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Development of a Multiantigen Panel for Improved Detection of *Borrelia burgdorferi* Infection in Early Lyme Disease

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The current standard for laboratory diagnosis of Lyme disease in the United States is serologic detection of antibodies against *Borrelia burgdorferi*. The Centers for Disease Control and Prevention recommends a two-tiered testing algorithm; however, this scheme has limited sensitivity for 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 positive results for two markers to designate a positive test. Ten markers were selected from our initial analysis of 62 *B. burgdorferi* 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 posttreatment visit than 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 *B. burgdorferi* 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.

Lyme disease is the most prevalent vector-borne disease in the United States and is caused by the spirochete bacterium *Borrelia burgdorferi sensu stricto* (*B. burgdorferi*) (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 areas where the disease is endemic (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 nonspecific to *B. burgdorferi* 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 *B. burgdorferi* 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 a positive second-tier Western blot, yielding an overall specificity of 99.5% (13, 14). Two-tiered testing has several limitations; specifically, it correctly identifies only 29 to 40% of patients presenting with an EM in the early stage of Lyme disease (10), and Western blot analysis is labor-intensive, subjective, and prone to 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 *B. burgdorferi* surface proteins has revealed novel peptides conserved across *B. burgdorferi* 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

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and specificity of the current technology by assessing antibody responses to multiple *B. burgdorferi* 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 *B. burgdorferi* 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-*B. burgdorferi* 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 the sensitivity of early *B. burgdorferi* 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 *B. burgdorferi* infection and aid diagnosis of early Lyme disease.

MATERIALS AND METHODS

Sample collection. Serum samples were collected after informed consent was obtained under protocols approved by institutional review boards at the institutions outlined below.

Cohort 1 included 79 patients who had been physician diagnosed with early Lyme disease based on the presence of an EM (see Table S1 in the supplemental material), and five patients with untreated late Lyme disease presenting with Lyme arthritis, with all samples being collected through Johns Hopkins University in a region of of Maryland where Lyme disease is endemic (27, 28). In this prospective study, patients meeting all entrance requirements were enrolled, prescribed a 3-week course of doxycycline at the baseline visit, examined 3 weeks later upon completion of antibiotic treatment (posttreatment visit), and then followed for four additional visits (one month posttreatment, 3 months posttreatment, 6 months posttreatment, and 1 year posttreatment). Twenty-six healthy controls with no known history of Lyme disease were followed for three visits (baseline, 6 months, and 1 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, with all samples being provided by Massachusetts General Hospital (29–31), which is located in a region of Massachusetts where Lyme disease is endemic. 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, with all samples being collected by Stanford University, which is located in a region of California where Lyme disease is not endemic, 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 being collected from regions of the United States where Lyme disease is endemic.

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, MA). Second-tier Western blotting was performed using MarDx *B. burgdorferi* Marblot Western blot systems (Trinity Biotech, Bray, Ireland), with IgG and IgM evaluated in patients with an illness duration of less than 1 month and only IgG evaluated if illness duration was 1 month or more. If a patient sample was negative by the two-tiered test at the baseline visit, a convalescent-phase serum sample from the posttreatment visit was sent for two-tiered testing.

Multiplex analysis of anti-*B. burgdorferi* antibody markers. We examined 62 candidate antigens, including seven *B. burgdorferi* surface proteins and 55 synthetic peptides based on *B. burgdorferi* antigenic epitopes. Several of the peptide antigens have been described previously (18–20, 34, 35). See Table S2 in the supplemental material 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 by "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, TX) running Bio-Plex Manager software v5.0 (Bio-Rad Laboratories, Hercules, CA), 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 for a set of 222 healthy-control samples reserved at random from cohort 3 (Fig. 1A). This definition of positivity was then used to select the two most sensitive markers at the baseline and posttreatment visits for a set of 39 early-Lyme-disease samples 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 from early-Lyme-disease patients. To test positive, a sample was required to be positive for two or more markers from this list of 10 (Fig. 1B), regardless of marker isotype.

Statistical analysis. The 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 were compared with those with single EM at each visit using the 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 interassay coefficient of variance for three controls tested across 10 independent runs (see Table S3 in the supplemental material).

