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Advances in Serodiagnostic Testing for Lyme Disease Are at Hand

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The cause of Lyme disease, *Borrelia burgdorferi*, was discovered in 1983. A 2-tiered testing protocol was established for serodiagnosis in 1994, involving an enzyme immunoassay (EIA) or indirect fluorescence antibody, followed (if reactive) by immunoglobulin M and immunoglobulin G Western immunoblots. These assays were prepared from whole-cell cultured *B. burgdorferi*, lacking key *in vivo* expressed antigens and expressing antigens that can bind non-*Borrelia* antibodies. Additional drawbacks, particular to the Western immunoblot component, include low sensitivity in early infection, technical complexity, and subjective interpretation when scored by visual examination. Nevertheless, 2-tiered testing with immunoblotting remains the benchmark for evaluation of new methods or approaches. Next-generation serologic assays, prepared with recombinant proteins or synthetic peptides, and alternative testing protocols, can now overcome or circumvent many of these past drawbacks. This article describes next-generation serodiagnostic testing for Lyme disease, focusing on methods that are currently available or near-at-hand.

Keywords. Lyme disease; *Borrelia burgdorferi*; tests; serology; diagnosis.

Lyme disease, the most common vector-borne illness in North America and Europe, is caused by tick-borne bacteria of the *Borrelia burgdorferi sensu lato* group. The annual incidence in the United States is approximately 300 000 cases, with more than 3 million diagnostic tests performed each year [1, 2]. After identification of the etiologic agent in 1983 [3, 4], antibody tests were developed, but specificity and reproducibility were poor [5]. To address this, the 1994 Conference on the Serological Diagnosis of Lyme Disease in Dearborn, Michigan, established a 2-tiered testing paradigm for serodiagnosis: a sensitive first-tiered test, now usually an enzyme immunoassay (EIA), followed by Western immunoblotting to increase specificity [6]. More than 2 decades later, despite drawbacks, this approach remains the standard for laboratory diagnosis of Lyme disease [7]. In the interim, technological advances have surmounted some of the drawbacks of earlier assay platforms, but other

important limitations remain. With the advent of whole-genome sequencing, and progress in mapping of immunodominant specific antigens, better-performing assays are being developed and some are at hand.

This article focuses on the technical aspects of current and near-at-hand platforms and assays for the laboratory diagnosis of Lyme disease. This is distinct from discussions about appropriate indications for the use of validated tests—that is, the question of when to request an assay for Lyme disease, or when testing is not needed. The article was developed after a 2016 Cold Spring Harbor Laboratory Banbury conference on the same subject. The conference included members of academia, industry, and public health agencies. Direct detection methods, designed to detect the infectious agent itself, were discussed along with indirect detection methods, which detect the host's immunologic response to infection. The current report is limited to indirect detection serologic tests, as they are the standard for diagnostic testing, and improvements to existing recommendations are more adoptable for immediate use. We emphasize serologic test insensitivity during the first several weeks of infection, and the technical and interpretive complexity of Western immunoblots. We focus on the US situation, because serologic testing is performed using uniform methods

and interpretive criteria, making comparisons with alternative methods more straightforward. The methodology applies similarly to Europe.

CURRENT APPROACH: STRENGTHS AND LIMITATIONS

The imperative at the 1994 Dearborn Conference was to address the poor specificity of serologic assays being used at the time, and to improve reproducibility by developing test performance and interpretation guidelines. The result was a 2-tiered serologic testing protocol [6], which retained the poorly specific but reasonably sensitive assays of the time—EIAs or indirect fluorescence antibody (IFA) tests—as first-tier tests. If the first-tier test is negative, no further testing is performed on the specimen, although follow-up testing to assess for seroconversion is sometimes indicated when early Lyme disease is suspected. If the first-tier test is positive or equivocal,

supplemental immunoglobulin M (IgM) and immunoglobulin G (IgG) Western blots are performed and interpreted according to standardized guidelines, requiring 2 of 3 particular bands for positive IgM blot results, or 5 of 10 particular bands for positive IgG blots. To maximize specificity, guidelines state that IgM reactivity alone should not be used to support the diagnosis of Lyme disease if signs or symptoms of early Lyme disease have been present longer than 1 month (the "1-month rule").

