Many diagnostic tests are based on the detection of specific antibodies in the blood of patients suspected of having a given infection. Such tests, especially those that have survived years of rigorous scrutiny, have been validated and approved for clinical use by the Food and Drug Administration (FDA) and endorsed by the Centers for Disease Prevention and Control (CDC). Thus, antibody-based tests have been invaluable in the control, diagnosis, and treatment of many infectious diseases. Notwithstanding, certain variables and limitations must be considered in evaluating the results. In this presentation, these issues will be considered with respect to the diagnosis of Lyme disease that has been defined as a tick-borne infectious disease caused by the spirochete, *Borrelia burgdorferi* (1).

Although sensitivity and specificity are decisive factors in judging the value of any antibody-based diagnostic test, several variables influence the assessment of each of these parameters. Of primary concern is the minimal amount of antibody that must be present in a test sample to give a positive test result. Here, an understanding of the concept of “antigen load” is useful. This refers to the least amount of antigen required for the immune system to produce a detectable antibody response.

The term “antigen” refers to any substance that stimulates the immune system to make antibodies in response to that antigen. For *B. burgdorferi*, this consists of an array of several well-characterized molecules that are present on its cell surface in extremely small amount. Some are proteins or lipoproteins that are specific for *B. burgdorferi* and may play a role in pathogenesis (2); such antigens are considered to have potential diagnostic significance. Others, antigens (e.g., flagellar antigens) are nonspecific for *B. burgdorferi* since they are also present on unrelated nonpathogenic bacteria. Antibodies against these shared or nonspecific antigens certainly are produced during Lyme disease; however, their presence alone is not diagnostically relevant since they are not produced only during the course of Lyme disease.

There is abundant evidence to indicate that the magnitude of an antibody response is dependent upon the amount of antigen available to stimulate
the immune system. As the amount (dose) of antigen is increased (antigen load), more antibody is produced in a dose-dependent manner until a peak level is attained. It is relatively easy to determine the optimal dose required to generate a detectable antibody response against an isolated purified antigen or a vaccine; however, it is much more difficult to do so when antigen is delivered in the form of live bacterial cells and when the total amount delivered is dependent upon the extent to which bacterial cells multiply (replicate) during the course of infection.

In contrast to rapidly growing bacteria such as Escherichia coli or Staphylococcus aureus that replicate by cell division once every 17-30 minutes (3), B. burgdorferi replicates much more slowly -- once every 12-24 hours (4). Although detectable amounts of antibody may be present in the blood within a few days after infection with rapidly growing pathogens, it may take several weeks before detectable amounts of antibody appear in the blood of patients infected with slow-growing B. burgdorferi. This influences the sensitivity of a diagnostic test and is a major limitation as to when, during the course of an infection, a given antibody-based tests can be used for diagnostic purposes, regardless of specificity.

The “bull’s eye “or erythema migrans rash (EM) is considered to be sufficiently diagnostic for Lyme disease to justify recommended antibiotic therapy, without the need for a positive serological test result (5). It develops typically within 7-14 days at the site an infected tick has taken a blood meal and detached (6,7). Usually, patients with EM are seronegative at the time of presentation; however, the probability of becoming seropositive increases with the duration of EM and as infection becomes more disseminated, i.e., as antigen load increases, and the amount of antibody produced reaches detectable levels in the blood (8,9,10). Although only 25-50% of patients with EM are positive with reference to the enzyme-linked immunosorbent assay (ELISA) during the early or acute phase of their infection, 80-90% of treated EM patients are seropositive by convalescence. This occurs, despite the fact that such patients were treated with an antibiotic that may have reduced “antigen load” to some degree, thereby lessening the magnitude of the resultant antibody response (10,11).

As one would anticipate and has been demonstrated by several investigators, the sensitivity of an antibody-based diagnostic test for Lyme disease increases
progressively with the duration of EM (10), and in patients with: (a) acute neurologic or cardiac abnormalities; and, with (b) arthritis or chronic neurologic abnormalities (11). Under such circumstances, and as infection becomes disseminated, both sensitivity and specificity range from 85% to 99% (11). Similar results were obtained when the same specimens were assayed in parallel to two-tier testing by another highly specific antibody-based procedure, the Vlse C6 peptide ELISA (10, 11).

