Clinical Infectious Diseases

VIEWPOINTS

Direct Diagnostic Tests for Lyme Disease

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Borrelia burgdorferi was discovered to be the cause of Lyme disease in 1983, leading to seroassays. The 1994 serodiagnostic testing guidelines predated a full understanding of key *B. burgdorferi* antigens and have a number of shortcomings. These serologic tests cannot distinguish active infection, past infection, or reinfection. Reliable direct-detection methods for active *B. burgdorferi* infection have been lacking in the past but are needed and appear achievable. New approaches have effectively been applied to other emerging infections and show promise in direct detection of *B. burgdorferi* infections.

Keywords. Lyme disease; *Borrelia burgdorferi*; tests; PCR; high-throughput sequencing.

Lyme disease is the most common vector-borne illness in the United States and Europe. *Borrelia burgdorferi* was identified as the etiologic agent of Lyme disease in 1983 [1, 2]. In 1994, a 2-tiered antibody testing strategy was adopted in which a first-tier test (usually enzyme-linked immunosorbent assay) was designed to be very sensitive in detecting new cases, and a second-tier test (Western blot) was intended as a more specific confirmatory step to rule out false positives [3]. However, serologic tests have been hampered by technical and biological shortcomings, the greatest being a time lag for the host to make detectable antibody by current tests. The other biologic hurdle is that a single antibody test can only indicate exposure, not active infection [4] or reinfection. The development of sensitive tests capable of directly detecting the organism in body fluids has proven challenging.

Borrelia burgdorferi infection begins as a local infection at the tick bite site. Hematogenous dissemination occurs in early disease in many, but not all, cases. However, even when it occurs, only low and transient levels of the bacteria are found in the blood [5]. Later, they have a predilection for certain tissues (heart, nervous system, joints). Nucleic acid amplification test (NAAT) methods that are efficient for the detection of other infections in blood have largely been ineffective for *B. burgdorferi* detection without measures to enhance their sensitivity.

Advances in molecular diagnostics over the past few years have led to the application of new technologies for many emerging infectious diseases. Now, soon after an emerging infection appears, diagnostic tests can be rapidly developed to detect the infection in patients, as well as monitor its incidence in the population. First-generation molecular tests can be quickly refined to attain higher sensitivity and specificity. Recent examples include the emergence of accurate diagnostics for severe acute respiratory syndrome–coronavirus, Middle East respiratory syndrome–coronavirus, Zika infection [6], and even 2 newly recognized tick-borne borreliae by *Borrelia mayonii* [7] and *Borrelia miyamotoi* [8]. Progress in our understanding of the biology of Lyme disease is allowing for the exploration of these promising new technologies in direct-detection diagnostics of the disease. As in any infection where there are low microbe copies, enhancement or enrichment methods will also be needed.

This article was developed after a September 2016 meeting at the Cold Spring Harbor Laboratory Banbury Center and subsequent discussions to assess current and potentially new laboratory tests for the diagnosis of Lyme disease. The participants were from industry, academia, and government, with extensive experience in clinical and laboratory aspects of Lyme disease and other infectious diseases, as well as participants with

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regulatory experience in the clearance of diagnostic tests. The emphasis of the meeting was on diagnostic testing platforms for early Lyme disease, since current serologic tests are insensitive during the first several weeks of infection. There was no intent to take a vote, advocate for one test product over another, or reach a consensus during the meeting; rather, there was discussion of research findings that support or challenge particular diagnostic concepts. What emerged was a recognition that improved approaches to serologic testing were now available [4] and that the technology for direct detection of bacterial proteins or DNA has advanced to the point that it is evaluable in Lyme disease. If the technology meets scientific rigor, these tests could become future diagnostic assays.

A wide range of direct diagnostic testing methods was discussed. The focus was on methods and approaches that may have practical use in the laboratory diagnosis of Lyme disease now and in the near future. Distinctions were made between assays that are currently available and being used for other infections and those that are in the developmental stages [9].

CURRENT APPROACH TO LABORATORY TESTING FOR LYME DISEASE

Serologic assays are the most frequently used and familiar tests for the laboratory diagnosis of Lyme disease. At present in the United States, all tests currently cleared for diagnostic use by the US Federal Drug Administration (FDA) are serologic assays. The current guidelines for serologic testing were adopted in 1994 [3]. The challenge to the 1994 Dearborn Conference [3] participants was to develop testing and interpretive guidelines that would standardize serologic testing for diagnostic purposes. The guidelines are geared to assess exposure to *B. burgdorferi* through the patient's antibody response to infection rather than direct detection of nucleic acid or protein from the microbe. The limitations of serologic testing and advances were recently reported in detail [4].

