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TICK-BORNE DISEASES EDUCATION

Applying Basic Concepts in Laboratory Testing to Serologic Testing for Lyme Disease

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The earlier that Lyme disease is diagnosed, the easier it is to treat. Missing the diagnosis can result in significant complications so it is important that all potential Lyme patients be identified; to do this requires a highly sensitive test. Yet, the inappropriate use of antibiotics can also be harmful so it is important to only treat patients who have Lyme; this requires a very specific test. Thus, Lyme is an illness where sequential testing, such as the CDC's two-tier protocol, makes sense.

Unfortunately, what looks good in theory falls apart in practice because serologic testing for Lyme disease is often inaccurate. This happens because both steps in the protocol – ELISA and Western blotting – lack sufficient sensitivity and their results are not highly reproducible. In addition, the tests on the market are FDA-cleared, not approved, and the distinction has real-world consequences.

In order to understand the pitfalls of serologic testing, it's important to know how these tests work individually, and in the CDC's two-tier protocol. ELISAs (or EIA) and Western blots for Lyme disease are indirect indicators of the infection because they measure antibodies to *Borrelia burgdorferi* and not the bacterium itself. Antibody production is a dynamic process and a single sample may not accurately reflect the clinical picture due to timing issues and patient variables. This means that while positive results are indicative of prior exposure to *B. burgdorferi*, they alone are not proof of a current infection. And, as will be discussed later, negative results are not proof that *B. burgdorferi* is not present.

General Characteristics of Diagnostic Tests

The goal of diagnostic testing is to clarify clinical uncertainty regarding potential diagnoses. Test selection requires an understanding of a test's underlying characteristics and should consider how results will be used.

Diagnostic tests can be described by several distinct characteristics – sensitivity, specificity and reproducibility. Sensitivity is the ability of a test to detect all of the patients who have the disease while specificity is the ability of the test to identify all those who do not have it. An ideal test would be highly sensitive and specific; unfortunately, ideal tests are rare.

Accurate tests give results that are true – a positive result is indicative of disease and a negative result means the disease is not present. Accuracy is directly related to a test's sensitivity and specificity. For positive results to be true, the test must be specific and for negative results to be true, the test must be sensitive.

The sensitivity and specificity for a given test are correlated and both values are cut-off dependent. Once a cut-off value for a positive test is set, results falling on one side are positive and those on the other side are negative. It's impossible to establish the perfect cut-off for a test. Although some cut-offs are clearly better than others, there will always be false positives and false negatives. The chosen cut-off ultimately favors the production of one over the other, and, subsequently, either raises sensitivity or specificity. We can think of sensitivity and specificity as opposite ends of a teeter-tooter, with the cut-off value acting as the fulcrum.

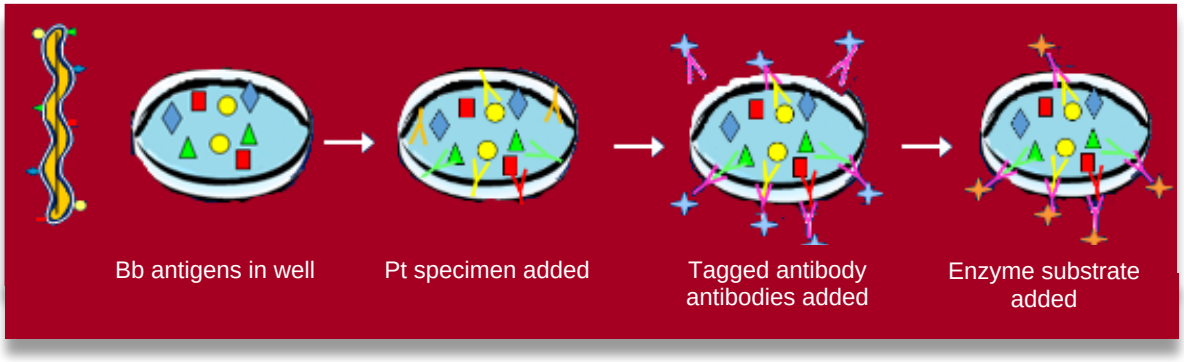
If the cut-off is shifted to raise sensitivity, then the specificity of the test will fall. And, when the cut-off favors specificity, then sensitivity fall.

Reproducibility or precision describes how likely it is that a test will give the same results on repeat testing. When a test is imprecise, this shortcoming undermines confidence in all of the results it produces.

Finally, an important principle in lab medicine is to select tests based on their intended use, which means that different needs require different tests. For example, epidemiologists who are tracking disease trends, need to avoid inadvertently including patients with other diseases in the study group. This requires using diagnostic tests with high specificity. In contrast, clinicians want to avoid letting treatable cases go undiagnosed, therefore they require tests with high sensitivity.

