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Laboratory Serodiagnosis of Lyme Borreliosis

Steven M. Callister, PhD and Ronald F. Schell†, PhD*

ABSTRACT

The variety of symptoms attributable to Lyme borreliosis is extensive; thus, a clinical diagnosis can be difficult to make. Because of this, laboratory tests are often used to provide evidence of infection with *Borrelia burgdorferi*. Serologic testing is the mainstay of the laboratory diagnosis of Lyme borreliosis. However, indirect fluorescent antibody and enzyme-linked immunosorbent assays suffer from a lack of specificity. Western blotting provides increased specificity but interpretative criteria are not standardized. The detection of borreliacidal antibodies is a highly specific test but the technology required for routine testing is not widely available. Despite these inadequacies, serodiagnostic testing for Lyme borreliosis has improved significantly during the past decade. The challenge for the clinician is to understand the strengths and weaknesses of the serologic testing procedures and appropriately interpret results.

Key words: *Borrelia burgdorferi,* Lyme borreliosis, serology

INTRODUCTION

During the early 1980s, the spirochetal bacterium, *Borrelia burgdorferi* sensu stricto, was implicated as the causative agent of an oligoarthritis epidemic in Lyme, Connecticut.[1] This illness, subsequently named Lyme disease, and now known as Lyme borreliosis, has become the most common tick-associated illness in the United States. Lyme borreliosis is a multisystem disorder that usually begins at the site of the tick bite with a localized skin infection called erythema migrans. Approximately 60% to 80% of infected individuals develop a noticeable skin lesion.[2] Constitutional symptoms such as fatigue, headache, mild stiff neck, arthalgias, myalgias, and fever are also frequently present. Dissemination of the spirochete by hematogenous or lymphatic spread can lead to clinical manifestations including secondary annular skin lesions, meningitis, Bell's palsy, radiculoneuritis, and atrioventricular heart blockage.[2-4] If the infection is left untreated, arthritis[5] or nervous system disorders[6] may develop after weeks to months. An additional sequela, acrodermatitis chronica atrophicans, is seen primarily in Europe.[7]

The clinical diagnosis of Lyme borreliosis is often based on exposure to *Borrelia* ssp.-infected *Ixodes* ssp. ticks, the presence of characteristic and protean clinical findings, and laboratory confirmation by detection of antibodies against *B burgdorferi*. However, clinicians can have difficulties diagnosing Lyme borreliosis based on cutaneous eruptions.[8] In addition, symptoms can resemble those of other clinical illnesses, including influenza, multiple sclerosis, aseptic meningitis, and rheumatoid arthritis, making an accurate clinical diagnosis of Lyme borreliosis even more difficult. Consequently, clinicians have relied heavily on the ability of the laboratory to detect antibodies against *B burgdorferi*. Detection of serologic evidence of infection may be the only option available to clinicians for establishing a Lyme borreliosis diagnosis. However, reliance on serologic confirmation has led to questions regarding both the accuracy and interpretation of test results. Unfortunately, inaccurate serodiagnostic

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assays combined with an inability to interpret conflicting results may have caused some clinicians to be reluctant or even refuse to request laboratory tests.

Bakken et al[9] reported that the specificity and sensitivity of Lyme borreliosis assays decreased from 1992 through 1995 among 516 laboratories participating in the National Lyme Disease Proficiency Survey sponsored by the Wisconsin State Laboratory of Hygiene, the public health laboratory for the State of Wisconsin, and the College of American Pathologists. However, this report did not address which Lyme borreliosis assays had sensitivity and specificity of 90% or more. Instead, to improve the quality of and confidence in testing methods, the authors recommended that the Food and Drug Administration apply more stringent criteria for continuing to approve commercially available kits for the serodiagnosis of Lyme borreliosis. Presently, kit manufacturers are reluctant to implement changes that would earn little return in an environment where poor test sensitivity and specificity are accepted. One way laboratories that use these kits can optimize the test quality is to carefully examine proficiency survey results and select a kit or kits that have consistently performed well over the duration of the survey.

To correctly interpret serologic test results, it is important to understand the human humoral immune response to *B burgdorferi* infection. Synthesis of anti-*B burgdorferi* antibodies commences shortly after infection, but these antibodies are generally not detectable by serological methods for 2 to 5 weeks. Therefore, early Lyme borreliosis may be difficult to detect by serodiagnostic testing. If serum was collected during this period, testing a convalescent serum or serum drawn 2 or 3 weeks later may be necessary. A request by the clinician to save the initial serum for retesting with the convalescent serum would help to improve the interpretation of Lyme borreliosis test results.