The most important contribution of Western blots is that they improve diagnostic specificity (Table 1). In addition, as the antibody response typically expands over time, the number and nature of reactive bands on Western blots may provide information about the duration of infection [8]. However, high specificity comes at the price of poor sensitivity in early disease. In patients with acute erythema migrans (EM), the most common early manifestation of Lyme disease, the Western blot is approximately half as sensitive as the first-tier (EIA) component

Table 1. Evolution of Serodiagnostic Testing for Lyme Disease in the United States

	Antigen Preparation	Strengths	Drawbacks	Possible Uses
Early generation EIAs	WCS of cultured *B.b.s.s.*	Very sensitive in noncutaneous LD Moderately sensitive in cutaneous LD	Suboptimal specificity Lack key antigens not expressed in cultured *Borrelia*	First-tier test, to be followed either by Western blots or different EIA
Next generation EIAs	Synthetic peptides, and/or chimeric or recombinant *B.b.s.s.* and/or *B.b.s.l.* proteins	More specific compared with early generation EIAs Similarly sensitive compared with early generation EIAs; might better detect infection by European *B.b.s.l.* strains Can be designed to include antigens only expressed *in vivo*	Significantly less specific, if used as stand-alone tests, compared with conventional 2-tiered testing with EIA followed by immunoblots	First-tier test, to be followed by Western blots or by an orthogonal EIA Second-tier test, to supplement a different first-tier test
Early generation immunoblots (Western blots)	Whole cell lysate of cultured *B.b.s.s.*	Very specific if scored according to CDC guidelines Provide detailed information about the antibody response to their target antigens	Less sensitive in early infection, compared with EIAs Subjective interpretation; presence of non-specific bands might complicate interpretation Technical complexity; relatively expensive Lack key antigens not expressed in cultured *Borrelia*	Second-tier test, to supplement early- or next-generation EIAs
Next-generation immunoblots (line blots)	Purified or recombinant *B.b.s.s.* proteins	Very specific if scored according to CDC guidelines Elimination of extraneous bands makes interpretation easier; often scored using objective (instrumented) processes Provide detailed information about the antibody response to their target antigens	Current assays recapitulate Western blots, using same antigens scored in CDC interpretive criteria that might include highly cross-reactive antigens	Second-tier test, to supplement early- or next-generation EIAs
Multiplexed serologic assays	Synthetic peptides, and/or chimeric or recombinant *B.b.s.s.* and/or *B.b.s.l.* proteins	Designed using simpler and more flexible platforms compared with immunoblots (eg, beads, microchips) Provide detailed information about the targets of antibody response Can be designed to include antigens expressed *in vivo*	Might require learning time for clinicians to become familiar with a new assay	Second-tier test, to supplement early- or next-generation EIAs Potential use as stand-alone tests without tiered approach

This table reflects the evolution of Lyme disease tests from the early (circa-1994) to the current/near-at-hand tests and strategies that developed along with advances in application of technologies and the new data that accompanied them.

Abbreviations: *B.b.s.l., Borrelia burgdorferi sensu lato*; *B.b.s.s., Borellia burgdorferi sensu stricto*; CDC, Centers for Disease Control and Prevention; EIA, enzyme immunoassay; LD, Lyme disease; WCS, whole-cell sonicate.

[a]Possible Uses. This is meant for illustration purposes and is not intended as a guideline or endorsement.

[8, 9], because adequate expansion of the antibody response to meet Western blot criteria takes more time. The overall sensitivity of any two-tiered algorithm is equal to its least sensitive component, and therefore the Western blot component reduces the overall sensitivity of the current 2-tiered algorithm for the diagnosis of early Lyme disease. Even in early disseminated infection, with neurologic abnormalities or Lyme carditis, false-negative results can sometimes occur when the 1-month rule of the Dearborn criteria is applied [10]. Such patients may have only a positive IgM Western blot, but not a fully evolved IgG antibody response, after 1 month of illness.

