Minireview

Quantitative multiplexed strategies for human Lyme disease serological testing

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Impact statement

The incidence of Lyme disease continues to increase each year, with recent estimates of nearly 500,000 new cases each year in the United States. Robust diagnostic tools and methods for monitoring response to treatment are needed to effectively combat this disease. We present a comprehensive overview of the multiplexed techniques that are currently employed for Lyme diagnosis, offer perspectives on their use for monitoring treatment response, and discuss how these methods may improve diagnostic accuracy and sensitivity.

Abstract

Lyme disease, which is primarily caused by infection with the bacterium *Borrelia burgdorferi* in the United States or other *Borrelia* species internationally, presents an ongoing challenge for diagnostics. Serological testing is the primary means of diagnosis but testing approaches differ widely, with varying degrees of sensitivity and specificity. Moreover, there is currently no reliable test to determine disease resolution following treatment. A distinct challenge in Lyme disease diagnostics is the variable patterns of human immune response to a plurality of antigens presented by *Borrelia* spp. during the infection. Thus, multiplexed testing approaches that capture these patterns and detect serological response against multiple antigens may be the key to prompt, accurate Lyme disease diagnosis. In this review, current state-of-the-art multiplexed diagnostic approaches are presented and compared with respect to their diagnostic accuracy and their potential for monitoring response to treatment.

Keywords: Lyme, diagnostic, multiplex, serology, Borrelia, disease

***Experimental Biology and Medicine* 2021; 246: 1388–1399. DOI: 10.1177/15353702211003496**

Introduction

Until recently, the only diagnostic approach recommended by the Centers for Disease Control and Prevention (CDC) for human Lyme disease diagnosis was a two-tiered scheme using Western blot analysis after a positive enzyme immunoassay (EIA or ELISA) result to detect serum antibodies against the causative agent, *Borrelia burgdorferi*.[1] This standard two-tier test (STTT) was shown to successfully diagnose 29–40% of Lyme disease cases within the first 30 days of infection.[2,3] For a disease with an estimated national incidence (in the United States) of 476,000 new cases per year,[4] false negatives can pose a significant risk to public health.[5]

Low sensitivity of testing during early-stage disease could mean that practitioners diagnose and treat patients based on clinical symptoms and without serological confirmation. Although the presence of one or more characteristic erythema migrans (EM) skin lesions is an acceptable way to identify Lyme disease in individuals, this clinical sign is sometimes not present or could appear atypically.[6–8] Antibiotic treatment has been shown to be effective by reducing bacterial load at various points of *B. burgdorferi* infection,[9–11] but this treatment has been associated with fewer symptoms and faster recovery when administered within the first month of infection,[12,13] thus supporting an ongoing need for better diagnostic strategies for early-stage disease.

Reliance on serological tests for diagnosing an active infection is less than ideal due to the indirect nature of antibody testing. There is an inherent delay between initial infection and a measurable immune response to specific antigens. The extent of antibody production and how that changes temporally during the disease course may also differ across patients. Further complicating this is the possibility of co-reactivity with other antigens as well as the difficulty in discerning a new infection in patients who were previously infected.

Replacement of the qualitative second-tier Western blot with a quantitative approach has shown promise. On 29 July 2019, the CDC approved a modified testing scheme

that uses an ELISA in lieu of the Western blot as the second-tier assay.[14] A recent comparison demonstrated that these modified two-tier tests, consisting of two quantitative ELISAs, are as specific as the STTT while yielding potentially higher sensitivity.[15,16] Quantitative and semi-quantitative assays avoid the subjective immunoreactive band interpretation of standard Western blot analysis, which may decrease both sensitivity and specificity.[17] A quantitative readout is typically standardized and used to objectively report target detection. Such data may also contribute to the design of multiplexed algorithms that incorporate each target as a diagnostic variable. To screen antibodies against *B. burgdorferi* as diagnostic biomarkers, serum reactivity to individual proteins can potentially be measured and used to identify targets that correlate with infection, recovery, or persistent disease manifestations.[17] Thus, multiplexed quantitative assays may allow for the development of better diagnostic strategies for early Lyme disease, as well as elucidate the etiology of post-treatment Lyme disease syndrome (PTLDS).[18,19]

This minireview summarizes some of the quantitative, multiplexed strategies that are currently under development for Lyme disease serology, as well as the diagnostic scoring algorithms used to predict infection status. We further describe a limited number of studies that investigated antibody titer changes throughout phases of infection and recovery. We focus here on antibody detection in serum samples, which is an indirect strategy limited by a time window between pathogen exposure and the host's immune system producing antibodies at detectable levels. Indirect detection is widely used because, despite the time window described, it has still been shown to be more sensitive and cost efficient than some more direct detection strategies that measure scarce amounts of bacteria and bacterial products.[17] Although quantitative strategies that directly detect targets using methods like PCR analysis,[20–23] chromatography/mass spectrometry,[24–27] and genomic sequencing[28–31] are available or in development, comprehensive exploration of each type of data and its analytical interpretation is beyond the scope of our discussion.

Quantitative multiplexed assays for predicting Lyme disease status

Serological tests for Lyme disease have been developed to detect IgG and IgM antibodies against proteins embedded on the surface of spirochetes involved in the infection. While Lyme disease is primarily caused by *B. burgdorferi* sensu stricto in the United States, in Europe there is increased prevalence of other Lyme disease Borrelia species, including *Borrelia garinii,*[32] *Borrelia afzelii,*[32] *Borrelia bavariensis,*[33] *Borrelia lusitaniae,*[33] *Borrelia valaisiana,*[33] *Borrelia bissettii,*[33] and *Borrelia spielmanii*.[33] Of these, *B. burgdorferi, B. garinii, and B. afzelii* are most frequently associated with the typical presentation of Lyme borreliosis.[32,34] Different serological targets have been used to diagnose the infection in the US compared with Europe,[34] although a comprehensive test that incorporates all Lyme disease bacteria in a multiplexed assay would be useful to correctly diagnose individuals who have traveled in these areas.

