and to the full set of 6 antigens, and a positive result was declared whenever 2 or more antigen measurements were above the cutoff.

The second determination of cutoffs was set by using a receiver operating characteristic (ROC) curve for each antigen being tested, using healthy non-endemic vs. each disease group and selecting the cutoff that maximized sensitivity while fixing specificity at 99%. Similar to the 2 SD method, a positive result was declared whenever 2 or more antigen measurements were above the cutoff.

A third determination of cutoffs was based on computing a “score” for each sample that combined the normalized ln(OD) values for all 6

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antigens, then using the resulting scores with ROC analysis to compute a cutoff. For each disease category compared to healthy non-endemic patient samples (n = 102), we computed the scores by finding the linear combination (i.e. weighted sum) of normalized ln(OD) values for all antigens that maximized the area under the ROC curve (AUC) (Huang et al., 2011). Generalized cross-validation (GCV) was used for each of these fits to provide a more robust estimate of the AUC for each linear combination. The linear combinations were ranked by their GCV-AUC values, and computed for each possible subset of antigens (63 possible sets of the 6 antigens). The top-ranked linear combination was the one with the highest GCV-AUC. Further technical details can be found in Huang et al. (2011). The coefficients (weights), $w_1, w_2, \ldots, w_6$, for this best-discriminating linear combination were used to compute the "score" as $w_1\, ln(OD_{bmp}) + w_2\, ln(OD_{flaB}) + \ldots + w_6\, ln(OD_{r73})$ for each sample. These scores were then used in ROC analyses to determine the cutoff that maximized sensitivity at 99% specificity.

Performances of the cutoffs were compared for each antigen and disease category combination by computing confidence intervals (CIs) for the differences between sensitivities or specificities among the 3 cutoff determination methods. These CIs were computed using the score CI of Tango (1998), and to account for the multiple tests per sample, the confidence levels were adjusted for multiple comparisons, using the Bonferonni adjustment. These were computed within a disease category. Binomial confidence intervals were computed using the standard Wilson (1927) score interval, with 95% confidence coefficient. Analyses were performed using the "ROCR", "optAUC", and "PropCIs" packages in R (R Core Team [2017], R: A language and environment for statistical computing [R Foundation for Statistical Computing, Vienna, Austria] https://www.R-project.org.).

3. Results

3.1. ELISA IgM evaluation of early Lyme disease patient serum samples against 6 antigens

3.1.1. Setting cutoff values at 2-standard deviations above the average

In our first analysis, we set the cutoff value for positive responses to each antigen at 2-standard deviations above the normalized average optical density absorbance readings for the healthy non-endemic patient control group after all data was transformed to the natural logarithmic scale. We first determined sensitivity and specificity using the original current 3 antigens, FlaB, BmpA, and OspC, where reactivity to ≥2/3 was considered positive, and then assessed sensitivity and specificity using 6 antigens, the original 3 antigens plus BBA65, BBA70, and BBA73 with reactivity to either ≥2/6 or ≥ 3/6 antigens considered positive.

Results showed an increase in sensitivity for early Lyme disease patient sample categories when scoring ≥2/6 antigens as positive over ≥2/3, i.e. early EM acute, 45% from 38% (a gain of 3 positive samples); early EM convalescent, 63% from 55% (a gain of 3 positive samples); early disseminated neuroborreliosis 100% from 89% (a gain of 1 positive sample); and early disseminated carditis remained equal for both at 57% (Table 1). Healthy endemic patient samples had a specificity of 99% for both ≥2/3 and ≥ 2/6, however when using ≥2/6, specificity for healthy non-endemic declined to 96% compared to 100% for ≥2/3 (Table 1). Therefore, the 3 additional positives scored in both early acute and early convalescent groups came at the expense of a decrease in specificity. It should be noted that the performance of the 2/3 antigen evaluation in this ELISA format is nearly equal in all categories to the published performance of commercially available, FDA approved, IgM Western immunoblots (Molins et al., 2016).

When scoring ≥3/6 as positive, sensitivities for early acute and convalescent samples were similar to the original ≥2/3 criteria (33% vs. 38% for early, and 53% vs. 55% for convalescent), but were reduced compared to ≥2/6 (Table 1). Neuroborreliosis and carditis samples were both reduced in sensitivity at 67% and 43%, respectively (Table 1). Specificities using all scoring criteria were similar for the non-Lyme disease categories except for mononucleosis which increased to 90% using ≥3/6 antigens (Table 1).