Like all serologic tests, Western blots cannot differentiate active infection from past exposure when performed on a single serum sample. Antibodies are the product of a host response to the infectious agent, and may persist for years after successful treatment. Occasionally serologic tests can be useful, if seroconversion can be documented by analyzing serial samples from an individual over a period of time if analyzed in the same assay run, as opposed to different runs even in the same laboratory. Such serial samples are rarely available. Also, prompt antibiotic therapy may dampen the immune response, further impacting the sensitivity of diagnostic tests early in disease. The net effect can be a reduction of antibody differences between pre- and post-antibiotic serum samples, likely associated with *Borrelia* killing [8].

Improvements in serologic testing methods or protocols will not address their inability to differentiate active infection from past exposure. Ideally it will be addressed through improved direct detection methods, because direct detection of the microbe is strong evidence of an active rather than a past infection. In addition, indirect tests that do not involve antibody detection, such as interferon-gamma release assays, could potentially play a role [11]. Ultimately, it will be advantageous to have both direct and indirect tests available, with direct detection methods favored in the evaluation of patients who present soon after initial infection, or who have been exposed multiple times and have a persistent antibody response, and indirect tests favored when clinical presentation of the primary infection occurs weeks or months after tick exposure.

Other limitations of Western blotting are related to the method itself. The precise antigens associated with some of the blot bands that are scored using the Dearborn criteria were not fully defined at the time the guidelines were formulated. It was later recognized that multiple proteins with similar molecular weights co-locate in one-dimensional electrophoresis of *B. burgdorferi* lysates. Thus, any immunoreactive band may represent antibody to more than one protein [12]. Also, it is now known that cultured *B. burgdorferi* does not express the full repertoire of antigens against which the host antibody response is mounted, and that some key antigens are only expressed *in vivo*. An important example is "variable major protein–like sequence, expressed" (VlsE), a surface lipoprotein of *B. burgdorferi* that is poorly expressed *in vitro*, yet becomes a major outer surface protein soon after mammalian infection is established. Epitopes contained within VlsE provoke an early antibody response that is not detectable using Western blots or, for that matter, EIAs prepared from whole-cell sonicates (WCS) of cultured *B. burgdorferi* bacteria, which are commonly used.

There have also been problems in achieving high reproducibility. When Western blots are scored by visual examination, the process is subjective and can be operator-dependent if faint bands or bands in incorrect locations are erroneously scored [13]. This is most often seen with IgM blots. These problems have been ameliorated with "line blots," in which recombinant or purified *Borrelia* proteins are placed in defined locations on the blot, and with densitometric blot analysis, which aids in determining whether bands are too faint to be scored [14]. However, many second tier tests are Western blot assays, which utilize cultured *B. burgdorferi* lysates and visual scoring. Furthermore, the complexity of Western blot protocols and interpretation prevents many clinical laboratories from offering the test on-site. Detailed information about responses to multiple *Borrelia* proteins may help in understanding complex cases but can also lead to confusion or misinterpretation of the results by health care providers or patients.

ALTERNATIVE SEROLOGIC TESTING METHODS AND STRATEGIES

Substitution of Next-generation Enzyme Immunoassays (EIAs) for Whole-cell Sonicate EIAs in 2-tiered Testing

A new generation of EIAs has emerged, in which the antigen preparation consists of recombinant proteins, synthetic peptides, or synthetically engineered chimeric proteins [9, 15–17]. Although whole *B. burgdorferi* sonicates have the advantage of containing numerous epitopes, the disadvantage is that some of the epitopes are cross-reactive with other, unrelated bacterial epitopes. Characterizing epitopes allows selection for immunodominance, conservation across *B. burgdorferi* genospecies, and minimal cross-reactivity with nonspecific antibodies [18, 19].

Many next-generation EIAs incorporate VlsE, or a portion of the molecule, as the sole antigen target or as one antigen among a small number selected for the assay. Among the best-characterized antigens of this type is the C6 epitope, a 25-mer oligopeptide (the "C6 peptide") corresponding to the sixth invariable region (IR6) within VlsE. This immunodominant epitope is partially conserved among *B. burgdorferi sensu stricto* strains, and among *B. burgdorferi sensu lato* genospecies [20–22]. It has some limitations as the basis for a serologic assay, namely, that the parent molecule (VlsE) is only expressed after the bacteria has been transferred from the tick to the host [23], and C6 assays do not detect IgM-class antibodies well [24]. However, IgG isotype switching to this protein occurs rapidly [15, 25], and the sensitivity of C6 EIAs is good in all but the earliest cases [24, 26].