Among the diagnostic antigens used to detect Lyme disease in the United States and in Europe, some are conserved across several Lyme disease Borrelia species (e.g. C6 peptide of VlsE),[35] while others have variants that can differ considerably across species (e.g. OspC).[36,37] Several proteins with diagnostic potential have been reviewed,[38] which include those used in the STTT.[39] Proteins that have been incorporated into quantitative diagnostic assays include outer fibronectin binding protein (BBK32), decorin-binding proteins (DbpA and DbpB), flagellin (FlaB), outer surface proteins (Osp proteins), OspEF-related proteins (Erp proteins), oligopeptide permease A2 (OppA2), and the vmp-like sequence expressed protein (VlsE). Besides full-length proteins, peptide sequences efficiently expose specific epitopes for antibody binding but could neglect antigenic qualities of protein folding.[40] A recent study screened 12-mer peptide sequences derived from 62 distinct *B. burgdorferi* proteins, including 10 sequences found most useful for diagnosing early-stage Lyme disease.[41] Use of recombinant sequences enables the incorporation of proteins that are normally expressed by *B. burgdorferi* within the infected host but not when grown *in vitro* (e.g. certain variants of VlsE and OspF),[42,43] thus expanding the repertoire of potential targets compared with the standard Western blot, and making it possible to utilize modified or truncated proteins.

The variety of antibodies that bind antigenic proteins found in Lyme disease suggests that patients develop an independent immune response (which can be quantified by antibody titer) to each antigen. Since an infected individual may produce detectable amounts of antibody against a subset of antigens, a multiplexed diagnostic approach could potentially maximize test sensitivity.[44] Moreover, establishing criteria for a positive result that involves multiple targets could increase test specificity.[45] The potential redundancy of antibody responses to bacterial antigens in multiplexed analysis may also provide reassurance against systematic errors caused by protein batch inconsistencies or assay-specific characteristics that affect the ability to analytical sensitivity. A comparison of the ViraChip and ViraStripe assays that measured similar targets in a microtiter well or a quantitative line blot, respectively, showed less than 100% agreement.[46] Inherent differences in assay matrix and detection mechanism may also contribute to this discrepancy. Tests that are designed to detect the same target can also differ in serum antibody affinity because of the antigen variant used (e.g. OspC A type vs. OspC K type).[44] By casting a wider net for targets and allowing for potential redundancy, multiplexed detection may achieve higher sensitivity and specificity than singleplex analysis.

When properly implemented, multiplexed assays provide options for measuring antibody titers and further using these data to generate a diagnostic result. The best approach to multiplexed assay design is not always obvious and it may be beneficial to try a few different strategies to select the one offering the highest sensitivity and specificity for a comparable sample.[47] By first determining signal cutoffs for each target, some assays are then able to establish optimal criteria for positive diagnosis when a specified

proportion of targets are detected.[47,48] Alternatively, positive diagnosis can be made using a weighted score combining the measured titers of each target based on a multivariate regression model.[45,49–51] Other multiplexed assays do not distinguish between targets, but instead directly measure a combined signal to predict infection status.[44,52] Cut-off and/or threshold establishment, whether it is for detection of individual targets or for scoring a positive sample, is another important decision in assay design. Using multiples of standard deviations above negative signal (a common standard is the mean plus three times the standard deviation) is a straightforward method that affords flexibility for controls, such as pooled negative samples, but could miss consistent subtle differences between groups. Moreover, pooled negative samples can potentially dilute or diminish low-level reactivity to targets, as it does not represent any individual sample. Cutoffs can also be established with receiver operating characteristic (ROC) analysis to determine the best threshold for classifying positive and negative outcomes.[53] This generally requires a large set of negative controls in balance with the positive samples and data analysis may be complex.

Table 1 summarizes several quantitative multiplexed strategies for Lyme disease diagnosis. These strategies often use one of three biosensing mechanisms: antigen-coated bead assays, protein microarrays, and standard ELISA (Figure 1). The general binding scheme entails antigen probes capturing IgG and/or IgM antibodies in serum and use either direct signal readout or attachment of a secondary label for detection. Automated line blot assays, interpreted using the same criteria as the standard Western blot, have also been used to decrease subjectivity in blot interpretation.[46,54] However, the interpretation software is adjustable by human technologists and the diagnostic strategy is not novel compared with the STTT, so these assays are excluded from our discussion.[54]

Bead-based quantitative assays have recently gained popularity for serological detection, since they can be optimized for simultaneous detection of multiple targets using existing commercial technologies, such as the Luminex xMAP system.[51,55] One study adopted this technology using sets of antigen-coated polystyrene beads to detect IgG against VlsE protein and IgM against a 10-mer peptide derived from OspC protein.[45] The authors tested several multivariate regression classifiers to combine the two targets into a bioinformatic score, where a value greater than 1.0 indicated a positive result. The resulting model was used as a second-tier test replacing the Western blot and provided potentially better overall diagnostic performance than the STTT.[45] Besides measuring single-antigen coated beads, other similar assays measure a combined signal output using beads coated with multiple antigens[44] or modified recombinant antigen hybrids.[52] In the bead-based assays mentioned here, cutoff values for a positive result were determined using several standard deviations above the mean signal obtained from negative control serum samples.