IgM antibodies against several *B burgdorferi* proteins are often detectable during early disease. Engstrom et al[10] showed that the predominant antibody response in patients with early Lyme borreliosis was against the 39 kDa periplasmic protein. Antibodies against the 41 kDa flagellar antigen and outer surface protein (Osp)C were often also present during early Lyme borreliosis.[10] The antibody response against OspC is especially important. Recently, Schwan et al[11] showed that OspC expression is upregulated by *B burgdorferi* in infected ticks soon after attachment to the host. Therefore, anti-OspC antibodies are among the first species-specific antibodies that could be detected with serodiagnostic tests. A major concern, however, is that *B burgdorferi* isolates incorporated in serodiagnostic tests may not express large amounts of this antigen, making detection of anti-OspC antibodies unlikely. Generally, IgM antibodies disappear 2 to 3 months after infection. Persistence of IgM responses have been documented, however, reactivity is directed against other *B burgdorferi* proteins.[12]

Weeks to months after infection, IgG antibodies may become detectable and increase to high concentrations during the course of the disease. *B burgdorferi* is comprised of at least 30 major antigens.[13,14] Antibody responses against most of these antigens have been detected in serum from patients with disseminated Lyme borreliosis.[15,16] During this period of infection, many commercial kits detect antibodies against *B burgdorferi* suggesting that many of the kit isolates have retained antigens that can detect antibodies produced during later stages of infection.

To further complicate serologic detection of infection, antibiotic therapy may severely blunt or abrogate the immune response.[17] The possibility of seronegative Lyme disease has also been raised.[18] *B burgdorferi* does not appear to multiply as well in vivo compared to its cousin, *Treponema pallidum*, the spirochetal agent of syphilis, or even the more slow growing bacterium, *Mycobacterium tuberculosis*. When mice or hamsters are irradiated or treated with immunosuppressive agents to prevent an immune response, *B burgdorferi* does not respond by increasing in number. In contrast, the numbers of *T pallidum* organisms and tubercle bacilli rapidly increase to 10^{10} or greater. *B burgdorferi* may control its numbers to subvert or deactivate the immune response. In addition, *B burgdorferi* organisms tend to disseminate to immunologically privileged sites. Thus, there may be little or no antibody production to detect.

Indirect fluorescent antibody and enzyme-linked immunosorbent assays

Indirect fluorescent antibody (IFA) assays are still commonly used in the serodiagnosis of this illness. IFA assays use whole spirochetes to detect anti-*B burgdorferi* antibodies. *B burgdorferi* organisms are fixed to a glass slide and a serial dilution of a patient serum is added. Bound antibodies are detected with anti-human IgM, IgG, or polyvalent antisera covalently labeled with fluorescein isothiocyanate (FITC). After washing away unbound antibodies, the spirochetes are examined by fluorescent microscopy and the degree of fluorescence is assessed. IFA assays have proved suitable tests for Lyme borreliosis, however, subjectivity can be a drawback since the intensity of fluorescence is determined visually. This method is also not conducive to large volume testing.

Enzyme-linked immunosorbent assays (EIA) overcome some of the drawbacks of IFA. Whole cells, sonicated

spirochetes, or purified or recombinant proteins are coated onto wells of 96-well microtiter plates. The test procedure is similar to that for an IFA, except that bound antibody reacts with an enzyme substrate and the degree of reactivity is determined by a spectrophotometer. The spectrophotometer eliminates subjectivity and allows for large volume testing. EIA has become the most commonly used technique for the serodiagnosis of Lyme borreliosis.

The sensitivity of IFA and EIA tests is lower during early localized disease than in later stages of the illness.[12,19,20] Russell et al[20] reported that with IFA and EIA tests, antibody was detected in only 17 (50)% of 34 patients with early Lyme borreliosis. Mitchell et al[19] also reported that, using IFA, IgM antibody was detected in 8 (42%) of 19 sera from patients with culture-positive primary EM lesions. Several investigators have developed modified IFA or EIA test methods to increase the potential for detecting antibody produced during early Lyme borreliosis. For example, Berardi et al[21] developed an antibody-capture EIA. Isotype-specific antibodies are coated onto microtiter plates so that IgM antibodies are retained when serum is added. *B burgdorferi* organisms, rabbit anti-*B burgdorferi* serum, and horseradish peroxidase labeled antirabbit serum are then added. Using this method, IgM antibody was detected in 67% of sera collected from patients with early Lyme borreliosis. However, despite the increased sensitivity, this procedure is not widely used because it is more complex and costly than commercial kits.