NEW METHODS ARE NEEDED

There is a lag period in most infections, and with *B. burgdorferi*, up to 3 weeks may be needed from infection to B-cell production of pathogen-specific antibody in sufficient quantity to be detectable by current serologic assays. Also immunoglobulin M reactivity is fraught with issues that affect utility. Culture of the pathogen, microbial nucleic acid, or protein may be detected in skin biopsies of erythema migrans (EM) before the development of a positive serology. However, culture is impractical for routine clinical use. It requires specialized media; *B. burgdorferi* grows slowly, requiring weeks before it comes detectable. Although improvements in serologic assays are foreseeable, current assays utilize antigen targets identified before a thorough understanding of the expression of the antigens or their epitopes. Key early antigen targets were missing from the assays, contributing to insensitivity of serology in early infection. Also, cross-reactive epitopes are found in virtually all of the antigens used in the currently approved seroassays.

ADVANTAGES AND LIMITATIONS OF DIRECT-DETECTION METHODS (*BORRELIA* PROTEINS AND DNA BY POLYMERASE CHAIN REACTION ASSAYS)

General

Early manifestations may range from asymptomatic without a known tick bite (impractical to test) to asymptomatic with tick bite, to nonspecific symptoms, to specific ones such as EM [2].

Serologic tests detect exposure to a pathogen indirectly via quantification of a specific antibody in blood, independent of whether infection is past or current. In contrast, direct tests are designed to detect the etiologic agent itself, or components of it. Such a test could be advantageous in the diagnosis of early Lyme disease. In the absence of EM, the signs and symptoms of Lyme disease are too nonspecific to be clinically diagnostic. In a patient who has the viral-like symptoms of early Lyme disease without EM, serologic tests may not yet be positive. A direct test could provide a definitive indication of active *B. burgdorferi* infection. Later in the disease, direct-detection methods may help confirm the presence of the pathogen.

Cultures as Direct Detection Are Time Consuming and Not Practical for Routine Use

Culture of *B. burgdorferi* requires special media. The organism grows slowly, taking up to 12 weeks before being seen [10], and has relatively low sensitivity. Culture continues to be a research tool as culture-confirmation techniques are beyond the capabilities of most clinical laboratories.

Detection of Bacterial Antigens

Antigen-capture assays provide one means of direct detection. Bacteria often shed or secrete antigens that can be detected in body fluids. For example, legionellosis, cryptococcosis, aspergillosis, and dimorphic fungal infections can be diagnosed using antigen tests applied to urine, blood, bronchoalveolar lavages, or cerebrospinal fluid. A direct test for Lyme disease is needed, but a sensitive and specific antigen-capture assay has been elusive. Even if the antigen is known to be present in a sample, past attempts to develop an antigen-capture assay had poor specificity, low sensitivity, and uncertainty about the choice of the capture antigen target [11].

The specificity and sensitivity of an antigen-detection assay is largely a function of the choice of the capture and reporter antigenic targets, as well as the affinity of the antibody used to capture or detect it [12]. Depending on the duration of the infection, the *B. burgdorferi* antigenic repertoire changes. In addition, because of the high sequence variability between *B. burgdorferi* strains, identification of conserved antigenic epitopes that span all strains at all times has been challenging

across genospecies. One can select 1 or more appropriate target antigens for any genospecies. Even OspC, known for its variability, has at least 2 well-conserved epitope targets found across the various serotypes.

Earlier attempts to create antigen-capture assays may have been hindered by lack of data for optimal selection of targets and antibodies [11]. Now, with advances in proteomics, specimen processing, and mass spectrometry, identification of pathogen-specific antigens is possible [13] and represents a path forward. Although some large commercial laboratories use mass spectrometry for diagnostic purposes (not Lyme disease), this methodology can be used to identify and validate the specific antibody–antigen complex so that simpler assays can be developed for use in routine laboratory settings or perhaps even closer to the point of care. In addition, emerging antigen concentration and enrichment methods [14, 15] have the potential to address another likely limitation—*B. burgdorferi*-specific antigens are probably in very low abundance in body fluids. Throughout the *B. burgdorferi* infection, there are few circulating organisms, and the organism burden in affected organs is low. Concentration methods can be categorized as follows: removal of solute or water [16], electrophoretic or chromatographic separation [17], and affinity capture [18–20]. Along with target enrichment, these methods may require exclusion of masking proteins such as common abundant proteins (frequently a preparative step prior to mass spectrometry detection of proteins).