Another type of multiplexed assay measures the signals of targets uniformly separated along a biosensing surface,[56] thus avoiding the limitation of size-based separation that occurs in Western blot analysis. One example is the grating-coupled fluorescent plasmonics (GC-FP) biochip developed by our research group,[57] which used a plasmonic fluorescence microarray to screen IgG antibodies against 17 proteins (DbpA, OspD, BBA73, RevA, BmpA, FlaB, BBA65, ErpL, VlsE, OspC, P45, BBA69, ErpG, DbpB, P58, BBA70, ErpY) for predicting Lyme disease status. The most predictive markers were determined using ROC analysis. Several combinations of targets where detection of 2 out of 3, 4, or 5 targets indicated a positive result yielded 90% sensitivity and 100% specificity for a small sample of human subjects.

The mChip-Ld is a different chip-based assay that detects serum antibodies against VlsE, pepVF (synthetic peptide combining regions of VlsE and FlaB), and OspC (K variant).[49] In developing this assay, 12 antigens were screened with EIA for their correlation with Lyme disease diagnosis using a serum panel from the CDC: Hsp90, ErpB, p45, p28, FlaB, p93/100, BmpA, DbpA, DbpB, OspC (K variant), VlsE, and pepVF. Out of these 12 antigens, the above 3 were deemed most predictive and incorporated into the mChip-Ld platform. ROC analysis of over 10,000 permutations of signal intensity weights for each antigen was conducted to develop the optimal quantitative algorithm for generating a diagnostic score, which is a linear sum of each weighted antigen signal.

Multi-antigen coated wells on microtiter plates represent another type of microarray. Using this technology, the ViraChip measures IgG and IgM antibodies against *B. burgdorferi* sensu stricto strain B31 and the SeraSpot assay does this for various European Lyme disease Borrelia.[58] The ViraChip is scored in the same way as STTT IgG and IgM Western blots, where detection of at least 2 out of 3 IgM targets or 5 out of 10 IgG targets indicates a positive result.[46] The SeraSpot assay scores positive with detection of at least two targets for IgG positivity or at least two targets (or detection of anti-OspC or anti-DbpA) for IgM positivity.[59] Recently, alternative interpretation criteria have been proposed for the ViraChip assay to increase sensitivity and specificity.[58]

Although not inherently a multiplexed platform, the standard ELISA is a versatile technique that can be used to measure antibody titer via colorimetric detection of an enzyme-activated labeling antibody on a microtiter plate. Different targets are typically not measured within the same well, but the large number of wells per plate affords analysis of several targets at once when reagent volumes permit it. ELISA was used to detect IgM against BBA65, BBA70, BBA73, OspC, FlaB, BmpA in early-stage Lyme disease.[47] A positive diagnostic result on this test required detection of at least two out of the six targets, which yielded higher sensitivity for early Lyme disease than the standard IgM Western blot. The authors explored different strategies to determine the cutoff values for antigen detection, including 2SD above the negative control, ROC analysis for best sensitivity while setting the specificity at $\geq$99%, and development of a weighted scoring metric.[47] In other study, IgM and IgG against *B. afzelii* flagella antigen was detected using ELISA. The resulting signals were then combined to generate an S score for predicting the risk for having Lyme disease.[50]

Table 1. Quantitative multiplexed serodiagnostic assays for predicting human Lyme disease.

Assay/Strategy	Antibody targets	Diagnostic criteria	Algorithm development	Study/Serum population
Bead-based:				
Mikrogen recomBead Hauser[58]	IgM or IgG against VlsE (different genospecies), p100 (*B. afzelii*), p58 (*B. garinii*), p39 (*B. afzelii*), OspA (*B. afzelii*), OspC (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto), p18 (*B. afzelii, B. garinii, B. spielmanii, B. burgdorferi* sensu stricto)	Final IgG or IgM result can be positive ($\geq$8 points), borderline (5–7 points), or negative (0–4 points)	A cutoff index (COI) was calculated by for each antigen. Individual target reactivities were categorized as negative, borderline, or positive. 1 point is given for each borderline antigen and 4 points for each positive antigen except OspC, which is given 8 points.	21 with erythema migrans, 33 with neuroborreliosis, 8 with late disease. 93 controls: 57 healthy, 31 potentially cross-reactive disease, 5 non-specific disease.
AtheNA multi-lyte test Porwancher *et al*.[45]	IgG against VlsE and IgM against pepC10 (OspC peptide)	Bioinformatic score $\geq$1	Signal cutoffs were 8SD > negative control for VlsE-IgG and 4SD >negative control for pepC10-IgM. The authors generated a bioinformatic score based on weighted linear combination of antibody levels.	242 patients at various points of disease and convalescence/antibiotic treatment. 794 controls.
Five-antigen multiplex assay Embers *et al*.[44]	IgG against OspC, OspA, DbpA, OppA2, and C6 peptide	For antigen beads mixed in a single well, a combined OD above the specified cutoff value was used to determine Lyme diagnosis.	3SD above the mean OD for negative controls on each plate was used as the cutoff value.	From CDC serum biorespository: 14 early/acute, 14 early/convalescent, 8 Lyme arthritis, 2 early cardiac Lyme, 6 neurologic Lyme. 80 controls including potentially cross-reactive disease and healthy individuals.
VOVO luciferase immunoprecipitation system Burbelo *et al*.[52]	IgG, IgA, and IgM against a repeated antigenic sequence of VlsE-OspC-VlsE-OspC	Geometric mean titer $\pm$95% confidence interval was compared with the specified cutoff value	Cutoff value: 5SD > negative control. The authors tested other antigens but determined that the repeated antigenic sequence yielded highest sensitivity and specificity.	122 patients with various manifestations of Lyme disease. 63 controls including potentially cross-reactive disease and healthy individuals.
Ten-antigen multiplex assay Lahey *et al*.[48]	IgM against OspC, FlaB, DbpB, P100, OspC (type K), pLA and IgG against Erp51, P35, OspF, OspC (Type K)	Detection of $\geq$2/10 targets, regardless of isotype	A marker was detected if its mean fluorescent intensity was > twice the 98th-percentile signal for healthy controls. Out of 62 screened markers, 2 markers were determined to be most sensitive for a set of early Lyme disease samples. Eight targets that tested positive when the top two markers tested negative were also added.	Cohort 1: 79 patients with erythema migrans, 8 untreated late Lyme arthritis in Maryland. 26 healthy controls. Cohort 2: 20 early Lyme, 107 late Lyme. 26 healthy controls. Collected in Massachusetts. Cohort 3: 175 chronic fatigue patients, 444 healthy controls.
Microarray/Spots:				
GC-FP Chou *et al*.[57]	IgG against DbpA, OspD, BBA73, RevA, BmpA, FlaB, BBA65, ErpL, VlsE, OspC, P45, BBA69, ErpG, DbpB, P58, BBA70, ErpY	Any of several combinations of 2 out of 3, 4 or 5 specified antigens detected	ROC analysis of individual antigens was used to set cutoff values and screen for predictive antigens. Various combinations of targets were tested for sensitivity and specificity and the ones with the highest sensitivity and specificity were selected.	11 early Lyme, 12 convalescent Lyme. Controls: 2 fibromyalgia, 1 multiple sclerosis, 8 non-symptomatic healthy individuals