Studies have demonstrated that EIAs utilizing the C6 epitope or VlsE protein are more specific than WCS EIAs [9, 10, 16, 27]. In a large study, its overall specificity was 98.9% (95% confidence interval [CI], 98.4–99.3%) compared with 95.2% (95% CI, 94.2–96.1%) for the sonicate preparation (*P* < .0001) [9]. When next-generation EIAs like the C6 EIA are used in the first tier of the conventional 2-tiered testing protocol, their higher specificity results in fewer second-tier tests [28]. C6 assays established the concept of using specific epitopes as antigen targets, and several next-generation EIAs are Food and Drug Administration (FDA)-cleared in the United States for use as first-tier tests in the standard 2-tiered testing protocol.

The Use of Next-generation Enzyme Immunoassays as Stand-alone Tests

Considering that some next-generation EIAs are more specific than WCS EIAs, their use as stand-alone tests—without a supplemental second-tier assay—has been proposed [9]. However, current FDA approved next-generation assays are significantly less specific than standard 2-tiered testing with Western blots. In 2 large studies, 2-tiered testing with a WCS EIA followed by Western blots was 99.5% specific, whereas the commercially available C6 EIA used as a stand-alone test was 98.9% specific (*P* = .05) [9] or 98.4% specific (*P* = .01) [10]. Similarly, a commercially available immunoassay using full-length, recombinant VlsE as the antigen target was 98.1% specific when used as a stand-alone test, compared with 99.5% using conventional 2-tiered testing (*P* = .003) [29]. Although the differences in specificity are small, they are statistically significant and can lead to profound differences in positive-predictive value, depending on the prevalence of Lyme disease in the population [10].

Two Enzyme Immunoassays Performed Sequentially in a 2-tiered Protocol Without Western Blots

Individual tests using different targets often are not susceptible to the same false-positive or false-negative effects. If a positive result from 2 tests is required to diagnose a particular disease, one expects greater specificity than would be obtained by either test alone. This principle is applied frequently in infectious disease diagnostics. For example, the sequential use of 2 different rapid tests for human immunodeficiency virus (HIV) infection has been recommended to improve the positive predictive value of point-of-contact testing [30]. In Lyme disease, the opportunity to apply this principle has arisen with the availability of next-generation EIAs. Next-generation EIAs are dissimilar from WCS EIAs and, in some cases, to each other. For example, the VlsE outer-surface protein and its C6 immunodominant epitope are not well represented in WCS EIAs. Thus VlsE or C6 EIAs are different from WCS EIAs.

Several groups of investigators have evaluated the sequential use of a WCS EIA and the C6 EIA in 2-tiered testing [31]. When these 2 tests are applied in 2-tiered testing, specificity is greater than that of either test alone and is equal to the specificity of standard two-tiered testing with EIA and immunoblotting

(Western blots or line blot) (Table 2). Moreover, studies show that this "2-EIA" protocol can be more sensitive in early Lyme disease than conventional 2-tiered testing (Table 3). In patients with EM, the advantage in sensitivity is due to the use of EIAs without the less-sensitive immunoblots. In patients with early disseminated infection, with acute neuroborreliosis or carditis, the 2-EIA protocol improves sensitivity by dispensing with the "1-month rule" of the Dearborn criteria. Of note, when applied in patients who acquired Lyme disease in central Europe, the 2-EIA protocol (using FDA-cleared assays designed for use in the US) was significantly more sensitive compared with standard 2-tiered testing (also using US assays; Table 3).

Although the most validated 2-EIA protocol involves a WCS EIA followed by a C6 EIA (or vice versa), alternative EIA combinations are promising. There are reports of other next-generation assays substituting for the C6 EIA in the 2-EIA protocol, with equivalent sensitivity and specificity [17, 27, 29]. Alternative paradigms such as the use of 2 different next-generation EIAs used sequentially in a 2-tiered testing protocol, without the use of WCS EIA, also show promise. In one study, the latter approach (using a VlsE assay followed by the C6 EIA) was significantly more sensitive than other 2-EIA algorithms evaluated [29]. The performance of any combination of assays depends on the degree to which the two assays are different from, or complementary to, one another.