Efforts to increase the EIA sensitivity have centered on using the 41-kDa flagellar antigen[22] or a flagellin-enriched preparation[23] instead of whole borrelial cells or sonicates since antibodies against the flagellar protein are synthesized during the primary immune response. These efforts have led to modest increases in sensitivity[24], although specificity has decreased. Non-specific antibodies in approximately 50% of sera from healthy individuals[13,15,25-28] react with the 41 kDa flagellar *B burgdorferi* protein.

The sensitivity of IFA and EIA tests on serum drawn from patients with later stages of Lyme borreliosis is high[20]; however, test specificity remains low. False-positive IFA or EIA reactions are common in patients with other illnesses.[13,29-31] In addition, antibodies directed against oral spirochetes, normal mouth flora in most individuals,[32] can also bind to *B burgdorferi* proteins. Thus, a large number of sera from individuals without Lyme borreliosis, including healthy persons, react positively in IFA or EIA tests.

Despite the low specificity, IFA and EIA are useful for confirming Lyme borreliosis in persons with epidemiologic evidence of infection. In these instances, lack of test specificity likely has little consequence; however, more specific assays should be used in less obvious cases.

Western blotting

Western blot (WB) tests are performed by electrophoresing *B burgdorferi* through an acrylamide gel, transferring the separated proteins to nitrocellulose, adding patient serum, and assessing any reactions. WB tests can provide information regarding the antibody response to individual *B burgdorferi* proteins. Antibodies recognizing the species-specific 22 kDa OspC,[33,34] the 31 kDa OspA,[33,35] the 34 kDa OspB,[35] the 39 kDa periplasmic polypeptide,[36] the 83 kDa major extracellular protein,[37] and/or the 94 kDa protoplasmic proteins[14] are commonly seen. Detection of these specific antibody-antigen reactions have been used to confirm Lyme borreliosis.[10,14,16,24,25,28] Many laboratories also report reactions that may be non-specific such as those to the 41 kDa flagellar protein or approximately 25 other proteins that are common to many bacteria including *B burgdorferi*.

WB tests are considered positive if a certain number of protein bands are observed, although positive test criteria vary among laboratories. For example, Dressler et al[16] recommended at least 2 of 8 bands after an IgM test or 5 of 10 bands after an IgG procedure before either could be interpreted as positive. Other investigators have recommended that there be significantly more or less bands.

WB test sensitivity for detecting antibody during early Lyme borreliosis varies among laboratories. Dressler et al[16] reported WB test sensitivity of 32% when patients with early disease were tested. Mitchell et al[19] found WB tests to be positive using serum from 2 (11%) of 19 patients with culture positive primary EM and 20 (62%) of 32 patients with culture positive secondary EM lesions (overall sensitivity 43%). Using a significantly less stringent criterion, Engstrom et al[10] reported a WB test sensitivity of 55% during early Lyme borreliosis. Collectively, these results are significantly less than ideal for confirmation of *B burgdorferi* infection, especially during early Lyme borreliosis.

When clinical signs and symptoms are highly suggestive of Lyme borreliosis or when individuals have developed antibodies to several species-specific proteins, WB tests are useful tools for confirming Lyme borreliosis. However, clinicians should be aware of problems with the procedure. Serum from patients with other illnesses may contain antibodies that react with *B burgdorferi* proteins.[13,28,31] This has caused a significant dilemma. Some laboratorians have increased the specificity by requiring additional species-specific antigen reactions before the test is considered positive. This approach is reasonable

since a confirmatory test must be specific. However, requiring more species-specific reactive bands allows confirmation of Lyme borreliosis only in patients with more severe late stage disease. This decreases the likelihood that WB tests will be positive during early illness. Some laboratories have compensated for this by including reactions against nonspecies-specific proteins in their diagnostic criteria. However, this can lead to decreased specificity from false-positive reactions. This problem is not trivial since up to 40% of uninfected persons may react with *B burgdorferi* proteins when subjected to WB testing.[26]

WB tests are also highly subjective laboratory procedures and widespread differences in diagnostic criteria amplify the subjectivity. The laboratorian must decide when to consider bands absent or present. This can be difficult, especially when reactions are weak. Even when antigen-antibody reactions are detected, their significance must be interpreted. To further complicate WB tests, accurate interpretation is dependent on adequate separation of *B burgdorferi* proteins. Engstrom et al[10] found it necessary to use a monoclonal antibody and gradient gels before reactions to the species-specific 39-kDa protein could be accurately distinguished from reactions to the nonspecies-specific 41 kDa flagellar protein. In addition, Bruckbauer et al[38] detected several minor *B burgdorferi* proteins of about 20 kDa that could easily be confused with the species-specific 22 kDa OspC.

