Laboratory Diagnosis of Lyme Disease

Advances and Challenges

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KEYWORDS

• Lyme disease • *Borrelia burgdorferi* • Laboratory diagnosis • Serology

KEY POINTS

- It is difficult to demonstrate *Borrelia burgdorferi* by direct techniques (culture and polymerase chain reaction [PCR]). The spirochete is more easily found in the skin and plasma samples of patients with early disease (erythema migrans), and in the synovial fluid of patients with Lyme arthritis (using PCR).
- The sensitivity of antibody-based tests increases with the duration of the infection. Less than 50% of patients with erythema migrans are positive at presentation. These patients should receive treatment based on the clinical diagnosis.
- Serologic tests are most helpful in patients with clinical findings indicating later stages of Lyme disease.
- Many tests for Lyme disease are being performed in patients with low likelihood to have the disease, a situation in which a positive result is more likely to be a false-positive.
- The current assays do not distinguish between active and past infection, and patients may continue to be seropositive for years.
- The use of nonvalidated Lyme diagnostic tests is not recommended.

OVERVIEW

Lyme disease, or Lyme borreliosis, is a multisystem illness caused by the spirochete *Borrelia burgdorferi* and it is the most common tick-borne illness in the United States and Europe. Newly revised estimates from the Centers for Disease Control and Prevention (CDC) suggest that there are likely to be around 300,000 new cases of

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Lyme disease per year in the United States.[1] *B burgdorferi* is transmitted by the bite of infected ticks of the *Ixodes ricinus* complex. In the United States, most cases of Lyme disease are caused by the blacklegged tick (*Ixodes scapularis*), occurring in the mid-Atlantic, northeast, and upper Midwest regions.

B burgdorferi is a gram-negative bacterium, and has the elongated and spiral shape of the spirochetes.[2] It varies from 10 to 30 μm in length and 0.2 to 0.5 μm in width. It has a linear chromosome and a variable number of circular and linear plasmids.[3] The *B burgdorferi* sensu lato group includes at least 20 genospecies.[4] Three genospecies are most commonly associated with human infections: *B burgdorferi* sensu stricto, which causes disease in North America and Europe; and *Borrelia afzelii* and *Borrelia garinii*, which occur in Europe and Asia.[5] Additional genospecies have been shown to at least occasionally cause human disease in Europe (eg, *Borrelia spielmanii* and *Borrelia valaisiana*).[5] There is some variation in the clinical presentation depending on the infecting genospecies, with *B burgdorferi* sensu stricto predominating in arthritis, *B garinii* in neurologic disease, and *B afzelii* in chronic skin manifestations.[6] Even within the same genospecies, there is variation in presentation and dissemination capability.[7,8]

For clinical purposes, Lyme disease is divided into early localized, early disseminated, and late stages. Lyme disease usually begins with the characteristic skin lesion, erythema migrans (EM), at the site of the tick bite.[9–11] After several days or weeks, the spirochete may disseminate and patients can develop neurologic, cardiac, and rheumatologic involvement.[12–15] The infection is characterized by low number of bacteria, which can persist in collagen-rich tissues. Although antibiotic therapy accelerates resolution of the disease, manifestations can spontaneously regress without antibiotic therapy. The resolution of disease is mediated by immune responses, which control the infection. However, without antibiotic therapy, it can recur and/or new manifestations can appear.[9,16,17]

The available laboratory methods for the diagnosis of Lyme disease are in 2 categories: direct methods to detect *B burgdorferi*, and indirect methods that detect the immune response against it (mainly the detection of antibodies against *B burgdorferi*). It is important to recognize that laboratory tests should be ordered and interpreted in the context of the clinical evaluation and the likelihood that the patient has Lyme disease. This article reviews the laboratory diagnostics for Lyme disease (with focus on the United States) and discusses current recommendations and new developments.