The diagnosis for Lyme disease is usually based on the results of a two-tier testing procedure recommended by the CDC (12). Here, an ELISA for Lyme disease is conducted which, if positive, is followed by an IgM or IgG Western blot. If a patient has symptoms for more than 30 days, the IgG Western blot is performed, whereas the IgM Western blot is used only for patients with symptoms less than 30 days. This is because there is a high rate of false positive test results when IgM Western blots are used for patients with symptoms of more than 30 days (13). It should be stressed that only the criteria recommended by the CDC should be used for interpreting the results of two-tier testing. The use of non-validated alternative criteria has been shown to result in a high incidence of false positives (14).

Because of the acknowledged low sensitivity of ELISA tests during the early acute EM phase, neither the CDC nor the Infectious Diseases Society of America (IDSA) advocate serological testing of such patients. Rather, they consider it appropriate to treat such patients with antibiotics on this clinical basis (4). Thus, the early or acute EM phase of Lyme disease is the only time during active infection when the sensitivity of two-tier testing is low.

Despite the above mentioned well-documented observations, some continue to discount the validity of two-tier testing as a diagnostic for Lyme disease, claiming that its sensitivity is “no better than that of a coin toss” (15). However, those who hold such a view ignore the fact that most -- if not all -- of the test results upon which their argument is based were derived from patients with EM, i.e., from patients with early acute Lyme disease when the immune response is just beginning and antibody levels are very low (see references cited in 15). Under such circumstances, low sensitivity is to be expected as has been reported by other investigators (8, 9, 10); this is why testing is not recommended under such
circumstances (5). Specificity is not an issue since whenever antibody is detected—albeit in small amounts—it is found to be 99-100% specific for *B. burgdorferi* (15).

The dissemination of such misleading information on the validity of two-tier testing (15) at best reflects ignorance of both the disease process and limitations associated with antibody-based tests when used for the diagnosis of Lyme disease. At worse, it is a manipulative attempt to selectively report only those observations that serve to discredit the validity of two-tiered testing. Neither is in the best interest of the public health or helpful to patients who suspect that they may have Lyme disease.

There is abundant evidence indicating that two-tier testing has performed well when applied under conditions when the probable risk of contracting Lyme disease is reasonably high (16, 17). Those competent and experienced in the use of diagnostic tests understand that the results obtained with antibody-based diagnostic tests are valid only when such tests are used when detectable amounts of antibody are likely to be present. Even the most sensitive and specific diagnostic test one can imagine is not going to show that a patient has Lyme disease, if that patient doesn’t have Lyme disease. Alternatively, if a patient has long-standing non-specific symptoms of a type often associated with Lyme disease but is seronegative, it is not prudent to treat such patients with an extended course of antibiotics for an infection that may not even exist. It makes more sense to consider other causes for their symptoms (see: [http://www.aldf.com/wp-content/themes/ALDF/pdf/The_Pain_of_Chronic_Lyme_Disease_FASEB_full_article.pdf](http://www.aldf.com/wp-content/themes/ALDF/pdf/The_Pain_of_Chronic_Lyme_Disease_FASEB_full_article.pdf)).

Certainly, continual efforts should be made to improve existing technology so that diagnostic tests are able to detect small amounts of antibody (or even antigen directly) early during the course of an infection; this would ensure that curative antibiotic therapy can be commenced as early as possible. This research is now being done. The recent establishment of a reference serum repository in which the results of newly developed diagnostic tests can be compared to those derived from existing procedures—using the same well-characterized panel of reference specimens in both cases—will greatly accelerate progress in that regard (18).
The ELISA format, with a sensitivity estimated to range from 0.01 to 0.1 nanograms of antibody per milliliter (19), seems to be ideally suited for early diagnostic testing. Its remarkable sensitivity is due largely to the ability of an enzyme conjugated to a second antibody to amplify the reaction between a single specific antibody molecule and its relevant ligand (or antigen), by a factor of 10,000-fold or more. The replacement of bacterial cell lysates often used in conventional ELISAs with well-defined peptides associated with specific antigens produced early in infection (17) reduces variability and greatly facilitates comparisons of results from different laboratories. The Vlse C6 peptide ELISA is but one example of just such an application. Parallel testing using this approach gives results comparable to those obtained with the conventional ELISA used in the standard two-tier test procedure. However, it should be noted, that there have been no reports of patients who are seropositive for Lyme disease by the Vlse C6 peptide ELISA, who are not also seropositive by the conventional ELISA (10,11). Although sensitivity appears equivalent in studies conducted to date (10,11), additional comparative studies are needed before this single diagnostic test can replace two-tier testing.