(continued)

Table 1. Continued.

Assay/Strategy	Antibody targets	Diagnostic criteria	Algorithm development	Study/Serum population
mChip Arumugam *et al.*[49]	IgG and IgM against Hsp90, ErpB, p45, p28, FlaB, p93/100, BmpA, DbpA, DbpB, OspC (K variant), VlsE, and pepVF (synthetic peptide combining regions of VlsE and FlaB)	The sum of weighted antigen titers was compared with a specified cutoff value (not reported).	The multiplexed diagnostic score was calculated using the sum of weighted signals for each antigen. The weights were determined using ROC analysis of over 10,000 different permutations of weights and choosing the most predictive combination.	112 early Lyme, 28 disseminated Lyme, 20 with post-treatment Lyme disease syndrome. Controls: 81, including sera from healthy individuals from Lyme endemic and non-endemic areas and those with potentially cross-reactive disease.
SeraSpot Hauser[58]	IgM or IgG against VlsE (*B. afzelii*), p39 (*B. afzelii*), p58 (*B. afzelii*), p100 (*B. afzelii*), p23, OspC (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto), and p18, DbpA (*B. afzelii, B. garinii, B. burgdorferi* sensu stricto)	For IgG, the diagnostic result can be positive ($\geq$2 targets detected, negative ($<$ 2 targets detected) or borderline (only anti-VlsE detected). For IgM, the results are positive (anti-OspC or anti-DbpA or $\geq$2 targets detected), negative (no targets detected) or borderline (1 target detected, except for anti-OspC and anti-DbpA).	Proprietary software was used to determine color intensity of antigen spots in microtiter wells. Antibody reactivity was detected if the intensity is $\geq$1.0 or negative if it is $<$1.0.	21 with erythema migrans, 33 with neuroborreliosis, 8 with late disease. 93 controls: 57 healthy, 31 potentially cross-reactive disease, 5 non-specific disease.
ViraChip microarray immunoblot Theel *et al.*[46]	IgM against P23, P39, P41 or IgG against P18, P23, P28, P30, P39, P41, P45, P58, P66, and P100	Detection of 2 out of 3 targets or 5 out of 10 targets for the IgM and IgG assays, respectively	The mean intensity per antigen was divided by the cutoff to determine a signal-to-cutoff ratio, where target antibodies are detected if the ratio $\geq$100.	411 serum samples that tested positive or equivocal based on C6 ELISA assay, 32 samples with confirmed Lyme disease (including 8 convalescent samples from patients treated with antibiotics, 2 with Lyme carditis, 3 with Lyme arthritis, 7 with neuroborreliosis). Controls: 71 samples collected from healthy individuals from Lyme-endemic area.
Standard ELISA:				
Early Lyme disease assay Brandt *et al.*[47]	IgM against BBA65, BBA70, BBA73, OspC, FlaB, BmpA	Standard ELISA-based detection of $\geq$2/6 markers above specified cutoff	The authors explored different methods to set the cutoff values, including 2SD above the negative control, ROC analysis for best sensitivity while setting the specificity at $\geq$99%, and development of a weighted scoring metric.	CDC serum biorepository: 40 early acute, 38 early convalescent, 9 neuroborreliosis, 7 Lyme carditis. Controls: 143 potentially cross-reactive diseases, 100 healthy endemic samples, 102 healthy non-endemic samples
IDEIA combined IgG and IgM assay Dessau *et al.*[50]	IgM and IgG against *B. afzelii* flagella antigen	S scores can be used to calculate % risk. A high-risk threshold of 20% indicates likely disease.	Scores (in standardized units, SU) for IgG and IgM reactivity were defined as 8 SU or 5 SU, respectively, for OD values above a specified cutoff. When OD $<$ cutoff, the SU was defined as a fraction of the cutoff. A logistic regression model combining the SU scores for IgG and IgM reactivity was used to obtain a final S score.	48 Danish patients with neuroborreliosis. 216 healthy donor controls.