WB tests are commonly used to confirm a Lyme borreliosis diagnosis and commercial WB kits are widely available. However, the problems discussed above highlight the need to obtain testing from a reputable laboratory. When results are obtained from a laboratory that understands the complexity of this procedure and takes steps to minimize shortcomings, WB tests can be useful. If not, WB test results can be difficult to interpret.

Flow cytometric borreliacidal antibody test

Another test method uses borreliacidal antibodies, antibodies that kill *B burgdorferi*, for the serodiagnosis of Lyme borreliosis. Inoculation of animals with whole *B burgdorferi* or its components induces antibody-mediated immunity against challenge.[39,40] *B burgdorferi,* or some of its components, induces borreliacidal antibodies that provide protection by specifically killing *B burgdorferi.*[40-46] Humans infected with *B burgdorferi* by natural means (tick bite) also develop borreliacidal antibodies;[47-55] however, the Lyme spirochetes are not eliminated. Presumably, *B burgdorferi* escape killing by mechanisms such as coating themselves with host proteins or sequestering in immunologically privileged sites. OspA,[44,48,50,52,56] OspB,[44,50,56-58] and/or OspC[55] induce highly specific[49-51,53] IgM and/or IgG borreliacidal antibodies in vivo shortly after infection with *B burgdorferi*. Detection of these antibodies with a borreliacidal antibody test (BAT) can be useful for confirming a Lyme borreliosis diagnosis.

Borreliacidal antibodies are detected by combining live *B burgdorferi* and complement with patient serum and determining whether the spirochetes are killed. The use of live organisms eliminates the detection of antibodies that bind to the spirochetes but are incapable of killing such as antibodies that recognize the 41 kDa flagellar protein. The specificity of BAT is superior to that of IFA and EIA tests. For example, serum from patients with rheumatoid factor, syphilis, and mononucleosis have been used to demonstrate the lack of specificity of IFA and EIA. In previous investigations,[50,51,53] 18 to 69% of these sera have been positive by IFA or EIA. In contrast, serum from these patients have not contained borreliacidal antibodies.[50,51,53]

The amount of borreliacidal antibodies in serum increases with length of illness and severity of symptoms[52] making antibody detection easier in serum from patients with later stages of Lyme borreliosis. Borreliacidal antibodies can be detected, however, in serum from patients with early Lyme borreliosis, but detection is dependent on two important variables. First, a sensitive BAT procedure must be used. Some BATs have relied on visual assessment of cell viability[50] or a pH color change of the *B burgdorferi* growth medium[48,49] to detect killing. These are subjective procedures that require large concentrations of borreliacidal antibodies to kill large numbers of *B burgdorferi*. The sensitivity of the BAT is greatly improved with a flow cytometer. As with other BATs, live *B burgdorferi* and complement are combined with the patient serum. Unlike other procedures, the spirochetes are stained with acridine orange after incubating for 16 to 24 hours. If the serum contains borreliacidal antibodies, acridine orange enters the dead spirochetes and the increased fluorescence intensity of individual organisms is detected by the flow cytometer. By decreasing the *B burgdorferi* concentration, smaller quantities of borreliacidal antibodies can be detected.

Second, detection of borreliacidal antibodies is dependent on the susceptibility of the *B burgdorferi* organisms. Anti-OspC borreliacidal antibodies are often present in high concentrations in serum from patients with early borreliosis. These borreliacidal antibodies, however, are only detectable when an isolate such as *B burgdorferi* 50772, which does not express OspA or OspB, is used.[55] Thus, it is necessary to use appropriate *B burgdorferi* isolates for detecting borreliacidal antibodies, especially when testing serum from patients with early Lyme borreliosis.