3.1.2. Setting cutoff values using receiver operator characteristic (ROC) curve analysis

We further analyzed the data by setting the cutoff values for IgM positivity by ROC curve analysis of the control serum (healthy non-endemic patient samples) vs. each disease group samples. Specificity was set at 99% when determining the ROC cutoff. Table 1 shows the sensitivities for each Lyme patient category with reactivity to ≥2/3 of the original antigens (FlaB, BmpA, and OspC) scored as positive. When adding the additional antigens and scoring ≥2/6 as positive, sensitivity for early acute samples increased to 33% from 28% (a gain of 2 positive samples), with a slight increase observed for early convalescent to 53%

Table 1
IgM sensitivity and specificity of early Lyme disease patient samples comparing 3 antigens vs. 6 antigens.

Patient Category	N	No. positive (% sensitivity) / [95% CI]					
		2 standard deviation cutoff			ROC cutoffs based on 99% specificity for healthy non-endemic samples		
		original 3 antigens	6 antigens		original 3 antigens	6 antigens	
Lyme disease		≥2 of 3	≥2 of 6	≥3 of 6	≥2 of 3	≥2 of 6	≥3 of 6
Early EM acute	40	15 (38) / [24–53]	18 (45) / [30–61]	13 (33) / [19–49]	11 (28) / [16–43]	13 (33) / [19–49]	7 (18) / [9–32]
Early EM convalescent	38	21 (55) / [40–70]	24 (63) / [46–78]	20 (53) / [36–69]	19 (50) / [35–65]	20 (53) / [36–69]	14 (37) / [23–53]
Neuroborrelosis	9	8 (89) / [57–99]	9 (100) / [63–100]	6 (67) / [31–91]	6 (67) / [35–88]	6 (67) / [35–88]	4 (44) / [19–73]
Carditis	7	4 (57) / [25–84]	4 (57) / [20–88]	3 (43) / [12–80]	4 (57) / [25–84]	4 (57) / [20–88]	3 (43) / [12–80]
		No. positive (% specificity) / (95% CI)					
Non-Lyme disease							
Other diseases		[a]	[a]	[b]	[a]	[a]	[b]
Syphilis	20	0 (100) / [84–100]	0 (100) / [80–100]	0 (100) / [80–100]	0 (100) / [84–100]	0 (100) / [80–100]	0 (100) / [80–100]
Mononucleosis	30	4 (87) / [70–95]	6 (80) / [61–92]	3 (90) / [72–97]	2 (93) / [79–98]	3 (90) / [74–97]	1 (97) / [83–100]
Periodontitis	20	1 (95) / [76–100]	1 (95) / [73–100]	1 (95) / [73–100]	1 (95) / [76–100]	1 (95) / [73–100]	0 (100) / [84–100]
Multiple sclerosis	21	0 (100) / ([85–100]	1 (95) / [74–100]	1 (95) / [74–100]	0 (100) / [85–100]	0 (100) / [81–100]	0 (100) / [81–100]
Fibromyalgia	31	0 (100) / [89–100]	0 (100) / [86–100]	0 (100) / [86–100]	0 (100) / [89–100]	0 (100) / [86–100]	0 (100) / [86–100]
Rheumatoid arthritis	21	1 (95) / [77–100]	1 (95) / [74–100]	1 (95) / [74–100]	1 (95) / [77–100]	1 (95) / [74–100]	1 (95) / [74–100]
Healthy controls							
Healthy endemic	100	1 (99(/ [95–100]	1 (99) / [94–100]	1 (99) / [94–100]	0 (100) / [96–100]	1 (99) / [94–100]	0 (100) / [95–100]
Healthy non-endemic	102	0 (100) / [96–100]	4 (96) / [90–99]	2 (98) / [92–100]	0 (100) / [96–100]	1 (99) / [95–100]	0 (100) / [95–100]

CI = confidence interval.
N = number of samples.
[a] specificity based on positivity to 1 or less antigens.
[b] specificity based on positivity to 2 or less antigens.