DIRECT METHODS FOR DETECTION OF *BORRELIA BURGDORFERI*

Laboratory tests for direct detection of *B burgdorferi* are hampered by very low numbers of spirochetes in most clinical samples. The lack of sensitive, easy, fast, direct tests for the presence of *B burgdorferi* is one of the main challenges in the laboratory diagnosis of Lyme disease. Although direct tests for *B burgdorferi* can sometimes be helpful, none are required for the diagnosis of the disease. The main direct test modalities used are culture and PCR. Histopathology has limited utility, being used mostly to exclude other diseases, and in the evaluation of suspected cases of borrelial lymphocytoma and acrodermatitis chronica atrophicans.[18,19] Detection of *B burgdorferi* is difficult and time consuming because of the extreme scarcity of organisms.[20–23] Warthin-Starry and modified Dieterle silver stains, focus-floating microscopy, as well as direct and indirect immunofluorescence assays with antiborrelial antibodies have been used, but can be difficult to interpret and require special expertise and careful use of controls.[24–26] At present, no antigen assays are recommended for the diagnosis of Lyme disease. A research test for detection of outer surface

protein (Osp) A has been used in cerebrospinal fluid.[27] An assay to detect antigens in urine has been shown to be unreliable.[28]

CULTURE

Culture is not a routinely available diagnostic method for the diagnosis of Lyme disease in clinical practice, because of its relatively low sensitivity, long incubation, and the requirement of special media and expertise. However, the ability to isolate and culture *B burgdorferi* is essential in Lyme disease research, and culture remains the gold standard to confirm the diagnosis. Methods that would improve sensitivity and simplify the procedure are needed to allow it to be adopted more extensively.

B burgdorferi has a limited metabolic capacity and requires a complex growth medium for cultivation. Media used for culturing *B burgdorferi* include variations of the Barbour-Stoenner-Kelly medium[29] and the modified Kelly-Pettenkofer medium.[30] Cultures are examined using dark-field microscopy or fluorescent microscopy after staining aliquots with acridine orange, but sensitivity is improved by testing aliquots with PCR methods.[31] *B burgdorferi* replicates slowly and cultures are kept for 8 to 12 weeks before being considered negative.[31]

The probability of culturing *B burgdorferi* depends on the specimen, the stage of the disease, and the expertise of the laboratory. It may also depend on the genotype.[32] Antibiotic therapy with agents effective against *B burgdorferi* (even a single dose) significantly affects the recovery rate.[33,34]

Culture of skin biopsies from EM has a sensitivity of 40% to 60%.[30,34–41] In the United States, where disease is caused by *B burgdorferi* sensu stricto, positive cultures are associated with shorter duration of the disease and smaller lesions.[35,42] Positive skin biopsy cultures in central Europe (where most of the isolates were *B afzelii*) were associated with larger lesions (up to about 15 cm in diameter) and increased duration (up to 30 days).[43] These findings are likely related to the different *Borrelia* species and the host immune response that eventually controls the infection. *B afzelii* causes slow-growing EM lesions with few systemic symptoms, whereas *B burgdorferi* sensu stricto is associated with more rapidly expanding skin lesions and more systemic symptoms.[11] Culture is moderately successful in skin biopsies of acrodermatitis chronica atrophicans lesions.[34]

Culture of 9-mL plasma samples from untreated patients with early and early disseminated infection has a sensitivity of around 40%, which can be increased to 75% by frequently testing culture aliquots with a sensitive PCR. Blood cultures are more likely to be positive in patients with multiple EM.[31,36] *B burgdorferi* is seldom cultured from the blood of patients with Lyme disease with later manifestations of the disease.[44,45] Culture of cerebrospinal fluid is rarely positive.[41,46–48] *B burgdorferi* has not been reliably cultivated from synovial fluid.[49]

There are serious concerns[50] regarding a new serum culture assay that claims a high positivity rate[51] and further validation is needed. Results from another culture assays reported as having high positivity rates in patients with chronic disease[52] could not be replicated.[53,54]