In a recent investigation using a flow cytometric BAT,[51] borreliacidal antibodies were detected in 34 (72%) of 47 sera from early Lyme borreliosis patients. This high sensitivity coupled with high specificity[50,51,53] highlights an increasing use for this serodiagnostic test. However, the technical expertise required to perform this assay, the instrumentation, and the use of live organisms make it appropriate for use only by an experienced reference laboratory. The BAT also has several other limitations. False-negative reactions may still occur since not all antibodies produced in response to infection are capable of killing *B burgdorferi*. In addition, some patients may fail to make borreliacidal antibodies. False-positive reactions may also occur. The BAT monitors killing of live *B burgdorferi* organisms; antimicrobial agents in serum will also kill the spirochete. Antimicrobial agents can be removed without affecting the borreliacidal antibody concentration (manuscript in preparation); however, the procedure is not widely known. Thus, clinicians should take extra precaution to insure that serum does not contain antimicrobial agents.

B burgdorferi heterogeneity

Lyme borreliosis spirochetes have been reclassified into genomic groups that include the human pathogens *B burgdorferi* sensu stricto, *B garinii,* and *B afzelii.* There is a strong relationship among these genospecies and the different clinical symptoms. *B burgdorferi* sensu stricto is most commonly associated with rheumatologic manifestations, while *B garinii* and *B afzelii* are frequently associated with neurologic and dermatologic complications, respectively.[59,60] Correspondingly, the heterogeneity of *B burgdorferi* is becoming an increasingly significant factor in Lyme borreliosis serodiagnosis, especially in Europe.

Fortunately, isolates from the United States are primarily *B burgdorferi* sensu stricto and intraspecies heterogeneity has not been a significant problem. In contrast, *B burgdorferi* genospecies are found throughout Europe and effects on serodiagnosis have been observed. For example, Dressler et al[15] demonstrated that sera from European neuroborreliosis patients were more WB reactive when *B garinii* was used as the antigen. Similarly, sera from patients with ACA were more reactive when *B afzelii* was used.[59] Hauser et al[61] also reported significant variations in EIA test results of European Lyme disease patients when using *B burgdorferi* sensu stricto, *B afzelii*, or *B garinii*.

Interspecies heterogeneity is also becoming increasingly important. Molecular analyses of *B burgdorferi* sensu lato isolates have demonstrated significant heterogeneity of species-specific Osps.[33,34,62-64] This has been most significant among European isolates, however, heterogeneity of OspA[62] and OspC[64] has been detected among North American isolates. Because of the increasing role of heterogeneity, it is likely that diagnostic testing by any method will be more reliable when an appropriate regional geographic isolate is used. Interspecies heterogeneity, especially among species-specific proteins, will also confuse the interpretation of WB reactivity because of variability in the molecular weight of these proteins. The BAT may circumvent this problem. When whole cells are used, borreliacidal antibodies against several Osps are detected. Lovrich et al[41,62] detected borreliacidal antibodies in a serum using isolates from throughout Europe and North America. In addition, anti-OspC borreliacidal antibodies are detectable in sera from throughout North America when only *B burgdorferi* isolate 50772 is used.[51,55] These results suggest that the Osp borreliacidal epitopes may be conserved, although additional studies are necessary.

B burgdorferi organisms have also exhibited several other characteristics that may continue to make some laboratory results confusing. Passage of the organism or differences in culture reagents can have profound effects on the reactivity of *B burgdorferi* with serum. Schwan et al[65] demonstrated that noninfectious *B burgdorferi* B31 (highly passaged) had reduced WB patterns compared to an infectious low passage isolate. In addition, the incubation temperature of the *B burgdorferi* organisms can have profound effects. Rousselle et al[55] demonstrated an increased ability to detect anti-OspC borreliacidal antibodies after incubation of *B burgdorferi* 50772 organisms at 35°C. These results highlight the need to insure that commercial kits perform similarly when different manufacturing lots are used.

CONCLUSIONS

Inaccuracies in Lyme borreliosis testing have contributed to misdiagnosis and overdiagnosis.[66,67] The inefficient use of these tests has generated considerable costs,[68] confused the general public, and made some clinicians wary of serologic testing. However, laboratory methods for detecting antibodies against *B burgdorferi* organisms have been improved. IFA, EIA, WB and BAT can all contribute valuable diagnostic information provided the clinician understands their advantages and disadvantages, utilizes an appropriate testing strategy, and understands the significance of the test results. In addition, recent studies of Lyme borreliosis testing have demonstrated wide variations of proficiency among laboratories. Thus, it is also important to utilize experienced diagnostic laboratories staffed by personnel familiar with the nuances of Lyme borreliosis serodiagnosis.