RESULTS

Generation of 10-antigen panel. To develop a multiplex panel with improved detection of anti-*B. burgdorferi* antibodies in the early phase of infection, we screened a spectrum of novel synthetic peptides and established proteins and selected 10 antigens (Fig. 1). The 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 in cohort 1 at the baseline visit, sensitivities of the training and validation sets were 56.4%

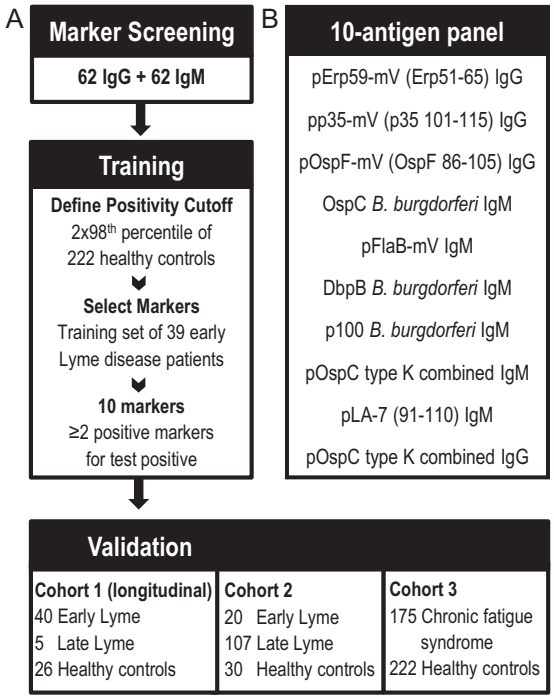

FIG 1 Training and validation of the multiantigen panel. (A) Using Luminex technology, 62 candidate antigen markers were assessed separately for IgG and IgM reactivity, for a total of 124 markers. A positivity cutoff for each marker was defined as twice the 98th-percentile MFI observed for 222 healthy controls. The sensitivity of each marker was evaluated in a training set of 39 early-Lyme-disease samples obtained at baseline and posttreatment 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 as testing 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.

and 55.0%, respectively. At the posttreatment 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 *B. burgdorferi* 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 regions where Lyme disease is not endemic (98.3% specificity), while one subject of the 50 healthy controls from regions where the disease is endemic 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 for cohort 3 are similar to those advertised by the manufacturer of the FDA-approved enzyme-immunoassay (EIA) discussed below (98.5% specificity in 200 controls from areas of nonendemicity) (36).

Comparison of the 10-antigen panel, commercial EIA, and two-tiered algorithm. The 10-antigen panel was compared to an FDA-approved EIA and to two-tiered serologic testing performed according to CDC guidelines in cohort 1 (Table 2). Whereas the 10-antigen panel identified 55.0% of samples from early-Lyme-disease patients at baseline in the validation set, the commercial EIA identified 52.5% as positive or equivocal within the same patient set, and the two-tiered algorithm was positive for 40% of these patients. By the posttreatment visit, detection using the 10-antigen panel had increased to 87.5%, the EIA had identified 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), but this individual tested negative by the same EIA at subsequent visits.

Thus, this novel 10-antigen panel detected *B. burgdorferi* infection in a larger proportion of early-Lyme-disease patients at baseline and posttreatment 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 with each Lyme disease type over time. Early-Lyme-disease patients in cohort 1 who tested positive by the two-or-more-positives criterion 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 immediately posttreatment and five markers at the 1-month-posttreatment visit (Fig. 2A). Beginning at the 3-month-posttreatment visit, some subjects who previously tested positive tested negative on the 10-antigen panel (see Fig. S1 in the supplemental material). 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 no markers being 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 (see Fig. S1 in the supplemental material). Additionally, healthy controls in cohort 2 tested exclusively negative, with one or no 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 (Fig. 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 versus 0.92 [$P < 0.001$] and 3.2 versus 0.92 [$P <$

TABLE 1 Performance of 10-antigen panel

Cohort and Subject type	*n*	10-antigen panel		
		No. positive	Sensitivity (%)	Specificity (%)
Training cohort 1 (early Lyme disease)				
Baseline	39	22	56.4	
Posttreatment	38	30	78.9	
1 mo posttreatment	36	29	80.6	
3 mo posttreatment	36	28	77.8	
6 mo posttreatment	36	26	72.2	
1 yr posttreatment	29	15	51.7	
Validation cohort 1				
Early Lyme disease				
Baseline	40	22	55.0	
Posttreatment	39	34	87.2	
1 mo posttreatment	35	30	85.7	
3 mo posttreatment	39	32	82.1	
6 mo posttreatment	38	29	76.3	
1 yr posttreatment	36	17	47.2	
Late Lyme disease				
Baseline	5	5	100.0	
Posttreatment	5	5	100.0	
1 mo posttreatment	5	5	100.0	
3 mo posttreatment	5	5	100.0	
6 mo posttreatment	5	5	100.0	
1 yr posttreatment	4	4	100.0	
Healthy controls				
Baseline	26	0		100.0
6-mo visit	25	0		100.0
1-yr visit	18	0		100.0
Validation cohort 2				
Early Lyme disease, culture confirmed	20	20	100.0	
Late Lyme disease, antibiotic responsive	54	53	98.1	
Late Lyme disease, antibiotic refractory	53	52	98.1	
Healthy controls	30	0		100.0
Validation cohort 3				
Chronic fatigue syndrome patients	175	2		98.9
Healthy controls				
Not in regions of endemicity	172	2		98.3
In regions of endemicity	50	1		98.0