POLYMERASE CHAIN REACTION

In general, sensitivity of PCR assays for detection of *B burgdorferi* DNA directly in skin or blood samples seems similar to culture, but there is more variation because of methodology, gene targets, and primer sets used.[34–38,42,45] When optimal culture methods are used, PCR seems to be less sensitive, particularly for plasma samples, which may relate to the smaller sample volume tested in PCR assays.[36] A new assay

using broad-range PCR and electrospray ionization mass spectrometry seems promising.[55] At this point, the main use of PCR assays is for evaluating synovial fluid samples in patients with Lyme arthritis, in whom *B burgdorferi* DNA can be detected in up to 70% to 85% of patients.[42,56,57] A positive PCR does not necessarily mean an infection is active.[42] Sensitivity of PCR in cerebrospinal fluid samples of patients with early neuroborreliosis is low (10%–30%) and even lower in late disease.[58]

INDIRECT METHODS

Indirect methods detect the immune response of the host against the causative organism. Most laboratory tests performed for Lyme disease are based on detection of the antibody responses against *B burgdorferi* in serum. Antibody-based assays are the only type of diagnostic testing for Lyme disease approved by the US Food and Drug Administration.

A major problem in laboratory diagnostics of Lyme disease is the appropriate use of tests. About 3.4 million Lyme serologic tests are done in the United States every year,[59] which is vastly more than the estimated number of 300,000 cases of the disease. It is likely that tests are being used in situations for which they are not recommended, including ruling out Lyme disease in populations with a low probability of having the disease. The predictive value of a test is determined by its sensitivity, specificity, and the prevalence of the disease in the population to be tested. Consequently, in a patient with low probability of disease, a negative test rules out the disease, whereas a positive test is more likely to be a false-positive.

To improve the specificity of serologic testing for Lyme disease, a 2-tier approach (**Fig. 1**) was recommended in 1995 by the CDC.[60] The first step uses a sensitive enzyme immunoassay (EIA) or, rarely, an indirect immunofluorescence assay. If the test is negative, there is no further testing. If the test is borderline or positive, the sample is retested using separate immunoglobulin (Ig) M and IgG Western blots (WBs; also referred to as immunoblots in the literature) as the second step. The WB is interpreted using standardized criteria, requiring at least 2 of 3 signature bands for a positive IgM WB, and 5 of 10 signature bands for a positive IgG WB. The IgM WB results are used only for disease of less than 4 weeks' duration. These recommendations apply to infection acquired in the United States, because other species within the *B burgdorferi* sensu lato complex can cause disease in Europe and Asia.

The use of specialty laboratories offering nonvalidated Lyme diagnostic tests, including unique interpretation of WB results, is discouraged. They offer no documented advantage in terms of sensitivity, whereas there is a large decrease in specificity.[61] The use of antibody assays in synovial fluid is not recommended.[62] There is little published information about use of WBs in cerebrospinal fluid for the diagnosis of neuroborreliosis.

The current 2-tier algorithm works well when used as recommended, but there are many areas for improvement. Problems include the low sensitivity during early infection, subjective interpretation of bands, and confusion by health care providers and patients regarding how to interpret results.

Most assays are based on whole-cell sonicate (WCS) derived from cultured *B burgdorferi*. WCS-based assays can have a significant number of false-positive results because of the presence of cross-reactive antigens.[63] Also, proteins expressed in culture can differ from antigens expressed *in vivo*. An example is the Vmp-like sequence, expressed (VlsE) lipoprotein, which causes a rapid and strong humoral response during infection, whereas there is minimal VlsE expression in cultured *B burgdorferi*. Adding VlsE to both first-tier and second-tier tests has improved their