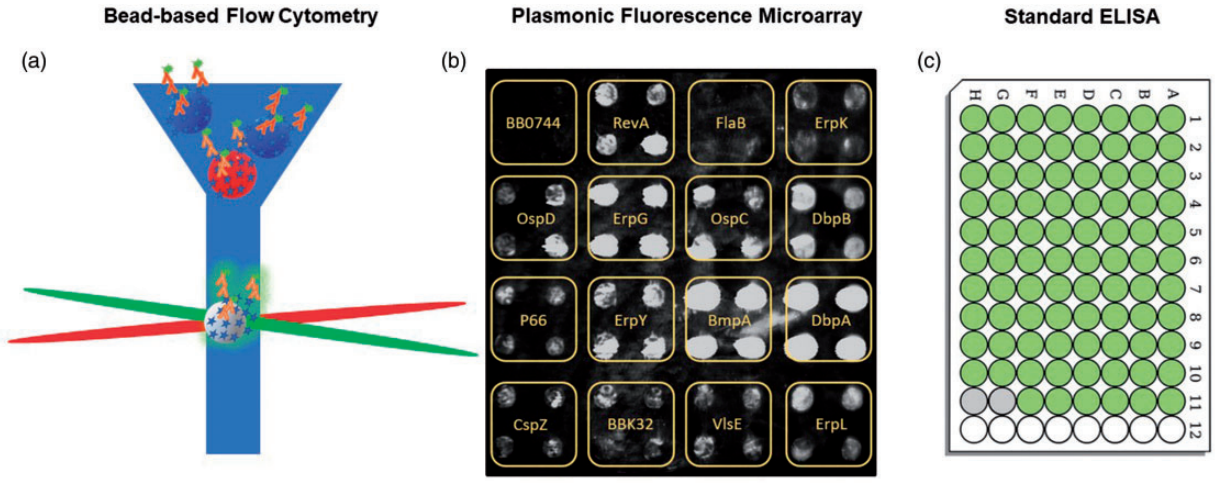

Figure 1. Quantitative multiplexed assays for Lyme disease serology use various strategies for multiplexed detection. Some recent work in this area has focused on: (a) bead-based flow cytometry, such as with the Luminex xMAP system, which uses lasers (e.g. red and green beams in the figure) to excite fluorescent tags that correlate with captured target analytes on individual beads and generates signal intensity readouts for each bead; (b) fluorescent microarray image analysis, such as with the GC-FP biochip, which captures target analytes passed through a lateral flow chamber for image-based quantitation via signal intensities of fluorescent labels; and (c) standard ELISA analysis, a common immunoassay platform conducted in microtiter plates and analyzed with specialized detectors that quantify targets via colorimetric or other chemical changes within wells. (A color version of this figure is available in the online journal.)

The diverse strategies for measuring serum antibodies and developing diagnostic algorithms have made the field highly interdisciplinary, harnessing the expertise of microbiologists, statisticians, and engineers alike. This would ultimately promote the development of a better diagnostic test that can be used to improve health outcomes. However, the general focus of algorithm development and optimization thus far has been on determining Lyme disease status in relatively uncomplicated samples, specifically patients with acute or disseminated active infection and those without a history of Lyme disease. The methods used to identify these two groups could potentially be applicable to more complex populations.

In addition to determining Lyme status, algorithms can be developed that may allow one to distinguish between past (treated) and current infections, which in regions of endemicity, would be useful for determining if a patient has been re-infected. Among patients who report persistent symptoms after a full course of treatment, an exploration of how antibody profiles may differ in these groups compared with treated groups with no long-term symptoms may lead to objective diagnostic algorithms for PTLDS or refractory Lyme disease. Lastly, because Ixodes (ticks) can be a vector for co-infectious organisms,[60,61] potentially causing some combination of Lyme disease, Ehrlichiosis, Babesiosis, and/or Anaplasmosis, multiplexed diagnostic algorithms that detect and distinguish the immune response in the presence of co-infections may be particularly useful.[41,60] Symptoms of active infection and post-treatment symptoms that result from co-infections manifest differently than simple Lyme infections in the absence of other tick-borne organisms.[60]

Quantitative immunoassays for diagnosis and monitoring of treatment response

There is currently no standard for monitoring the serological response to Lyme disease treatment, which could be useful to diagnose re-infection or PTLDS. Early studies on antibody titer changes focused on either a limited subset of antibody markers or the general antibody titer against whole-cell lysate.[62–65] To shed light on the temporal dynamics of specific antibody titers during infection and treatment, researchers examined antibody biomarkers with affinity to C6 peptide, flagellin, OspC, VlsE, OspA, OspB, DbpA, Arp, and *Borrelia* whole-cell lysate. Table 2 summarizes various findings involving human patients.[63–70] Ultimately, a screening effort to select biomarkers and design algorithms for predicting treatment prognosis, such as the case for diagnostic assays, has remained elusive.