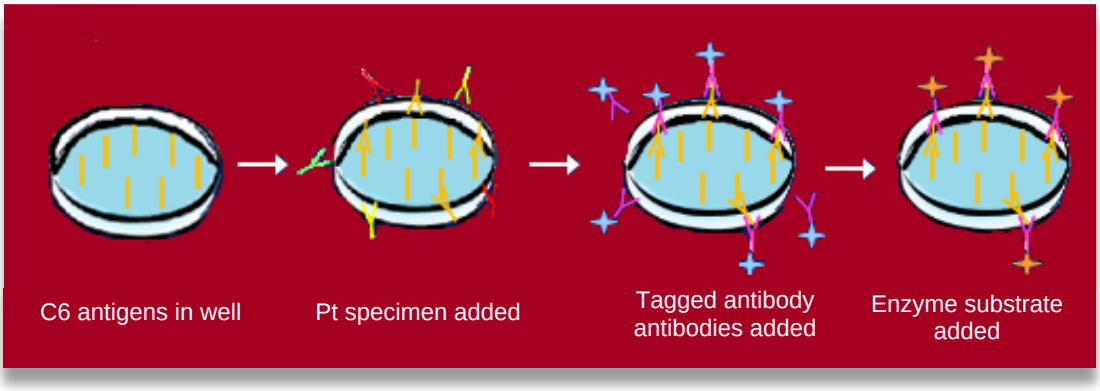
ELISA Tests

The original whole cell sonicate test is shown in Diagram 1. *Borrelia burgdorferi* is broken into individual antigenic components via sound waves and the components are adsorbed onto the test well. The patient specimen is added and allowed to incubate with the antigens so that free antibodies to the bacterium have time to bond with their matched antigens. The well is washed to remove unbound patient antibodies and tagged anti-human antibody antibodies are added and allowed to incubate. These secondary antibodies are conjugated to a substrate-specific enzyme. The unbound secondary antibodies are washed away and the substrate is added. The substrate is converted by the enzyme on the secondary antibody to produce a color change that can be read by a spectrophotometer.



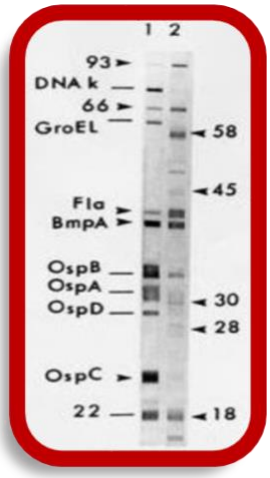
Other ELISAs use purified or synthetic/recombinant antigens. Diagram 2 depicts the C6 ELISA. The procedure mirrors that of the whole cell sonicate except that the C6 ELISA uses a single synthetic protein as its antigen. This protein mimics a specific portion of *B. burgdorferi's* VlsE antigen called invariable region 6. Multiple copies of the antigen are embedded in the well and patient blood is added just as it was in the whole cell sonicate test. Although other antibodies to *B. burgdorferi* may be present, there won't be anything for them to attach to so they'll get washed away, leaving only the antibodies to the C6 antigen for measurement.

Diagram 2: C6 ELISA



ELISA tests measure all antibodies as a single group. Dozens of ELISA test kits from various manufacturers have been cleared by the FDA. These tests vary in terms of their sensitivity and specificity. The majority of tests measure either IgM or IgG antibodies to the test antigens as a single group. In contrast, the C6 ELISA measures the total of IgM and IgG antibodies to a single antigen.

Western blots



http://www.cdc.gov/lyme/diagnosistesting/LabTest/TwoStep/WesternBlot/index.html
accessed 7/19/18

Western blots provide a different perspective on the antibody response. These tests identify which specific anti-Borrelia burgdorferi antibodies are present in a patient specimen but do not provide a quantitative measurement of the antibodies. Western blots look for one immunoglobulin class, IgM or IgG, at a time.

In this test, *B. burgdorferi* antigens are placed on a gel and an electrical current is used to separate them by weight, forming a ladder-like antigen array. The antigens are then transferred to a membrane while preserving their gel alignment. Patient specimens and controls are added and allowed to incubate so that antibodies present in the samples can bond to their corresponding antigens. Conjugated secondary antibodies are added and allowed to incubate. A substrate is added which allows the antigen-antibody bands to be visualized. There are dozens of potential bands and the intensity of the individual band is scored as "0", "indeterminate", or 1-4. The positive bands produced on Western blots can be interpreted in many ways and several interpretation schemes have been used in the past. These schemes differ over the relative importance of individual bands and their relative sensitivity and specificity for identifying cases of Lyme disease.