Beyond improved sensitivity, the 2-EIA protocol offers several advantages compared with standard 2-tiered testing. The results are obtained objectively by an instrument system, and the information provided to the clinician is straightforward (i.e., the patient is either seropositive or seronegative), with an interpretation that is less complex than immunoblotting. The sequential use of 2 EIAs may cost less compared with protocols involving immunoblotting [28], and the methodology—ELISAs or similar assays—is accessible to most clinical laboratories,

Table 2. Specificity of Standard and Modified Serologic Testing Protocols in Control Subjects

Sample Size and Reference	Specificity (%)		
	Standard 2-tier	2-EIA	C6 EIA Alone
N = 1300; Ref [10]	99.5 (98.9–99.8)	99.5 (98.9–99.8) *P* = 1.0	98.4 (97.5–99.0) *P* = .01
N = 2208; Ref [9]	99.5 (99.1–99.7)	99.5 (99.1–99.8) *P* = 1.0	98.9 (98.4–99.3) *P* = .05
N = 100; Ref [36]	100 (95.6–100)	100 (95.6–100) *P* = 1.0	100 (95.6–100) *P* = 1.0
N = 347; Ref [27]	98.3 (96.2–99.3)	98.3 (96.2–99.3) *P* = 1.0	96.5 (94.0–98.1) *P* = .2

The numbers in parentheses represent the 95% confidence intervals surrounding the specificity values.

The *P* values pertain to the comparison with results obtained by standard two-tiered testing (whole-cell sonicate enzyme immunoassay (EIA) followed by immunoglobulin M and immunoglobulin G Western blots). The values provided for the 2-EIA protocol refer to results obtained using a Food and Drug Administration (FDA)-cleared, commercially available whole-cell sonicate EIA followed by an FDA-cleared, commercially available C6 EIA.

Table 3. Sensitivity of Standard and Modified Serologic Testing Protocols in Patients With Lyme Disease

	Sample Size and Reference	Disease Manifestations	Patient Location	Sensitivity (%)		
				Standard 2-tier	2-EIA	C6 EIA alone
Early Lyme Disease	N = 140; Ref [10]	EM, ENB, LC	US	48 (40–56)	61 (53–69) P = .03	64 (56–72) P = .008
	N = 318; Ref [9]	EM, ENB	US	41 (36–46)	60 (55–66)[a] P < .0001	66 (60–71) P < .0001
	N = 35; Ref [36]	EM, ENB	Europe[b]	29 (16–45)	74 (58–86) P = .0003	77 (61–88) P < .0001
	N = 57; Ref [27]	EM, ENB, LC	US	54 (42–67)	61 (48–73) P = .57	68 (55–79) P = .18
	N = 55; Ref [29]	EM	US	25 (16–38)	38 (26–51) P = .22	65 (52–77) P < .0001
Late Lyme Disease	N = 29; Ref [10]	LA, LNB	US	100 (86–100)	100 (86–100) P = 1.0	100 (86–100) P = 1.0
	N = 122; Ref [9]	LA, LNB	US	96 (91–98)	98 (93–99) P = .7	98 (94–100) P = .4
	N = 15; Ref [36]	LA	Europe[b]	60 (36–80)	93 (68–100) P = .08	100 (76–100) P = .02
	N = 29; Ref [27]	LA	US	100 (86–100)	100 (86–100) P = 1.0	100 (86–100) P = 1.0

The numbers in parentheses represent the 95% confidence intervals surrounding sensitivity values. The *P* values pertain to the comparison with results obtained by standard two-tiered testing (whole-cell sonicate enzyme immunoassay [EIA] followed by immunoglobulin M and immunoglobulin G Western blots). The values provided for the 2-EIA protocol refer to results obtained using an Food and Drug Administration (FDA)-cleared, commercially available whole-cell sonicate EIA followed by an FDA-cleared, commercially available C6 EIA.

Abbreviations: EM, erythema migrans; ENB, early neuroborreliosis (Neurologic Lyme disease); LA, Lyme arthritis; LC, Lyme carditis; LNB; late neuroborreliosis (Neurologic Lyme disease); US, United States.