New developments to stabilize proteins in samples prior to analysis may be suitable for several assays [14].

Detection of DNA: Nucleic Acid Amplification Tests

For infectious agents that are difficult to visualize or cultivate, nucleic acid amplification tests (NAATs) can identify specific pathogen DNA. Many pathogens are easily detected using NAATs, especially with high enhancement or enrichment methods when present. Many polymerase chain reaction (PCR) assays have been geared to analyze small volumes of blood, plasma, or other sample types. In Lyme disease, outside the EM in skin, the infectious agent resides in very low numbers in tissue and body fluids, and standard PCR methods, as applied to *B. burgdorferi* detection, have suffered from poor sensitivity, especially in blood and cerebrospinal fluid [21–25]. An illustrative strategy to increase PCR sensitivity without diminishing specificity is to start with a larger specimen volume and/or use target enrichment methods [26]. It appears possible to detect *B. burgdorferi* in the blood with higher sensitivity [27] in early Lyme disease. In addition, there are a variety of PCR-based assays in development that use unconventional signal detection or amplification methods [27, 28], with the potential to achieve a limit of detection (LOD) that is substantially lower than with standard PCR methods.

Today's advanced direct-detection diagnostic tools were not available at the time of the 1994 Dearborn recommendations. In the ensuing years, PCR and high-throughput sequencing have matured and are now routinely used to detect many new pathogens [29]. Such techniques can be applied successfully to Lyme disease diagnostics. For an improved Lyme disease assay, performance should be equal to or better than the existing serologic 2-tier enzyme immunoassay followed by Western blot. The advantage of a NAAT is that it is a measure of the presence of the microbe and therefore active infection, especially when found in circulating fluid, instead of host antibody responses to *B. burgdorferi*.

PCR strategies have performed well in blood tests for causative agents of sepsis [30] and Ebola [29]. These infections have many genomic copies detectable by methodology geared to small volumes of blood or plasma. In contrast, Lyme disease has very low microbe numbers in the majority of clinical samples. With enhancement and enrichment methods, detection of *B. burgdorferi* in the blood is increasingly possible. Multiple displacement amplification [26] and similar strategies in which DNA is exponentially amplified by isothermal amplification increases the yield of *B. burgdorferi* by at least 200 times above normal [27]. This makes a PCR detection assay far more sensitive. In other studies, the sensitivity of PCR-based assays was increased, on the order of 16 000-fold, by combining 3 consecutive methods [27]. Similar to the approach used for blood cultures for sepsis where multiple large volumes of blood are taken, the first step was to start with a larger volume of whole blood, 1.25 mL (more than 0.5 mL of plasma or serum are often requested by clinical laboratories). The second step was to increase the relative content of *B. burgdorferi* compared to human or other microbial DNA by using targeted isothermal amplification. In vitro experiments resulted in a 200- to 2000-fold increase in the targeted DNA. In a set of clinical EM samples, the number of cases detected was 7-fold greater than without this step. The third step that increased sensitivity with 100% specificity was the use of multiple primers. It should be noted that there appear to be skin-restricted genotypes, which may be the reason for negative results in blood. We should also consider enrichment or concentration of a pathogen to increase the target concentration for nucleic acid extraction followed by PCR analysis.

An additional hopeful point for future acute NAAT development is that some acute Lyme disease studies have documented that the number of organisms or their DNA in the blood may have been higher than appreciated [5].