Western blotting is a detailed, technician-dependent procedure. Getting the antigens to separate well can be tricky and determining whether a band is present, and its intensity are subjective calls. Looking at the Western blot shown here, some bands are very dark and wide making them easy to read while others are very faint. It's easy to see how different technicians might read the blot differently. Some labs use optical scanners to perform this step but scanners have their own set of problems. The procedural concerns raise the possibility that the test's performance could be influenced by the technician's level of experience with Western blots. Another drawback of Western blots is that the turnaround times for results are fairly prolonged. Although most labs use test kits that come with prefixed antigens to avoid some of the technical problems, others perform the entire process in-house.

As previously noted, several Western blot interpretation criteria, with varying sensitivity and specificity have been proposed. The interpretation criteria used in the CDC surveillance case definition were adopted at the Second National Conference on Lyme Disease Testing which was held at Dearborn, MI, in 1994.[1]

The IgM criteria were based on a study by Engstrom and colleagues and require the presence of at least 2 of 3 antibodies to the 23-25kd, 39kd and 41kd.[2] The IgG Western blot criteria were based on a study by Dressler and colleagues. In the Dressler study, the researchers identified the 10 most commonly seen antibodies in a relatively small group of Lyme disease patients.[3] These antibodies were to the 18kd, 23-25kd, 28kd, 30kd, 39kd, 41kd, 45kd, 58kd, 66kd, 93kd antigens. They then carried out a detailed statistical analysis to determine how many bands needed to be present in order to achieve a specificity of ≥ 95% without drastically reducing sensitivity, thus arriving at the minimum 5 of 10 band criteria.

It is important to note that antibodies to the 31kd and 34kd antigens are not included in the CDC's group of 10 antibodies. These antibodies tend to occur later in the infection, which may explain why they were not seen more frequently in the Dressler subjects. However, they are highly significant indicators of Lyme disease when both appear on a Western blot.[4] Additionally, the CDC interpretation criteria fail to account

for differences in banding patterns between various *B. burgdorferi sensu lato* species and strains, including common European species.

ELISA and Western Blot Sensitivity and Specificity

The sensitivity of ELISA and Western blot testing is strongly affected by timing issues. The process of antibody formation develops over weeks, infected people who are tested too early in their infection will produce falsely negative results. This is not the fault of the tests per se because they can't measure what isn't there.

False negatives may also arise when people are tested too late in the disease. Individual case reports suggest that the immune response to *B. burgdorferi* wanes over time such that infected patients, even those who were never treated for the infection, may have negative results. In a human observational study of Lyme encephalopathy, 17% of the subjects were had negative serologic results and their enrollment was based on other parameters.[5] This phenomenon of seronegative late disease is well documented in nonhuman primates infected with *B. burgdorferi*. Untreated animals were originally C6 ELISA positive but over the next two years became C6 negative even though bacteria were readily apparent when the animals were sacrificed and their tissues examined for *B. burgdorferi*.[6] For obvious reasons, a similar study hasn't been undertaken in humans. The symptoms of disseminated Lyme disease are generally nonspecific and often wrongly attributed to other causes. Thus, many patients go undiagnosed for several years. For those with a waning immune response and subsequently negative serology, it makes it that much more difficult for clinicians to piece together the correct diagnosis – Lyme disease.

In some instances, the administration of antibiotics very early in the infection can result in actively infected patients who are negative on serologic testing.[7] The hypothesis to explain this phenomenon is that a significant reduction in the bacterial load leads to a premature halt in the antibody formation process. In other instances, antibodies may be present but unmeasurable because they are bound to bacterial antigens and not free to interact with test antigens.[8]

These are some of the papers documenting the insensitivity of ELISA and Western blotting for Lyme disease.[9,10,11,12]

Although the IgG Western blot criteria was 99% specific in Dressler's retrospective study, his prospective study, using his well-characterized patients, demonstrated that the sensitivity of the 5 of 10 criteria varied by clinical presentation.[3] The criteria identified up 96% of the patients with Lyme arthritis but missed 28% of the patients with active neuroborreliosis. Of note, the C6 ELISA also appears to be more sensitive for detecting Lyme arthritis than neuroborreliosis,[13] suggesting that the antibody response varies by clinical presentation.

Although ELISA and Western blot tests are generally highly specific, false positive do occur Some antibodies to *B. burgdorferi* are cross-reacting, meaning that their production may have been due to exposure to a different infectious agent and not *B. burgdorferi*. For example, antibodies to Epstein Barr virus or cytomegalovirus can cross-react with the antigens used in Lyme disease tests.[14] Additionally, antibodies to *B. burgdorferi* can linger for a long time after the infection has cleared. The duration of the antibody response is highly variable and impossible to predict for a given individual.[15] With regard to patients who were recently treated and who are currently asymptomatic, positive results are more likely to reflect the prior infection and not ongoing infection. That said, it seems prudent to consider positive results in those who remain symptomatic as potentially indicating ongoing disease.