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from 50% (a gain of 1 positive sample). Neuroborreliosis and cardiac sample sensitivities remained unchanged (Table 1). Because the early acute Lyme disease group was a primary focus for this study, the cutoff value obtained for this group was used to determine specificity for the non-Lyme disease category ROC results shown in Table 1. Specificities for other diseases patient serum using ≥2/3 antigens were at 100% except for rheumatoid arthritis, periodontitis, and mononucleosis at 95%, 95%, and 93%, respectively. Specificities using ≥2/6 antigens were at 100% for fibromyalgia, syphilis, and multiple sclerosis samples, 95% for rheumatoid arthritis and periodontitis, 90% for mononucleosis (Table 1). Specificities for the healthy control sets of samples were 100% using ≥2/3, and 99% when using ≥2/6 antigens.

Scoring IgM positives using ≥3/6 antigens, the sensitivities for all Lyme disease sample categories were reduced compared to the ≥2/3 and ≥ 2/6 scoring with specificities remaining equal or better among the non-Lyme disease controls (Table 1).

3.1.3. Setting cutoff values by linear combination of antigen normalized Ln ODs maximizing the ROC AUC

The third method of deriving cutoffs, i.e., using the scores derived from the linear combination of normalized ln(OD) values that maximized the AUC then ROC analysis, gave the coefficients (weights) corresponding to the highest GCV-AUC shown in Table 2, listed for each Lyme disease category. The estimate of the AUC is also shown, as is the positivity cutoff-value determined using the ROC curve derived from the score value for each sample, using the coefficients shown. The estimated sensitivity using the positivity cutoff given is also shown in Table 2. Early acute Lyme disease was evaluated and results demonstrated that the subset of antigens (FlaB, OspC, BBA65) resulted in the highest GCV-AUC of 0.7768, and the corresponding coefficients (weights) for these antigens were (0.2627, 0.7854, −0.5604). The positivity cutoff value for the scores computed using these coefficients was 0.7841, which resulted in an estimated sensitivity of 30% (Table 2). Sensitivity for early Lyme disease convalescent samples was 68%, an increase over the 2 standard deviation ≥2/6 (63%) and the ROC ≥2/6 (53%), a gain of 2 and 4 positive samples, respectively. Sensitivity for neuroborreliosis samples was 100%, which matched the 2-standard deviation calculation, and was higher than the ROC ≥2/6 value of 67%. Sensitivity for carditis samples was 71%, an increase over the 57% found with the other methods. Again, the cutoff value obtained for the early acute Lyme disease group was used to determine specificity for the non-Lyme disease category shown in Table 2. Specificities were exceptional for the non-Lyme disease control samples with all at 100% except for healthy non-endemic (99%) and mononucleosis (97%) (Table 2).

3.1.4. Comparison of the 3 methods for Ln(OD) antigen cutoffs

For each disease category, the differences in sensitivities or specificities were compared among the 3 methods. Within the 2 SD and the ROC methods, we compared using all 6 antigens (≥2 or ≥ 3) to ≥2 out of the original 3 antigens. We then compared the linear combination method (optAUC) to the outcomes from 2 SD ≥2/6 and ROC ≥2/6. Results are shown in Table 3. While no differences are statistically significant, and the available sample sets result in CIs that are relatively wide (especially adjusted for multiple comparisons), the results suggest that the optAUC method compares favorably in sensitivity in the early EM convalescent and carditis groups.

3.2. Breakdown of individual early EM acute and early EM convalescent Lyme disease patient serum sample reactivity to each of the 6 antigens

To examine the representation of the additional 3 antigens among all 40 early EM acute Lyme disease patient serum samples, the frequency of reactivity to each combination of antigens was performed using the scoring criteria derived from ROC cutoff values. The analysis revealed 12 combinations representing at least 1 serum sample (Table 4). Supplemental Table 1 breaks down reactivity to each of the 6 antigens for each early EM acute Lyme disease patient serum sample.

For the early acute Lyme disease samples, reactivity against BBA65 (6/40), BBA70 (8/40), and BBA73 (7/40) was equal to or better than BmpA (6/40). OspC and FlaB were most representative at 45% and 37.5% of patients in this group (Table 4 and Supplementary Table 1). Interestingly, 2 samples were positive for 4 antigens (5%) and 4 samples were positive for all 6 antigens (10%) for a total 6/40, 15%, indicating that the additional antigens are supportive and could add assurance when scoring prospective samples (Table 4, Supplemental Table 1). Moreover, when scoring ≥2/6 antigens, 2 additional samples were scored positive that would have scored negative by the IgM immunoblot criteria (Table 4, Supplemental Table 1).