Genomic Sequencing

High-throughput sequencing (HTS), also known as next-generation sequencing (NGS) [31], has been a breakthrough method for identifying and characterizing nucleic acids from diverse microbes. However, *B. burgdorferi* is particularly challenging, in part, because it is present in low abundance in circulating fluids and because it has an unusual genome [32]. The approximately 1.7 Mb *Bbl.* genome is AT-rich (approximately 30%), and nearly half of it consists of linear

and circular plasmids. *Borrelia burgdorferi* isolates an average of 17 such plasmids, with up to 21 for the B31 type strain [33]. Thus, *Borrelia* genomes are among the most complex and challenging bacterial human–pathogenic genomes to sequence and analyze. These difficult-to-sequence plasmids carry antigen-encoding genes and are often required for propagation of the bacteria in ticks and/or mammals. The recent development of long-read sequencing technology [34] (eg, Pacific Biosciences), in combination with the use of HTS technologies with relatively very low sequencing error rates (eg, Illumina Novae or MiSeq platforms) [34–36], has allowed resolution of the repeated sequences that are the hallmark of *Borrelia* genomes and of other sequencing challenges commonly found in the critically important Borrelia plasmids.

In addition to its use in characterizing the *B. burgdorferi* genome, NGS could potentially be used as a direct-detection method in clinical diagnostics [37]. Targeted deep sequencing (eg, enrichment for desired genomic regions prior to sequencing via either hybridization or PCR amplification techniques) is proving to achieve adequate sensitivity; emerging approaches that sequence cell-free DNA from plasma show promise [38]. Sequencing also allows for a much better resolution of species and strains, either through multilocus sequence typing–like sequencing strategies or whole-genome sequencing approaches [39].

The rapidly decreasing cost to perform sequencing may make this a viable and high-throughput (multiple samples characterized at the same time) method for detection in the clinical laboratory when appropriate sensitivity can be established. Challenges remain, however, including our incomplete knowledge of the full breadth of *Borrelia* genomic diversity, that is, of the genes that might be shared by all isolates (also called the "core genome") and those that might be unique to specific species (also called the "accessory genome") [32]. Without this critical knowledge, it would be challenging to determine precisely which genes or antigens should be targeted by a selective diagnostic test.

PCR and HTS technology have value in authenticating that a sample is actually infected with the organism. This proof of infection is valuable for any assay development including improved serology and research.

It is assumed but yet to be proven that there is a preferential window of nucleic acid detection in blood at the earliest points of the infection, well before serological assays tend to be positive. It may be that in the untreated patients, *Borrelia* DNA may be detected with deep sequencing or hybrid targeted enrichment or background subtraction [38].

It is important to realize that certain molecular techniques may be too expensive for routine clinical use, at least currently, and may require curated genomic or proteomic databases of higher quality than those currently publicly available. Other limitations of genomic testing, particularly on a large scale, require attention to the risk of exogenous contamination; degradation of the nucleic acid during transport to the laboratory; complexity of the technique, which can make the testing of hundreds of samples in 1 day more difficult; and cost-benefit factors. However, using currently available tools, researchers should be able to help define the parameters, such as the optimal nature and required volume of the sample, the degree of targeted amplification, sequence capture, host background suppression needed, and the LOD that must be achieved. This knowledge could help drive the development of economically viable assays that could eventually go through the regulatory review process for routine clinical use. It will require concentrated research followed by product development and regulatory approval before robust, affordable, and reliable direct-detection assays are available for routine diagnostic use.

As with any new or improved assay that one is contemplating for FDA clearance, it is advisable to initiate early, direct communication with FDA, including participation in the Qsub program, during which a developer may receive assay-specific feedback [40].

CONCLUSIONS

New technical advances and knowledge demonstrate that direct tests for early active infection are ready for practical assessment in Lyme disease. Future tests are also likely. When testing guidelines for Lyme disease were adopted in 1994, they were intended to make the best use of available assays, which were antibody tests that provided indirect evidence of infection. Although the guidelines improved standardized serologic testing, aspects are amenable to improvement in light of new data and technologies. Continued reliance on serologic testing for early Lyme disease is suboptimal given the common 2–3 week seronegative window with current methods and the need for a biomarker of active infection.

The goal of an active-infection diagnostic test is now technically achievable. It can be evaluated for practical performance in clinical settings (Table 1). Direct nucleic acid and protein detections would complement improving indirect serological tests for more comprehensive diagnosis of Lyme disease.

Table 1. Characteristics of an Ideal* Direct Test for *Borrelia burgdorferi* Infection (Lyme Disease)

High sensitivity and specificity soon after a tick bite and/or infection at or before the time of symptom onset
High sensitivity and specificity in later stage of disease when extracutaneous infection has been established
Short turnaround time (within 24 hours)
Applicable to easily obtained sample types such as blood, urine, and saliva
Nonreactive when active infection is absent

*Distinct but complementary molecular and serological approaches may be necessary.

Notes

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