[a]Sensitivity of the 2-enzyme immunoassay protocol was not included in the referenced article as published, but was determined upon post-publication re-analysis of the data and provided as a personal communication.

[b]In this study, patients who acquired Lyme disease in Europe were evaluated using serologic tests designed for use in the United States, a situation that arises for travelers.

without special expertise, decreasing turnaround time, especially if performed concurrently.

The main limitation of this strategy is that by removing the immunoblot component from the 2-tiered algorithm, without a substitute, information about the extent and maturity of the antibody response is lost. For routine cases, in which the patient has objective signs compatible with a common Lyme disease manifestation, and no history of previous *B. burgdorferi* infection, a "positive" or "negative" result is likely all that is needed. In endemic areas, a background seropositivity of 5% or more can be found, making interpretation of any serologic test more complicated [32, 33].

Other Testing Platforms

Besides Western blots and line blots, there are other effective platforms upon which multiplexed serologic assays are designed to provide information about antibody responses to multiple *Borrelia* antigens, including bead-based assays or microchip configurations. As a starting point, the *Borrelia* antigens can include all of those currently used in Western blots [14, 18, 34], allowing detection of each antigen-specific antibody response by objective processes. A major advantage of these versatile platforms, however, will be the ability to add informative antigens [35] or subtract less informative or cross-reactive antigens, potentially improving performance in comparison to assays that simulate conventional Western blotting.

Mapping linear B-cell epitopes of key *B. burgdorferi* antigens has identified sensitive, specific epitope antigen targets [18, 19, 35] from both *in vivo* and *in vitro* expressed antigens. Peptides containing specific epitopes, with or without recombinant antigens, are uniquely suited as antigen targets in multiplex platforms. As an example, there was an evaluation of a multiplexed bead-based assay including 10 separate *B. burgdorferi* antigens, most of which were peptides composed of specific epitopes and different from those scored in Western blots using the Dearborn criteria. In that evaluation, the antigen panel was more sensitive in early Lyme disease than the standard 2-tiered algorithm while maintaining high specificity [35]. In a cohort of 40 patients with EM, 22 of 40 (55%) were seropositive at baseline using the multiplex assay, whereas 16 of 40 (40%) were seropositive by standard 2-tiered testing (*P* = .05), and specificity was 100% using both methods. This approach offers the prospect of a single tier assay in the near future and lends itself to adaptations as new data are discovered.

PATHWAY FORWARD

For routine cases of suspected Lyme disease, only a "positive" or "negative" result is likely needed, and this can be provided a multiplex assay or by 2 different EIAs performed sequentially or concurrently, without the use of Western blots. Published data suggest that this strategy could provide sensitivity and specificity equivalent to, or better than, current 2-tiered

testing with EIA and immunoblotting [10, 17, 27–29, 36], and the 2 EIAs can be done reliably in most clinical laboratories [28]. Although several next-generation EIAs are FDA-cleared as first tier assays, none is currently cleared as a second-tier test in place of immunoblotting. Currently, the Centers for Disease Control and Prevention (CDC) recommends that only laboratory tests cleared or approved by FDA be used to aid in the routine serodiagnosis of Lyme disease [37]. Thus, an important next step for widespread adoption will be for assay developers to provide performance data establishing that their assay is equivalent to, or better than, the current reference standard, which at the moment is two-tiered testing with immunoblots. Assay developers who desire FDA clearance should contact the FDA directly for early guidance [38–40].

CONCLUSIONS

For the past 2 decades, the concept of a 2-tiered strategy aimed at high sensitivity and specificity has been widely used, but the current approach is insensitive during the first weeks of Lyme disease, and Western blotting can be complex to perform and interpret. Recent studies suggest that a single multiplex or 2-tiered strategies involving 2 different EIAs perform as well or better than 2-tiered testing with EIA and Western blots. Using this approach, one can obtain a positive or negative result quickly in a standard laboratory setting. The capability to diagnose Lyme disease at various time points during the course of infection will also likely be complemented by new methods, including direct detection assays. Advances in our understanding of key *B. burgdorferi* antigens and the antibody response to them, coupled with improvements in assay design and development, have brought us to a turning point where new diagnostic approaches can deliver better performance than current methods.

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