The same analysis was performed for each early EM convalescent Lyme disease patient serum samples and revealed 18 antigen combinations of reactivity (Table 5, Supplemental Table 2). At this later stage of infection more samples were positive for a greater number of individual antigens indicative of the increased antibody response as a function of time. The only antigen with reduced seroreactivity was BBA65 with 5 samples scoring positive down from 6 in the early acute samples. OspC and FlaB were the antigens with the highest representation of reactivity among early convalescent patients at 68% and 47%, respectively. BBA70 and BBA73 increased in representation of reactivity for convalescent samples from the acute samples to 32% and 21%, respectively. When scoring ≥2/6 antigens, one additional sample was positive

Table 2
Linear combination giving the highest cross-validated AUC.

Patient category	N	Sensitivity (%)	AUC	Coefficients						Score cutoff
Lyme disease		No. positive (% sensitivity) / [95% CI]		BmpA	FlaB	OspC	BBA65	BBA70	BBA73	
Early EM acute	40	12 (30)/ [18–45]	0.7768		0.2627	0.7854	−0.5604			0.7841
Early EM convalescent	38	26 (68)/ [53–81]	0.8657	0.067		0.9137	−0.3508	0.1811	0.0694	0.8935
Neuroborrelosis	9	9 (100)/ [70–100]	1		0.6419	0.6105	−0.2665	−0.3003	0.2326	0.1111
Carditis	7	5 (71)/ [36–92]	0.916		0.2507	0.8007	0.5433	−0.0369	−0.0097	0.7482
Non-Lyme disease[a]		**Specificity (%)**								
Other diseases		No. positive (% specificity) / [95% CI]								
Syphilis	20	0 (100)/ [84–100]								
Mononucleosis	30	1 (97)/ [83–100]								
Periodontitis	20	0 (100)/ [84–100]								
Multiple sclerosis	21	0 (100)/ [85–100]								
Fibromyalgia	31	0 (100)/ [89–100]								
Rheumatoid arthritis	21	0 (100)/ [85–100]								
Healthy controls										
Healthy endemic	100	0 (100)/ [96–100]								
Healthy non-endemic	102	1 (99)/ [95–100]								

[a] The cutoff value obtained for the early acute group was used to show consequential specificity for non-Lyme disease category.

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Table 3
Confidence intervals (95%) for differences in sensitivity or specificity between the 3 cutoff methods, for each disease category. Intervals are adjusted for multiple comparisons.

Patient Category	N	SD ≥ 2/6 to SD ≥ 2/3	SD ≥ 3/6 to SD ≥ 2/3	ROC ≥ 2/6 to ROC ≥ 2/3	ROC ≥ 3/6 to ROC ≥ 2/3	optAUC to SD ≥ 2/6	optAUC to ROC ≥ 2/6
		Percent difference between methods (95% CI)					
Lyme disease							
Early EM acute	40	7.5 (−8.4, 25.7)	−5.0 (−25.3, 14.8)	5.0 (−10.5, 22.4)	−10.0 (−28.9, 6.3)	−15.0 (−38.2, 9.1)	−2.5 (−26.2, 21.4)
Early EM convalescent	38	7.9 (−8.8, 26.9)	−2.6 (−19.6, 13.3)	2.6 (−13.3, 19.6)	−13.2 (−33.3, 4.4)	5.3 (−15.5, 26.4)	15.8 (−2.1, 36.4)
Neuroborrelosis	9	11.1 (−37.3, 54.9)	−22.2 (−64.3, 31.1)	0.0 (−43.6, 43.6)	−22.2 (−64.3, 31.1)	0.0 (−43.6, 43.6)	33.3 (−24.8, 72.6)
Carditis	7	0.0 (−49.9, 49.9)	−14.3 (62.5, 42.7)	0.0 (−49.9, 49.9)	−14.3 (62.5, 42.7)	14.3 (−42.7, 62.5)	14.3 (−42.7, 62.5)
Non-Lyme disease							
Other diseases							
Syphilis	20	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)
Mononucleosis	30	−6.7 (−28.4, 13.4)	3.3 (−16.1, 23.9)	−3.3 (−23.9, 16.1)	3.3 (−16.1, 23.9)	16.7 (−5.3, 40.3)	6.7 (−16.4, 30.3)
Periodontitis	20	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)	0.0 (−25.8, 25.8)	5.0 (−22.1, 32.7)	5.0 (−22.1, 32.7)	5.0 (−22.1, 32.7)
Multiple sclerosis	21	−4.8 (−31.5, 21.3)	−4.8 (−31.5, 21.3)	0.0 (−24.9, 24.9)	0.0 (−24.9, 24.9)	4.8 (−21.3, 31.5)	0.0 (−24.9, 24.9)
Fibromyalgia	31	0.0 (−18.3, 18.3)	0.0 (−18.3, 18.3)	0.0 (−18.3, 18.3)	0.0 (−18.3, 18.3)	0.0 (−18.3, 18.3)	0.0 (−18.3, 18.3)
Rheumatoid arthritis	21	0.0 (−24.9, 24.9)	0.0 (−24.9, 24.9)	0.0 (−24.9, 24.9)	0.0 (−24.9, 24.9)	4.8 (−21.3, 31.5)	4.8 (−21.3, 31.5)
Healthy controls							
Healthy endemic	100	0.0 (−6.5, 6.5)	0.0 (−6.5, 6.5)	0.0 (−6.5, 6.5)	0.0 (−6.5, 6.5)	1.0 (−5.6, 8.3)	0.0 (−6.5, 6.5)
Healthy non-endemic	102	−3.9 (−12.6, 2.7)	−2.0 (−9.7, 4.6)	−1.0 (−8.1, 5.5)	0.0 (−6.4, 6.4)	2.9 (−4.7, 11.8)	0.0 (−7.2, 7.2)