According to some previous studies, early markers for infection include antibodies against OspC, DbpA, Arp, VlsE, and Borrelia sonicate.[64,70] During late-stage infection, OspA and OspB may be detectable, although antibodies present during early disease may also be detectable during disseminated disease or for some time after initiating treatment.[65] Researchers have reported conflicting results regarding antibody persistence after the standard course of treatment was completed. One study using serial serum samples from 74 patients with Lyme arthritis reported decreased antibody levels against several antigens 4–6 months following treatment,[70] while other studies with sample sizes ranging from 79 to 128 patients with varying disseminated Lyme disease manifestations reported persistently elevated antibody levels years following treatment,[65,69,71,72] which were independent of clinical symptoms. It has also been shown across human ($n = 120$–131) and rhesus macaque ($n = 7$) studies that C6 antibody titer exhibited a $\geq$4-fold decrease following treatment, especially if the patient is treated early.[13,66,73] For human patients, these results were in accordance with clinical symptoms, such that those who exhibited a $\geq$4-fold decrease in C6 antibody titer were also clinically asymptomatic at the last point of serum collection. Furthermore, the rare patients who had persistent arthritis or

Table 2. Studies that investigated the temporal dynamics of serum antibody titer against Lyme disease bacteria during treatment and recovery.

Antigen/Antibody isotype	Serological course	References
Flagellin/ IgG and IgM	IgM and IgG decreased over 10 years following treatment, but some study subjects remained seropositive without associated symptoms	Hammers-Berggren *et al*.[63]
C6 peptide/ IgG and IgM	In a clinically cured group, antibody titer decreased either fourfold or to undetectable levels in most patients within 6 to 12 months post treatment	Philip *et al*.[66]
VlsE (*B. garinii* strain PBi)/ IgG	Using the LIAISON Borrelia Screen assay, all IgG-positive patients became seronegative at 2 to 6 months post treatment, although these patients remained seropositive based on a comparable Anti-*Borrelia* Plus VlsE ELISA	Marangoni *et al*.[67]
OspC7 peptide/ IgG and IgM	Of IgM-positive patients, 22% and 50% had a declining or negative titer, respectively, 2 months post treatment. Of IgG-positive patients, 60% and 40% had a declining or negative follow-up titer, respectively.	Jobe *et al*.[68]
OspC/ IgG and IgM	Early IgM response occurred in patients with EM or meningitis. Increased IgG titer 3 months to 4 years after disease onset may coincide with arthritis episodes.	Fung *et al*.[64]
OspA/ IgG	Western blot showed increasing IgG reactivity towards later disease, which coincided with arthritis episodes	Kalish *et al*.[65]
OspB/ IgG	Western blot showed IgG reactivity 2–6 years following disease onset, which coincided with some arthritis episodes.	Kalish *et al*.[65]
Flagella (*B. afzelii*, strain DK-1)/ IgG and IgM	Patients with EM fit three profiles at post treatment follow-up ~1+ year later: persistent positive titer, persistent negative titer, or decrease of a positive pretreatment titer to negative signal.	Glatz *et al*.[69]
B. burgdorferi sonicate/ IgG	Low antibody reactivity in early disease with increased titer months later. Antibody titers remained high up to 3 months after starting antibiotic therapy and declined by 4–6 months.	Kannian *et al*.[70]
DbpA/ IgG	Antibody reactivity was sometimes present during early disease and titers increased considerably months later. Median antibody titers remained steady or increased 1 to 3 months after starting antibiotic therapy and decreased by 4–6 months.	Kannian *et al*.[70]
OspA/ IgG	No detected antibody reactivity during early disease, but some patients developed increased antibody titer later on. Antibody titer tended to be higher in antibody-refractory arthritis patients, including 4 to 6 months after starting treatment.	Kannian *et al*.[70]
Arp/ IgG	Antibody reactivity was sometimes present during early disease and some of the patients treated for Lyme arthritis demonstrated low antibody reactivity, which decreased in patients who were responsive to therapy but increased slightly before decreasing by 4–6 months in patients with antibiotic refractory arthritis.	Kannian *et al*.[70]
VlsE C6/ IgG	Moderate antibody reactivity observed early in disease. Antibody titers decreased within the first 3 months of starting antibiotic treatment and further declined during additional follow-ups.	Kannian *et al*.[70]

neurological symptoms associated with the Lyme disease were also more likely to also have persistently high (i.e. <4-fold decrease) C6 antibody titer.[13,73]

To explain some of the conflicting evidence about antibody levels against Lyme disease Borrelia following return to a healthy state, researchers posit that the ability of some antigens to be stored in folliculodendritic cells as immune complexes may stimulate B-cells to produce antibodies for up to several years, resulting in the maintenance of detectable titers independent of active infection.[13,74] Such antigens would be abundant and stable during active infection, with low turnover rate. Other proteins that undergo frequent turnover, such as VlsE, would be unlikely to reach the folliculodendritic cells at levels enough to generate long-term B-cell immunity.[75,76] Another potential explanation for differential maintenance of specific antibody levels is the T-cell dependency of each antigen.[77] It has been shown that several *B. burgdorferi* surface antigens elicit T-cell independent responses and that such antigens may also elicit relatively weak B-cell humoral immunity.[78] Altogether, one may hypothesize that among the larger repertoire of Lyme disease diagnostic antigens, there exists a subset of diagnostic antigens useful for monitoring treatment response and help elucidate the etiology of persistent symptoms in PTLDS. These may include antibodies that decrease after treatment or, alternatively, tend to be elevated in patients who experience successful recovery compared with patients that report persistent symptoms.[19] In the latter disease paradigm, researchers found that a robust plasma B cell response contributes to inhibition of bacterial growth and disease clearance.[19]