0.01]) and significantly higher average IgG AI (18.3 versus 1.9 [$P < 0.001$] and 28.2 versus 1.9 [$P < 0.01$]). Among early-Lyme-disease patients with illness duration of 0 to 6 days, 7 to 13 days, or 14 or more days and late-Lyme-disease patients, there was a trend toward 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 (Fig. 2C). The average number of positive markers in the multiple EM group was significantly elevated at baseline compared to the single-EM group for IgG and IgM (2.5 versus 1.2 [$P < 0.001$] and 2.5 versus 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, posttreatment, 1-month-posttreatment, and 3-month-posttreatment 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 observed only at the baseline visit (2.4 versus 1.0 [$P < 0.01$]).

DISCUSSION

We developed a novel multiplex bead array for detection of *B. burgdorferi* infection on the premise that discrete assessment of multiple anti-*B. burgdorferi* antibodies may improve sensitivity and specificity compared to existing testing formats. Indeed, our 10-antigen panel yielded more sensitive identification of samples from early-Lyme-disease patients at either baseline or posttreat-

TABLE 2 Comparison of 10-antigen panel versus commercial two-tiered testing

Cohort and subject type	Total	10-antigen panel		VIsE1/pepC10 EIA[a]		CDC 2-tiered algorithm	
		No.	% of total	No.	% of total	No.	% of total
Training cohort 1 (early Lyme disease)	39						
Seropositive at baseline		22	56.4	20[b]	51.3	16	41.0
Seropositive posttreatment		9	23.1	12	30.8	11	28.2
Seronegative at both		8	20.5	7	17.9	12	30.8
Validation cohort 1							
Early Lyme disease	40						
Seropositive at baseline		22	55.0	21[c]	52.5	16	40.0
Seropositive posttreatment		13	32.5	14	35.0	11	27.5
Seronegative at both		5	12.5	5	12.5	13	32.5
Late Lyme disease	5						
Seropositive at baseline		5	100.0	5	100.0	5	100.0
Healthy controls	Total	No. positive	Specificity (%)	No. positive	Specificity (%)	No. positive	Specificity (%)
Baseline	26	0	100.0	1	96.2	0	100.0
6 mo	25	0	100.0	0	100.0	0	100.0
1 yr	18	0	100.0	0	100.0	0	100.0

[a] The number of EIA seropositive results includes both positive and equivocal test results.
[b] One subject out of 20 had an equivocal test result.
[c] One subject out of 21 had an equivocal test result.

ment visits than two-tiered testing in cohort 1. Importantly, the 10-antigen panel in combination with a two-positive rule achieved specificity comparable to that of 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 for 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 *B. burgdorferi* in mammalian hosts, are early antigen targets of the mammalian immune response to *B. burgdorferi* infection (38–40). Members of the OspEF-related protein family, termed Erp, bind both complement inhibitor factor H and plasminogen, allowing *B. burgdorferi* to evade complement-mediated killing, bind host endothelium through plasminogen receptors, and gain surface protease activity (40, 41). The p35 protein binds fibronectin, enabling *B. burgdorferi* 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-*B. burgdorferi* antibody response at any visit with 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 *B. burgdorferi* from EM lesion biopsy specimens was unfortunately not available for cohort 1. For 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 (STARI), for which the etiologic agent is unknown, can present an EM-like rash and has been reported in Maryland, where cohort 1 was located (43). Future studies testing serum from early-Lyme-disease cohorts from the upper Midwest in the United States, 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 *B. burgdorferi* infection. Alternatively, these patients were infected with *B. burgdorferi* and the laboratory detection of anti-*B. burgdorferi* 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 *B. burgdorferi* 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-*B. burgdorferi* antibodies in early-Lyme-disease patients in the United States. While these findings must be validated in larger cohorts and in patients infected with *Borrelia* genospecies from