that would have been negative by IgM immunoblot criteria (Table 5, Supplemental Table 2). Like the early acute samples, several individual patients (n = 16) were seroreactive to 3, 4, 5, or all 6 antigens indicating that additional antigens in an IgM assay can be useful for providing confirmation of test results.

4. Discussion

Current serologic testing for antibodies against *B. burgdorferi* in patients with early Lyme disease with EM, i.e. from 2 to 4 weeks or less, is of limited sensitivity commonly resulting in a negative test. We hypothesized that specific *B. burgdorferi* antigens synthesized in vivo during tick transmission or soon after deposition into the host's skin elicit antibody responses that could be targets for serologic testing. We also hypothesized that detection of the IgM antibody response, which is generated earlier than the IgG response, might allow earlier confirmation of infection.

In the present study, we assessed the sensitivity and specificity of BBA65, BBA70, and BBA73 in combination with the standard antigens currently used for IgM second-tier immunoblot testing (FlaB, BmpA, and OspC) reasoning that inclusion of additional antigens expressed early in infection would increase the sensitivity for early detection.

We tested each serum sample for reactivity against each of the 6 antigens by ELISA. Cutoff values for positive samples by optical density measurements were set by 3 separate statistical criteria with the aim of generating data using different analyses for comparative purposes thereby yielding more information for future test evaluations.

Table 4
Twelve antigen combinations of IgM seroreactivity for the 40 individual early EM acute Lyme disease patient samples.

combination	BmpA	FlaB	OspC	BBA65	BBA70	BBA73	frequency
0 antigens	0	0	0	0	0	0	17
1 antigen	0	1	0	0	0	0	4
	0	0	1	0	0	0	6
2 antigens	1	1	0	0	0	0	1
	0	1	1	0	0	0	2
	0	0	1	0	1	0	1*
3 antigens	0	1	1	0	1	0	1
	1	1	1	0	1	0	1
	0	0	1	1	0	1	1*
4 antigens	0	1	1	1	0	1	1
	0	1	1	0	1	1	1
6 antigens	1	1	1	1	1	1	4

* Scored negative by current IgM immunoblot criteria.

Our first analysis set the cutoff value for positive responses for each antigen at 2-standard deviations above the average ln optical density absorbance readings of the healthy non-endemic patient control group. This statistical method for cutoff determination has historically been used and was included for comparative purposes against the more sophisticated analyses included in this study. We found that using ≥2/6 antigens, sensitivity was increased over the current ≥2/3 for both the early EM acute and convalescent Lyme disease groups, resulting in 3 additional samples scoring positive in each group. Although specificity was decreased, 96% vs. 100% in the healthy non-endemic group, specificity in the healthy endemic group was 99%. We found that using ≥3/6 antigens as the scoring criteria in both early acute and convalescent samples resulted in similar sensitivities as the current ≥2/3 with a 98% specificity compared to 100%.