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Infection Rates of *Ixodes* Ticks with the Lyme Disease Spirochete, *Borrelia burgdorferi,* in a New Jersey Park

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ABSTRACT

Lyme disease (LD) is the most common vector-borne disease in the United States. It is an infection caused by the bacterium *Borrelia burgdorferi,* which is introduced into the host by the bite of the black-legged tick, *Ixodes scapularis,* and other species of ticks in the *I ricinus* complex. Determining infection rates in vector ticks is one of the most economical ways to define an area at risk. We have collected ticks of the genus *Ixodes* and tested them for infection with *B burgdorferi* using the polymerase chain reaction (PCR) and direct fluorescent antibody analysis (DFA). Our results have demonstrated that approximately 50% of the questing adult ticks in Watchung reservation (Union County, New Jersey) are infected with *Borrelia burgdorferi.* Female *Ixodes* exhibited higher infection rates than males. These data should assist public health officials and park superintendents in establishing policy towards reducing the risk to those who work and recreate in this park. Educating the public is a primary means of keeping the incidence of LD low.

Key words: *Ixodes*, Borrelia, Lyme infection rates

INTRODUCTION

Lyme disease is the most frequently reported vector-borne disease in the United States. The bacterium, *Borrelia burgdorferi*, which causes Lyme disease (LD), is transmitted to humans via the bite of the black-legged tick, *Ixodes scapularis* and related species of the *I ricinus* complex.[1] Although this disease is rarely fatal, it causes significant morbidity and economic loss. Over 16,000 cases were reported in the United States to the Centers for Disease Control and Prevention in 1996.[2] The New Jersey Department of Health reported 2,190 cases of LD to the CDC in 1996. New Jersey routinely reports greater than 10% of total U.S. cases.

In New Jersey, intense foci (case incidence) of LD occur in some counties whereas other counties have very low incidences. For example, Hunterdon County, which is rated as one of the high-incidence counties in the U.S.,[2] reported 610 cases in 1996. In contrast, Union County tallied only 15 cases in 1996. This variability could be because of ecological and demographic differences.

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One approach to defining the risk in a Lyme-endemic area is to conduct tick surveys. Unfortunately, published tick infection rates do not exist for some counties, while others have been extensively surveyed. In New Jersey, Monmouth County is the most characterized in terms of tick infection rates. This is probably because of the first cases of LD in NJ were reported here, and it has a consistently high incidence of LD (110 cases in 1996). Published infection rates of adult ticks in Monmouth County range from a high of 79%[3] to a low of 33%.[4] Other surveys of ticks in Monmouth county have yielded infection rates of 39% in a spring sampling and 50% in a fall sampling.[5] A multisite survey for infected ticks in Monmouth County revealed a range of infection of 33% to 57%.[4] In the above surveys darkfield microscopy (DM), DFA, or culture in artificial medium were used to identify infected ticks. Recently, Varde et al, used PCR to show that 43% of adult *I scapularis* in Hunterdon County are infected with *B burgdorferi.*[16]

In one instance, tick surveys identified a newly evolving focus of LD.[7] Within an area of low LD incidence in Monmouth County (Perrineville, NJ) tick infection rates determined by darkfield microscopy, were compared to an area of high incidence (Naval Weapons Station, Earle, NWSE) in the same county. NWSE had tick infection rates of 50% in the fall and 33% in the spring while Perrineville

had 53% and 66% adult tick infection rates in the fall and spring respectively. Perrineville reported 4 cases of LD in 1984. By comparison, NWSE reported 29 cases from 1981 through 1982.[8] This study identified an area with high tick infection rates, yet low LD incidence, and thus defined an emerging focus of LD.

Risley and Hahn[9] used PCR and DM to survey *Borrelia*-infected *Ixodes* in various New Jersey counties. They found infection rates of 6% in Bergen, 24% in Morris, and 0% in Passaic and Essex counties. Interestingly, in one of the more rural counties, Sussex, no ticks were collected even though there is a large white-tailed deer population. White-tailed deer is the preferred host for adult *I scapularis*.