Recent studies using the Luminex multiplex assay system with 10 markers were developed from an analysis of 62 *B. burgdorferi* surface proteins and synthetic peptides; this involved assessing the binding of IgG and IgM antibodies to each and then comparing samples from Lyme disease patients to those from controls (20). In a validation set, this 10-antigen panel identified a higher proportion of early Lyme disease patients as positive at the baseline or post-treatment visit compared to two-tiered testing (87.5% and 67.5%, respectively, p<0.05). Equivalent specificities of 100% were observed in 26 healthy controls. Upon further analysis, positivity with respect to the novel 10-antigen panel was associated with longer illness duration and multiple EM rash. The improved sensitivity and comparable specificity of the 10-antigen panel compared to two-tiered testing in detecting early *B. burgdorferi* indicates that multiplex analysis, featuring this next generation of new markers, could advance diagnostic technology significantly, resulting in better diagnosis and treatment of early Lyme disease.

As noted above, the procedure recommended by both the Centers for Communicable Disease Control (CDC) and the Food and Drug Administration
(FDA) for the diagnosis of Lyme disease is a two-step procedure that includes an ELISA test which, if positive for Lyme disease, is confirmed by either an IgM or IgG Western blot. Because the Western blot is not a quantitative procedure, it is only able to detect the presence of antibody specific for *Borrelia*, not the amount of such antibody. Consequently, a Western blot -- as usually performed -- is unable to distinguish between the relatively large amounts of different types antibodies produced during active infection and the smaller amounts of those same antibodies that might be present for as long as 10-20 years after patients have been given recommended antibiotic therapy, i.e., background antibodies(22). Bypassing the ELISA to proceed directly to Western blot testing increases the chances of false positive results based on the presence of minute amounts of antibody that would not be representative of an active untreated infection. Obviously, in the absence of clinically objective evidence of active infection, it makes no sense to treat with antibiotics until a patient becomes seronegative – or until symptoms attributed to Lyme disease disappear. Alternative possibilities should be considered (see: http://www.aldf.com/wp-content/themes/ALDF/pdf/The_Pain_of_Chronic_Lyme_Disease_FASEB_full_article.pdf).

Although the basis for the persistence of such background antibody is not known, it may be the result of prolonged antigenic stimulation.Since *Borrelia* are known to produce several different integrins or adhesion molecules that enable them to bind tenaciously to various mammalian cells (23), the ability of intact but dead *Borrelia* -- or their remnants-- to persist in mammalian tissues for long periods of time after successful antibiotic treatment and cure might be sufficient to stimulate the immune system to produce detectable amounts of background antibodies. It also has been suggested that these same nonviable *Borrelia* or their remnants may possess pharmacologically active lipoproteins that could contribute to either immunologic or metabolic changes driving persistent symptoms despite cure of the infection by antibiotic therapy (24).

A step toward easier diagnosis of Lyme disease that avoids misinterpretation of results would be advanced by the use of quantitative ELISAs in conjunction with well-defined ligand specific for *Borrelia* that would be vastly superior to Western blots with the counting of individual bands. Such a replacement test might better assess the efficacy of antibiotic therapy, as well as enable one to
distinguish between high levels of antibody characteristic of active infection and low levels of background antibodies that might be found in some patients who have been successfully treated in the past for their infection and microbiologically cured. The time is long past due to move the diagnosis of Lyme disease in that direction. The establishment of a highly recommended Lyme disease reference specimen repository by the CDC, in collaboration with the NIH and FDA, should facilitate and hasten progress in that regard (http://www.aldf.com/2nd-banbury-conference/).

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Phillip J. Baker, Ph.D.
Executive Director
American Lyme Disease Foundation

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