We further analyzed the data for these 2 groups of samples by setting the cutoff for positive responses using ROC curve analysis and setting the specificity at ≥99%. We found that ≥2/6 antigens outperformed the standard ≥2/3 in sensitivity at this level of specificity for both early acute (resulting in 2 additional positive samples) and convalescent samples (resulting in one additional positive sample), whereas ≥3/6 resulted in decreased sensitivity. These results suggest that the best IgM scoring criteria for sensitivity uses 2 of these 6 antigens over 3 of 6, and is better than the current 2 of 3.

Table 5
Eighteen antigen combinations of IgM seroreactivity for the 38 individual early EM convalescent Lyme disease patient samples.

combination	BmpA	FlaB	OspC	BBA65	BBA70	BBA73	frequency
0 antigens	0	0	0	0	0	0	10
1 antigen	0	1	0	0	0	0	1
	0	0	1	0	0	0	6
	1	0	0	0	0	0	1
2 antigens	0	1	1	0	0	0	2
	0	0	1	0	1	0	1*
	1	0	1	0	0	0	1
3 antigens	0	1	1	0	1	0	3
	0	1	1	0	0	1	2
	1	1	1	0	0	0	2
4 antigens	1	1	1	0	1	0	1
	0	1	1	0	1	1	1
	0	1	1	1	0	1	1
	1	0	1	0	1	1	1
5 antigens	1	1	1	1	1	0	2
	1	1	1	0	1	1	1
	0	1	1	1	1	1	1
6 antigens	1	1	1	1	1	1	1

* scored negative by current IgM immunoblot criteria.

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Using ≥2/6 antigens resulted in correctly scoring one additional patient sample from the neuroborreliosis group in the 2 standard deviation statistical analysis, but the sample number in this group was small (n = 9). Sensitivities scored for the carditis group were equal regardless of the cutoff analysis or specificities for ≥2/3 vs. ≥2/6, with again a small number of samples (n = 7). For proper statistical evaluation for these stages of Lyme borreliosis, more sample numbers will be required, however the trend suggests that IgM testing with the additional antigens would be beneficial for improved diagnosis.

Specificities for non-Lyme diseases were similar regardless of the method for setting cutoffs and whether scoring ≥2/3, ≥2/6, or ≥3/6. The mononucleosis group consistently scored the lowest specificity among all analyses, including commercial IgM tests (Molins et al., 2016). Comparison of the serum panel evaluations in this study with the performances by commercial IgM assays shows similar sensitivities and specificities with our combination 6 antigen findings (Molins et al., 2016). A significant difference between these assays and the ones used in this study is that our evaluation was based on quantified ELISA optical densities using recombinant antigens whereas the commercial IgM assays were performed by immunoblot, which is subjectively or densitometrically scored when using whole cell lysates as the fractionated antigens.

A more sophisticated statistical analysis involved creation of "scores" for each sample as weighted averages of the ln normalized sample ODs, where the weights were computed by maximizing the cross-validated AUC. These scores were then used with the resulting ROC curve to set the positivity cutoff by maximizing the sensitivity with specificity fixed at ≥99% and this cutoff was used to determine sensitivity for each Lyme disease category. Although sensitivity for the early acute Lyme disease sample group was reduced compared to the other analyses, sensitivity for the early convalescent Lyme disease sample group was the highest among the evaluations at 68%, and the neuroborreliosis group was detected at 100%. This methodology could be useful with a sufficiently large data set where such an algorithm has the potential to maximize the value of the data for sensitivity and specificity by finding the best combination of antigens for each disease category. We showed resultant specificities for the non-Lyme categories based on the early acute cutoff score as one example of the usefulness of this methodology. In this analysis model, specificities for non-Lyme disease categories would be re-calculated based on cutoffs for each Lyme disease sample category.

Collectively, these results suggest that IgM scoring of 2/6 antigens has an increased sensitivity for early EM Lyme disease acute and convalescent patient samples compared to the current 2/3 scoring (albeit at a reduced specificity for healthy non-endemic patient samples) when setting positive cutoff values at 2-standard deviations from the mean. When the cutoffs were set using ROC at ≥99%, scoring of 2/6 antigens resulted in higher sensitivities than 2/3 scoring for early EM acute, early EM convalescent, with early disseminated neuroborreliosis and early disseminated carditis equal in both. A 3/6 scoring did not result in greater sensitivities for these patient groups. In this exploratory study, the addition of new antigens resulted in combinations that indicated increased sensitivity with high specificity, and use of additional antigens correctly identified samples in this well-characterized data set previously scored as negative by current IgM immunoblot testing.