There are inherent problems with each of the various methods used to detect infected ticks. For example, PCR is very sensitive and thus subject to target contamination. DM cannot differentiate between different species of spirochetes. Finally, DFA is sometimes not sensitive enough to detect spirochetes in infected ticks and also cannot discern different spirochete species. Nonetheless, one can get an appreciation of the *Borrelia* burden in a population of ticks using these various methodologies. For example, in a study comparing DFA and *ospA*-PCR,[12] it was shown that in 15 field-collected *I dammini* (=*I scapularis*) from Massachusetts, 100% concordance was achieved between the two methods. It is conceivable that concordance rates may have declined if larger sample sizes were used. Wittenbrink et al,[11] analyzed 100 *I ricinus* from Germany for *B burgdorferi* using culture, DM and PCR. The PCR target was the flagellin gene, which is believed to be ubiquitous in all *B burgdorferi*. In addition, the authors used DM as opposed to DFA. They showed that of the14 ticks positive by DM and culture, 10 were positive by PCR, a concordance of 71%. Culture is considered to be the ultimate test for infection since PCR can provide positive results even when there are no viable organisms. However, culture contamination often complicates results. Kahl et al,[12] have investigated *B burgdorferi* infection rates in field-collected *I ricinus* using DFA and PCR. Kahl et al,[12] have shown that 25% of nymphs (n=206) were infected as determined by DFA and that 16% of nymphs (n=254) were infected as determined by PCR. These authors concluded that laboratories determining *Borrelia* carriage rates should employ quality control protocols in their studies.

Taking these inherent diagnostic problems into consideration we have surveyed a frequently used public park in Union County New Jersey and determined the infection rate of adult *Ixodes* with *B burgdorferi* by using DFA and PCR. The surveillance data presented here has relevance in that New Jersey residents should be made aware that they may be at risk of contracting Lyme disease even in counties with historically low incidences.

METHODS

Tick collection and processing

Adult ticks were collected from 1994 through 1996 from Watchung Reservation, Union County, New Jersey by the flagging method[13] and identified to the genus level. Although a statistical sampling was not performed, other parks in Union County (Lenape and Nomahegan) were sampled to gain an approximation of tick abundance in these parks. Tick collection efforts were concentrated at sites bordering field and/or wooded environments, on vegetation approximately 0.5 to 1.0 m from the surface of the soil.

Ixodes collected in the current study were not assumed to be of the *scapularus* taxon since other *Ixodes*, such as *I dentatus*, may be found in the same niche. This work preceded tick identification training of the authors and as result, *Ixodes* were not identified to the species level. However, approximately ten ticks were submitted to the United States Tick Collection in Statesboro, GA and New York State Department of Health and all were identified as *I scapularis*. Adult specimens were chosen because the original purpose of this study was to develop a novel-*Borrelia* detection method. Presumably, the larger specimens (if infected) would harbor more spirochetes than infected nymphs, facilitating the development of an assay.

Live ticks were surface sterilized, by submersing twice in 70% ethanol/0.1% NP-40, followed by a 70% ethanol rinse, and finally a rinse in sterile dH_20. Individual ticks were then placed in a microfuge tube containing 10 µL TEN (10 mM Tris pH 7.5, 1 mM EDTA, 0.1% NP-40). These ticks were then homogenized using a flame-sealed pipet tip that when in the molten state was dipped in sterile glass beads. After homogenization, TE was added to bring the final liquid volume of the solution to 100 µL. Two aliquots of the homogenate was removed for analysis using DFA microscopy and PCR.

Direct fluorescence antibody staining

Ten microliters of tick homogenate was placed on a microscope slide, allowed to air-dry, and was then fixed using cold (4°C) acetone. Twenty microliters of diluted (1:10) fluorescein isothiocyanate (FITC)-labeled goat polyclonal IgG anibody against *B burgdorferi* (Kirkegaard and Perry, Gaithersburg, MD) was added to the smear. The slide was overlayed with a coverslip and placed in a humidified incubator at 37°C for 30 minutes. The coverslip was removed and the slide was rinsed 3× with PBS, 1× with dH_2O and then allowed to air dry.[14] A drop of immersion oil (Cargille, Cedar Grove, NJ) was added to the slide, which was then overlayed with a coverslip and sealed with clear lacquer. Slides were examined for fluorescence for 5 minutes using a 100× oil immersion objective and ultra-violet illumination with a Zeiss Axioscope (Zeiss, New York, NY).