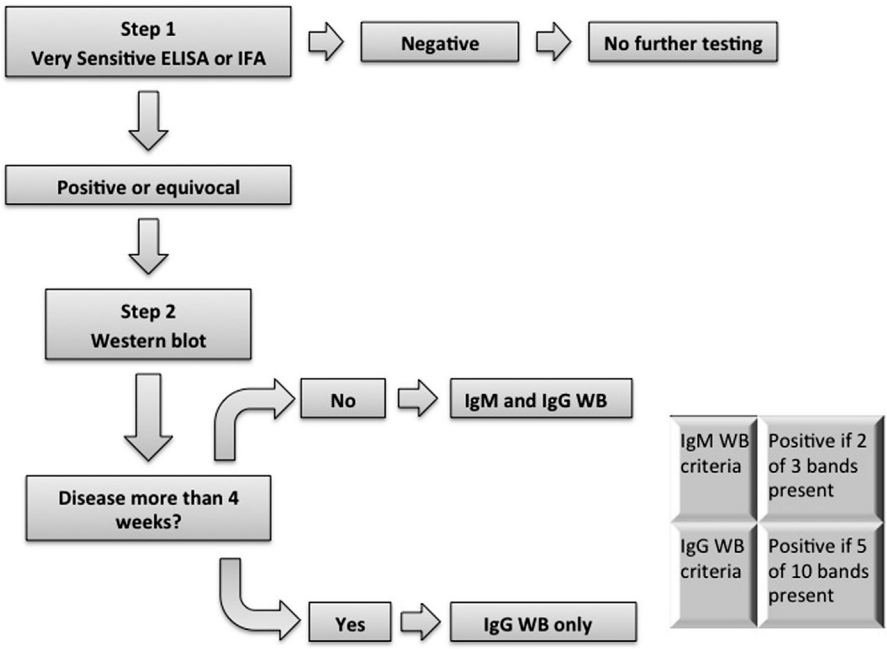

Fig. 1. Current CDC recommendations on serologic diagnosis of Lyme disease: 2-tier algorithm. Both immunoglobulin (Ig) G and IgM Western blot (WB) results are reported, but an IgM WB-positive result is only significant for patients who have been ill for less than a month. ELISA, enzyme-linked immunosorbent assay; IFA, immunofluorescence antibody assay. (*Adapted from* CDC. Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. MMWR Morb Mortal Wkly Rep 1995;44:590–1.)

performance.[64] Tests using the C6 peptide (a 26-amino acid peptide derived from invariant region 6 of VlsE) have comparable sensitivity with WCS-based EIAs, with significantly improved specificity, most markedly in patients with other diseases.[64–72] The C6 enzyme-linked immunosorbent assay (ELISA) can also be used in patients who acquire the infection in Europe, because it is able to detect antibody responses elicited by other *B burgdorferi* sensu lato species, and can be used as a stand-alone diagnostic strategy when such cases are evaluated in the United States.[70,73] A variety of other recombinant and synthetic antigens have been evaluated for use in serodiagnosis of Lyme disease, including antigens combining portions of different proteins. Conserved regions of OspC, an antigen recognized early during the course of infection by *B burgdorferi*, have been explored to develop diagnostic peptides, which are used as single-peptide or as part of multipeptide assays.[66,68,74–77]

The sensitivity of antibody-based tests increases with the duration of the infection, and there is a lag from initial infection until the time when there are sufficient levels of antibodies to be detected. Patients who present very early in their illness are more likely to have a negative result. Less than 50% of patients with EM are positive at presentation, and these patients should receive treatment based on the clinical diagnosis. Serologic tests are most helpful in patients with clinical findings indicating later stages of Lyme disease.

Fig. 2 shows how the duration of illness substantially affects the results of antibody-based tests. In a large study comparing the C6 ELISA with a WCS ELISA and the 2-tier