Adjusting for multiple comparisons, no statistically significant differences were discerned between the 3 statistical methods employed to determine sensitivity and specificity, however the results provide a useful guideline for future studies regarding appropriate methodology for calculation. Further, the use of the quantitative measurement values (ln ODs) and weighting them for classification, as with the Huang, et al., method, can provide insights unavailable using the methods that use the binary classifications for the measurements. Other researchers have investigated different statistical methods for classification in Lyme disease diagnostics. For example, Dessau, et al. use logistic regression fitted with maximum likelihood and automated stepwise selection to minimize the AIC for model selection, which is a common approach (Dessau et al., 2015). The method of Huang, et al. that we use differs from this approach in 2 ways. First, the parameters or weights are estimated here by finding those which maximize the AUC, which is a natural objective function in this setting. Further, model selection here uses generalized cross-validation, rather than stepwise AIC, which is expected to characterize predictive performance better, while AIC is a measure of fit. While larger sample sets are going to be necessary to make firm recommendations, we find the weighting of ln OD values a promising approach warranting further investigation.

The breakdown of individual patient serum reactivities against each antigen (using the data collected from ROC cutoffs) showed a distribution of BBA65, BBA70, and BBA73 that was similar to BmpA and validated that FlaB and OspC are dominant antigens that elicit early antibody responses in Lyme disease patients. These results confirm that BBA65, BBA70, and BBA73 are well represented as seroreactive antigens in early Lyme disease and are relevant for inclusion in formulating new algorithms for IgM serodiagnosis. Examining individual early acute samples for reactivity to each antigen showed that addition of these 3 antigens scored 2 more positives that would be scored as negative using the current IgM immunoblot algorithm. Similarly, one more sample was scored positive in the early convalescent Lyme disease group that would have been scored negative by current IgM immunoblot criteria. Given the large number of Lyme disease serology tests conducted, such a small percentage gain could conceivably extrapolate into a significant increase in positive serodiagnoses among patients tested for Lyme disease.

Several patients were positive for 4 to 6 total antigens indicating that the inclusion of additional antigens in test scoring criteria have value for identifying a positive infection in the early stages or for supporting subjective calls in the current immunoblot format. Furthermore, we envision the use of recombinant antigens as a replacement for the current immunoblots that utilize borrelial whole cell lysates to eliminate the limitations of subjectivity in the scoring criteria. Multiplex platforms that incorporate multiple antigens are available for quantitative analysis and could be developed into a one-step test (Embers et al., 2016).

By including these antigens in our study, it must be noted that not all Lyme borreliae possess the genes encoding these proteins, e.g. strain 297 does not contain *bba73* (Hughes et al., 2008; Wywial et al., 2009). Also, the OspC sequence is heterogeneous between strains (Earnhart et al., 2005; Hughes et al., 2008; Wilske et al., 1993; Wywial et al., 2009) which could complicate accurate serological detection with this antigen, although there are common epitopes among invasive strains (Earnhart et al., 2005; Wilske et al., 1993). Therefore, the use of multiple antigen targets may be advantageous to detect infections caused by different strains or species.

In conclusion, IgM class antibodies are produced within days of infection and could be advantageous for assay development. We hypothesized that serological detection of Lyme borreliosis in the early duration of illness can be improved by identifying additional novel antigens expressed in the tick or human host that are the first targets to be recognized for antibody production as has been shown for OspC, FlaB, and BmpA. The results of this study indicate that addition of 3 in vivo expressed antigens for IgM testing of early Lyme disease increased sensitivity while maintaining specificity. Based on this analysis, we would expect that addition of novel or currently characterized in vivo expressed antigens would enhance serological testing for patients in the early stages of Lyme disease. Furthermore, IgG class antibodies are produced later, but can be present against certain antigens within 2 weeks of infection and represent additional targets for early detection. Our ongoing work is focused on the evaluation of novel antigens to add to the repertoire for IgG early detection to be used in conjunction with IgM testing to enhance sensitivity and specificity for Lyme disease testing for early illness.

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Disclaimer

The findings and conclusions in this report are those of the authors and do not necessarily represent the official position of the Centers for Disease Control and Prevention.

Declarations of interest

none.

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