Clinical Validity of ELISA and Western Blot Tests

The procedures for creating and performing ELISAs and Western blots have not been standardized. Of the more than 70 ELISAs and Western blot tests sold in the US, none are FDA-approved. The tests are merely FDA-cleared and the distinction is important. Approved tests need to demonstrate clinical validity, that they

actually work in clinical situations, but cleared tests only need to demonstrate that they're comparable to what's on the market. Many newer tests claim very high sensitivity based on how they perform against reference panels. However, many of the samples in those panels were chosen on the basis of their having produced positive results on other serologic tests. Their inclusion results in a preselection sample bias towards positive results so the reported sensitivity should be viewed with some skepticism.

Reproducibility

The other issue that bears on the overall accuracy of the tests is their tendency to be imprecise, such that results on the same specimen often disagree with each other. Western blotting is especially prone to imprecision. For example, one paper reported on Western blot sensitivity as performed by a single lab using three different IgG Western blot kits and known reference samples.[16] The documented sensitivity ranged from 43.6% - 74.3%.

CDC's two-tier protocol

The concept of applying sequential testing to Lyme disease makes sense but in reality, this approach compounds the problems that occur with ELISAs and Western blots. Although Western blot specificity is quite high, 98% or higher, the overall sensitivity of two-tier testing is no more than 70% -80%. And, in some settings, far less. Neuroborreliosis can be especially difficult to diagnose clinically because the signs and symptoms of late neurologic disease are highly variable and often overlap with symptoms from other illnesses. Unfortunately, the two-tier approach may offer little help to clinicians.[17] In the study by Bacon and colleagues the C6 was 73% sensitive and as discussed earlier, the 5 or more of 10 IgG Western blot interpretation criteria is only 72% sensitive. This yields a two-tier sensitivity of 53%.

The study by Ang and colleagues highlights several concerns regarding two-tier testing.[18] Samples from 31 well-characterized cases of highly suspected Lyme disease were tested with eight different ELISAs and five different Western blots, producing 40 distinct two-tier combinations. Some of the results were surprising. For example, many Western blots were more sensitive than the ELISAs. And, the lack of agreement between IgG Western blot results were quite significant. Ultimately, the authors concluded that the choice of the ELISA-Western blot combination highly influenced the ability to obtain positive results. In other words, whether or not a patient with Lyme disease meets the lab criteria for it has less to do with their infection status and more to do with which test combination was used. Given that clinicians can't control which test manufacturers their lab uses, findings from this paper suggest they should be wary of the results, especially those that are negative.

In 2019, the CDC accepted a modified two-tier testing approach using a sequence of 2 ELISA tests that were specifically designated for this purpose.[19] This approach is said to offer improved sensitivity for detecting cases of early Lyme disease while maintaining high specificity.[20] Whether this approach holds up in large-scale clinical use remains to be seen.

It is important to recall that the two-tier laboratory criteria adopted by the CDC at the 1994 conference were chosen on the basis of specificity so that they could be incorporated into the surveillance case definition of Lyme disease.[1] Surveillance case groups are very select; every effort is made to exclude from the group all who do not have the illness. Epidemiologists desire to follow an illness over time, by geographic region and by treatment outcome; surveillance groups make this possible. However, as a consequence of the selectiveness of the surveillance case definition for Lyme disease, especially the lab criteria, many people who are actively infected will not satisfy the definition, which is why the CDC and the Council of State and Territorial Epidemiologists has repeatedly stated that the surveillance criteria were not intended to be used for clinical diagnosis.[21]

It is possible for both epidemiologists and clinicians to use Western blots to identify their patients of interest. Referring back to the teeter-totter concept of sensitivity and specificity and using antibody band

interpretation criteria as the fulcrum, the two groups simply need to select criteria that shift the fulcrum in the appropriate direction for their specificity/sensitivity needs. Substituting other Western blot interpretation criteria for the CDC's, as others have suggested,[22,23] might provide the sensitivity clinicians need without forfeiting too much specificity.

Summary

Serologic testing for Lyme is inaccurate. While the inadequate sensitivity of ELISA and Western blot tests is the primary problem, imprecision and the lack of clinical validity contribute to the poor performance of two-tier testing in clinical settings. Although the high specificity of the CDC two-tier strategy works well for epidemiologic purposes, the testing sequence reduces the overall sensitivity, thereby limiting its clinical effectiveness. While positive results on two-tier testing in an untreated patient who has symptoms of Lyme disease would confirm the clinical diagnosis (and it would be a mistake to label such results as "false positives"), negative results do not rule out Lyme disease.

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