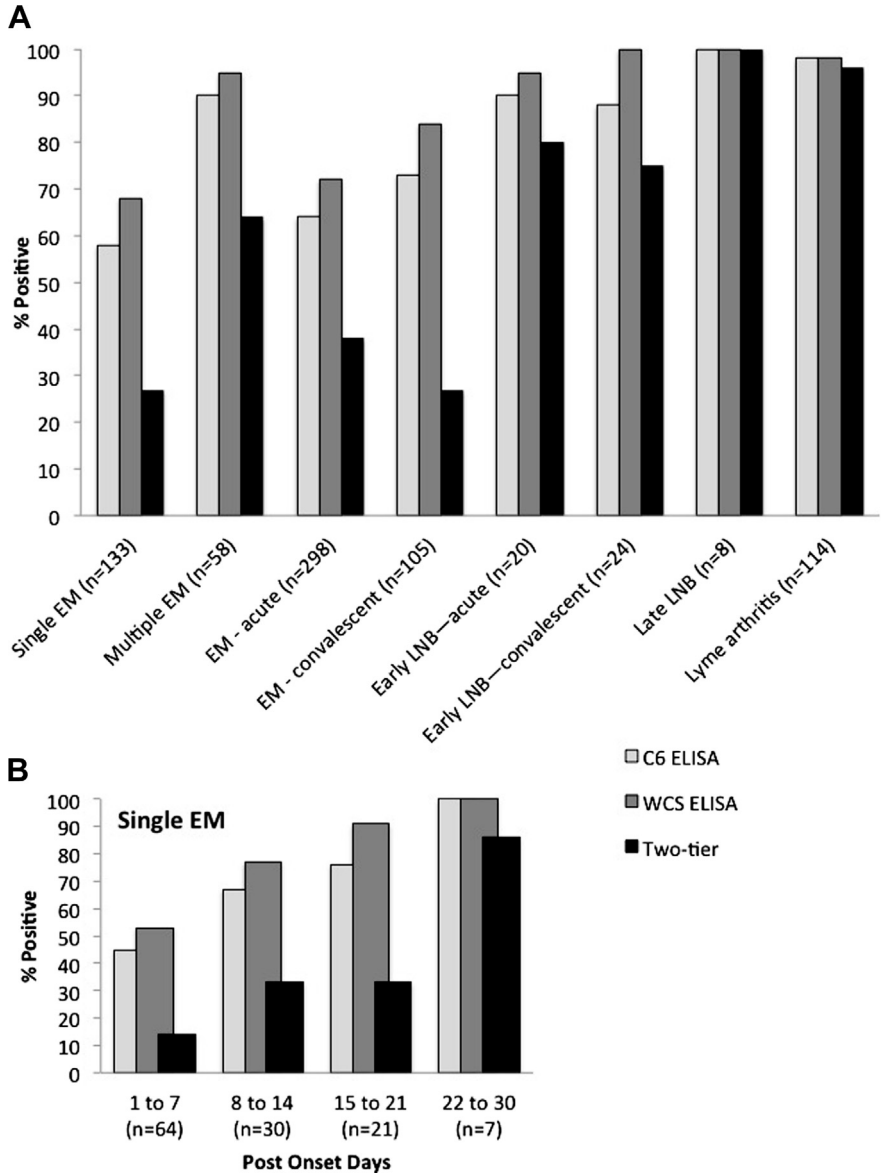

Fig. 2. Serologic results, clinical presentation and duration of illness. (*A*) Rates of seropositivity for the C6 ELISA, WCS ELISA, and 2-tier algorithm in relation to disease presentation and time of sample (acute and convalescent). (*B*) Rates of seropositivity in relation to duration of disease in patients with a single EM. LNB, Lyme neuroborreliosis. (*Data from* Wormser GP, Schriefer M, Aguero-Rosenfeld ME, et al. Single-tier testing with the C6 peptide ELISA kit compared with two-tier testing for Lyme disease. Diagn Microbiol Infect Dis 2013;75:9–15; and Wormser GP, Nowakowski J, Nadelman RB, et al. Impact of clinical variables on *Borrelia burgdorferi*-specific antibody seropositivity in acute-phase sera from patients in North America with culture-confirmed early Lyme disease. Clin Vaccine Immunol 2008;15:1519–22.)

algorithm,[69] patients with single EM lesions were less likely to be seropositive than patients with multiple EM, and patients in the convalescent phase were more likely to be positive than patients in the acute phase. Most patients with Lyme arthritis or late neuroborreliosis were positive (**Fig. 2**A). In another study,[78] less then 50% of patients with a single EM were positive by WCS ELISA or C6 ELISA and only 14% were positive by 2-tier testing when the patients were tested within the first week of illness, but the sensitivity of the tests increased with each weekly time point thereafter (see **Fig. 2**B).