A thorough effort to identify potential biomarkers associated with active infection or persistent symptoms would benefit from detailed documentation of the time points

from infection to treatment, specific patient characteristics, and the prescribed treatment regimen. It has been shown that a longer interval between exposure and treatment may lead to more severe disease (e.g. multiple EM, neurologic symptoms, and arthritic symptoms), which is further associated with higher and more expansive antibody reactivity that lasts longer compared with patients treated at earlier stages of the disease.[48,66,69,79] Individual differences in genetic background and other circumstances can also affect how a patient responds to infection and antibiotic treatment. Thus, patients may be prescribed one of several variations of the standard treatment. The Infectious Diseases Society of America recommends the following as first-line treatment for early localized or early disseminated Lyme disease (i.e. EM in the absence of neurological symptoms nor atrioventricular heart block): oral doxycycline (100 mg twice daily) or amoxicillin (500 mg 3 times daily) for 14–21 days.[10] For patients intolerant of these treatments or present to the clinic with more serious disseminated disease symptoms, including Lyme arthritis and neurological involvement, IV administration of other antibiotics is recommended.[10] Additional treatment for patients with persistent symptoms is not recommended by the Infectious Disease Society of America guidelines; however, some studies involving patients with PTLDS report inconsistent results of retreatment.[13,80–82] Ultimately, the "standard treatment" for Lyme disease is not a one-size-fits-all protocol that induces the same response in everyone. An understanding of the differences within the patient population, as well as supplementing the existing and novel testing procedures with information about patient medical histories would allow for more personalized and potentially accurate predictions of treatment prognosis.

Novel diagnostic approaches may provide additional information about serum antibody levels and the profile of antibody response against different antigens for acute and convalescent disease. Using a highly sensitive microarray-based detection platform called GC-FP, our laboratory measured IgG antibody levels against 17 *B. burgdorferi* antigens (BBA65, BBA69, BBA73, BmpA, DbpB, ErpG, ErpL, ErpY, OspC, OspD, P41, P45, P58, RevA, VlsE) in acute and convalescent serum pairs from two patient cohorts, in conjunction with our prior study on GC-FP for Lyme diagnostics (Figure 2).[57] As a caveat for this analysis, the time interval between samples collected before and after initializing treatment differed across patients as well as cohorts and the total number of paired acute/convalescent samples is limited. The first cohort ($n = 3$) consisted of patient samples from the Lyme Disease Biobank taken during early Lyme disease and again 76 to 99 days after initiating the standard course of antibiotic treatment. Convalescent stage patients in this group may have reached at or neared clinically asymptomatic disease resolution.[57] This may explain the observed general decrease in IgG antibody titers below detectable levels using GC-FP analysis. Although patient serum samples from the CDC Lyme Serum Repository were also taken at the early Lyme disease stage for the second cohort ($n = 4$), the convalescent stage sample was drawn much earlier than in the first cohort, at 10–35 days after initiating treatment.[57] At this stage of early convalescence, patients may have been in the process of mounting a peak humoral immune response to the infection.[66] Thus, there was a measured titer increase for IgG antibodies to several antigens at the convalescent stage. The baseline titers of early acute samples may further be affected by the time interval between infection and initial serum collection, individual genetic differences, and whether samples were decontaminated with heat shock treatment (as was done for the CDC samples).[83] Thus, several factors including the temporal dynamics of infection and treatment, as well as sample handling conditions, need to be standardized and accounted for to accurately predict Lyme disease status based on the circumstances of each patient (and their serum samples). Nonetheless, these initial results demonstrate the potential to measure differential antibody profiles during acute and convalescent phases of infection, which could provide insight into a patient's stage of disease or response to treatment. Along the same line of investigating temporal changes in immune response to Lyme disease, another study measured levels of various cytokines and chemokines associated with T-helper cell (Th1 and Th17) function.[84] The authors reported several differences across serum samples from patients with early disease with or without antibiotic treatment, versus late-stage Lyme arthritis. Analysis of these inflammatory mediators can provide information about how heterogeneity of immune responses may correlate with disease stage, Lyme symptoms manifestation, and possible autoimmune phenotypes,[84] potentially supplementing antibody data in determining Lyme disease prognosis.

Outlook

The ongoing challenges of diagnosing early Lyme disease and understanding the treatment response have been important areas for research. Current test sensitivity for early Lyme disease was estimated to be 60 times lower than comparable HIV serological tests.[5] Currently there is no standard method for confirming that a patient has fully recovered from Lyme disease or has been re-infected after being diagnosed and treated previously. Among proposed solutions, quantitative multiplexed serological assays have emerged to be relatively simple and powerful techniques that have been shown to be comparable or better than the current STTT scheme.[44,49] Besides accessing quantitative information about the extent of specific antibody response to spirochete proteins, a layer of information not typically permitted with qualitative methods such as the Western blot, such assays also provide data that can be used to discover patterns in serum reactivity and enable us to predict disease status based on these patterns. For example, we recently used both ROC analysis and machine learning algorithms to develop diagnostic thresholds for COVID-19 antibody detection in a GC-FP based assay.[85] This has been done to diagnose Lyme disease using several quantitative multiplexed assays described in this minireview. We further propose that similar techniques using predictive modeling and machine learning can be adapted to differentiate active Lyme infection in patients who have complex