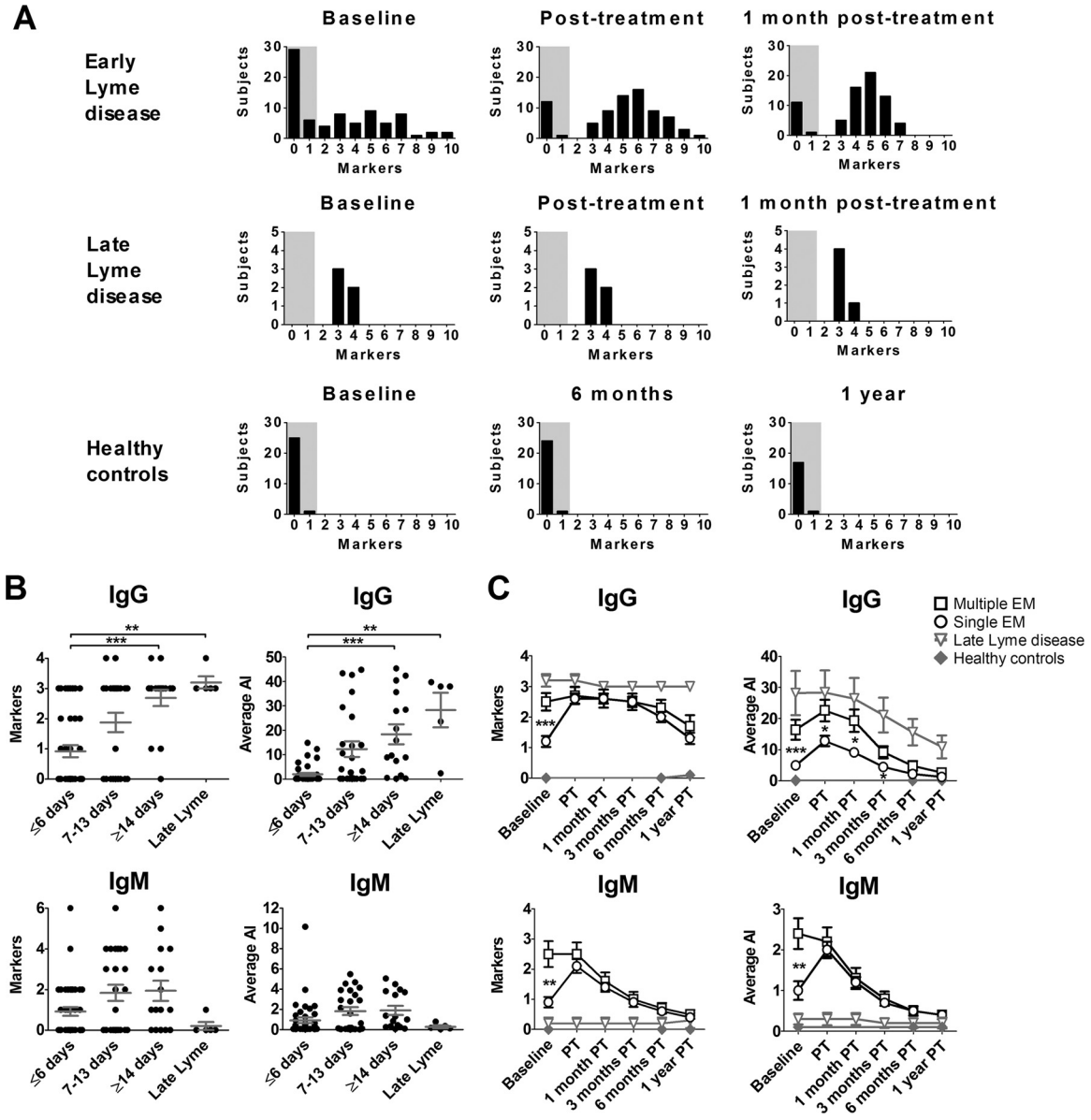

FIG 2 Distribution of antigen markers detected on the 10-antigen panel and marker positivity as a function of illness duration and dissemination of infection. (A) The number of subjects testing positive for a given number of markers on the 10-antigen panel was plotted as frequency distributions for cohort 1. Subjects in the gray 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 means ± standard errors of the means (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 are displayed for reference. Symbols represent means ± SEM. PT, posttreatment. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (all panels).

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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