As shown in many studies,[64,69,71,72,78] the additional IgM WB step decreases sensitivity in early disease, the only situation in which its use is indicated. Positive IgM results for *Borrelia* can occur in more than 40% of parvovirus B19 infections[79] and have been observed in patients with human granulocytic anaplasmosis,[80] Epstein-Barr virus infections, and patients with autoimmune diseases. In addition, false-positive IgM WBs are common in commercial laboratories,[81] and there is misinterpretation of positive IgM results in those patients with symptoms for longer than 4 weeks. Therefore, there is a need to change the testing algorithm for early Lyme disease, avoiding the use of the IgM WB. Possible strategies include the use of the WCS ELISA followed by the C6 ELISA,[71,82] the addition of the VlsE band,[64] and the use of multipeptide assays.[75,76]

Future developments that are needed include point-of-care tests. These tests would be particularly useful in evaluating patients with stage 2 manifestations of Lyme disease, like facial palsy or carditis. At present, if these patients do not have other manifestations of Lyme disease or a suggestive history, the diagnosis may depend on serologic tests results, resulting in a delay in appropriate therapy.

Current assays do not distinguish between active and inactive infection, and patients may continue to be seropositive for years, including an IgM response, even after adequate antibiotic treatment.[83,84] It is hoped that, with further studies using new, promising immunoassay techniques, a combination of multiple antigens can be developed that will help in early diagnosis, inform on the stage and disease manifestations, and on the presence of active versus past infection.[85–87]

INTRATHECAL ANTIBODY PRODUCTION

The concomitant analysis of serum and cerebrospinal fluid is used to show selective production of anti–*B burgdorferi* antibodies in the central nervous system. Measuring the antibody concentration only in the cerebrospinal fluid can be misleading, because a positive result may be caused by passive transfer of antibodies from the serum. Evidence of intrathecal antibody production is considered a gold standard for the diagnosis of Lyme neuroborreliosis in Europe, where most studies originate and where *B garinii* is the species most often associated with neurologic disease. There are many difficulties in the interpretation of results from these studies, because of the lack of a gold standard; the use of different case definitions, different assays, and interpretative criteria; retrospective evaluation; and little comparison among assays and among laboratories. Overall, the sensitivity of intrathecal antibody production in acute Lyme neuroborreliosis is around 50%.[41,46,88–95] Intrathecal antibody can persist after therapy.[96,97] Although there are few studies, positive intrathecal antibody production seems to be found less frequently in patients with neuroborreliosis in the United States.[14,27,93]

CXCL13

CXCL13 is a B lymphocyte chemoattractant chemokine that is increased in the cerebrospinal fluid of patients with acute Lyme neuroborreliosis and may be helpful in

certain clinical settings, but its diagnostic value remains to be established.[46,98,99] At this point, this test is not routinely available to clinicians.

OTHER TESTS

The clinical usefulness of cell proliferation assays, Enzyme-Linked ImmunoSpot (ELISPOT) assays, cytokine measurements, complement split products, and lymphocyte transformation tests has not been established, and these tests should not be used for the diagnosis of Lyme disease. Natural killer cell measurements (CD57) are not helpful.[100]

Xenodiagnosis, using the natural tick vector (*I scapularis*) to detect evidence of infection in Lyme disease, is an experimental test, and its clinical applications depend on the results of future studies. Although xenodiagnosis is unlikely to be used in routine practice, it can offer researchers a tool to develop new tests for the disease.

SUMMARY

Major advances in laboratory testing for Lyme disease have occurred in recent years, but there is need for further progress. Improvements of several aspects of the currently recommended testing algorithm are needed. These aspects include making the algorithm simpler, possibly as a single test or procedure; using objective, quantitative data; having greater sensitivity in early disease; and being independent of disease duration. The use of the current IgM WBs should be avoided, because it decreases the sensitivity in the clinical situations for which it is recommended (early Lyme disease), and has lower specificity than tests for IgG antibody generally. There is a need to improve direct methods for detection of *B burgdorferi*, and to develop accurate, sensitive, and rapid diagnostic tests for early Lyme disease; preferably point-of-care tests. No current test can be used to follow the response to antibiotic therapy; the development of biomarkers for active infection would be a major advance.

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