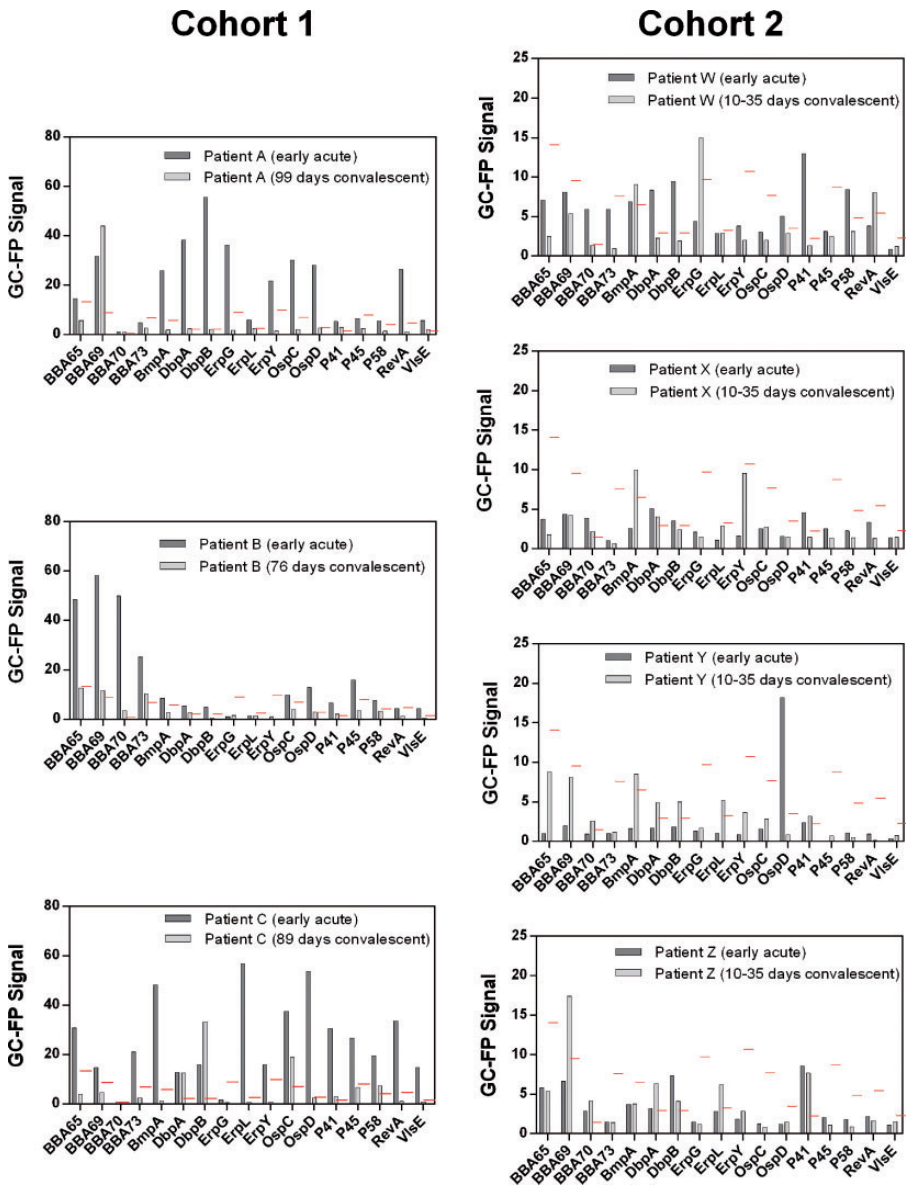

Figure 2. GC-FP analysis was conducted to measure IgG antibody levels against 17 *B. burgdorferi* antigens (BBA65, BBA69, BBA73, BmpA, DbpB, ErpG, ErpL, ErpY, OspC, OspD, P41, P45, P58, RevA, VlsE) in acute and convalescent serum pairs from two cohorts. Cohort 1 consisted of samples from three patients, collected by the Lyme Disease Biobank and Cohort 2 consisted of samples from four patients collected by the CDC Lyme Serum Repository. Samples from both cohorts included an early Lyme disease sample (early acute) and a sample collected after initiating treatment (convalescent), with the days after initial dose of treatment specified. The mean GC-FP signal intensity for each antigen is reported for each patient. The red lines indicate cutoff values for detectable signal, which were determined from measurement of negative control serum samples. For a given sample and antigen, GC-FP signal above the red line indicates measurable antibody concentration above the background signal measured in a negative control sample. (A color version of this figure is available in the online journal.)

Lyme disease statuses, such as past infection, PTLDS, and co-infection.

In these cases, when disease status is complicated, randomized training and test sets could be useful in making cutoff decisions and designing diagnostic algorithms to prevent model overfitting. To accomplish this, a large-scale effort to collect and consolidate a variety of data from negative serum control sets representative of local and general populations could be used to identify active disease in areas of different prevalence for Lyme disease antibodies and where people may have other look-alike or cross-reactive conditions.[83] Although they may be difficult to obtain and confirm, negative controls from successfully treated Lyme disease patients would be particularly useful in identifying re-infections. Data from patients with confirmed treatment failure, while also difficult to obtain, would help in elucidating why a subset of patients exhibit PLTDS.[13,86] Data from these populations may contribute to novel diagnostic algorithms that address the current gap in scientific knowledge and move Lyme disease diagnostics closer to a quantitative analysis of the immune response to disease, and thus promote better ways of tackling tick-borne illness in the clinic.

AUTHORS' CONTRIBUTIONS

EC performed writing and data analysis, AM provided editing and assistance with writing, and NCC contributed to writing and editing.

ACKNOWLEDGMENTS

The authors thank Liz Horn and Stephanie Gervasi for reviewing the manuscript.

DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

FUNDING

There was no funding to support this minireview.

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