Table. Analysis of adult Ixodes *for* B burgdorferi *infection using PCR and DFA.*

Sampling Year	PCR No. Pos/No. Tested (% Infected)	DFA No. Pos/No. Tested (% Infected)	PCR-DFA Concordance No. Agrmnt./No. Test. (% Concordance)
1994-5	50/95 (52.6)	42/69 (60.8)	54/69 (78.2)
1996	16/28 (57.1)	14/28 (50.0)	24/28 (85.7)
Totals	66/123 (53.7)	56/97 (57.7)	

Molecular techniques and polymerase chain reaction

Tick homogenates were assayed for *B burgdorferi* infection by *ospA* PCR[10] using Taq polymerase and, in later experiments, Elongase (Life Technologies, Gaithersburg, MD). The plasmid, pTRH43, which contains the cloned *osp*AB genes of *B burgdorferi* was used for a PCR positive control. This plasmid was grown in *Escherchia coli* (DH5 alpha) in Luria-Burtani broth and plasmid DNA was isolated using Quiagen columns (Chatsworth, CA). PCR primers were synthesized on the Applied Biosystems 394 DNA synthesizer with the trityl-on synthesis and purified with OPC columns (PE Applied Biosystems, Foster City, CA). Primers PospA2(+) and PospA(2-) were chosen from the literature.[10] Initial PCR was performed by using 0.5 µg of each primer (1 µg/µL), and as template, either 0.1 µg of control plasmid, 5 µL of tick extract, or water (negative control). Taq polymerase was used as directed by the manufacturer (Life Technologies). A 50 µL reaction contained in addition to the primers and template, 5 µL of 10× Taq PCR buffer, 2 µL of 5 mM dNTP's, 3 µL of 50 mM $MgCl^2$, 0.5 µL Taq DNA Polymerase (5 U/µL), qs to 50 µL with Millipore purified dH_2O and 1 drop of mineral oil (Sigma, St. Louis, MO.). Initial cycle conditions were as follows: 1) combine reagents except enzyme and oil, heat at 95°C for 4 minutes; 2) spin in microfuge approximately 20 seconds, add Taq polymerase, oil; 3) heat for 72°C for 3 minutes followed by; 4) 35 cycles at 94°C for 30 seconds, 45°C for 45 seconds, 72°C for 2 minutes. The thermal cycling was performed in an OmniGene thermal cycler (National Labnet, Woodbridge, NJ). To further improve upon detection of *Borrelia* DNA in tick extracts, 10 µL of extract was used and the number of cycles was increased to 45. A band of 156 bp was expected upon analysis using 1% or 2% agarose gel electrophoresis and ethidium bromide visualization. Because of the initial inconsistent amplification, we modified our PCR protocol and used Elongase Taq polymerase (Life Technologies).

Control ticks

To validate our PCR system, we tested 20 control ticks: 13 known replete, infected nymphs, and 7 uninfected, unfed nymphs. DFA was performed on the 20 control ticks to test our ability to obtain similar results between PCR and DFA. Ten microliters of the tick homogenate was used for each analysis.

RESULTS

Our PCR analysis identified 12 out of 13 infected nymphs as positive and 7 out of 7 uninfected nymphs as negative. One infected nymph came up negative in our PCR. Perhaps blood and digested blood components inhibited the PCR reactions of the PCR-negative infected tick as has been suggested by others.[15] DFA analysis identified 13 out of 13 infected nymphs as infected and 7 out of 7 uninfected nymphs as negative.

Over 300 *Ixodes* were collected from Watchung Reservation. To illustrate the effort involved, a 30 minute flagging session, which took place October 29, 1995, yielded 32 adults. Although not performed on the same day, similar flagging efforts in nearby Nomahegan or Lenape parks (in Union County) routinely proved to be less successful.

We tested 95 adult *Ixodes* that were collected in 1994 and 1995 and found an infection rate of 52% when using PCR (Table). A subset of those PCR-tested ticks (n=69) were assayed by DFA and 60% were found to be infected. In this subset of 69, 15 ticks did not give concordant results between PCR and DFA. Of these discordants 11 were negative by PCR yet found to be positive using DFA; the remaining 4 were found to be positive by PCR yet negative by DFA. Using PCR to determine infectivity, 33 of the 52 females (63%) from the 1994-95 lot tested were found to be positive for *B burgdorferi*, while 17 of 43 males (39%) were infected as determined by PCR. This observation was significant (X^2=5.4, P=0.02). An example of amplification of the *ospA* target in questing adult ticks from the 1995 sampling is shown in the Figure. The *ospA* bands are somewhat diffuse due to the low percentage of agarose (1%). A similar survey was performed in 1996 where we tested 28 adult ticks by PCR and found 16 (57%) were infected with *B burgdorferi* (Table). All 28 of these were tested by DFA and 14 were found to be

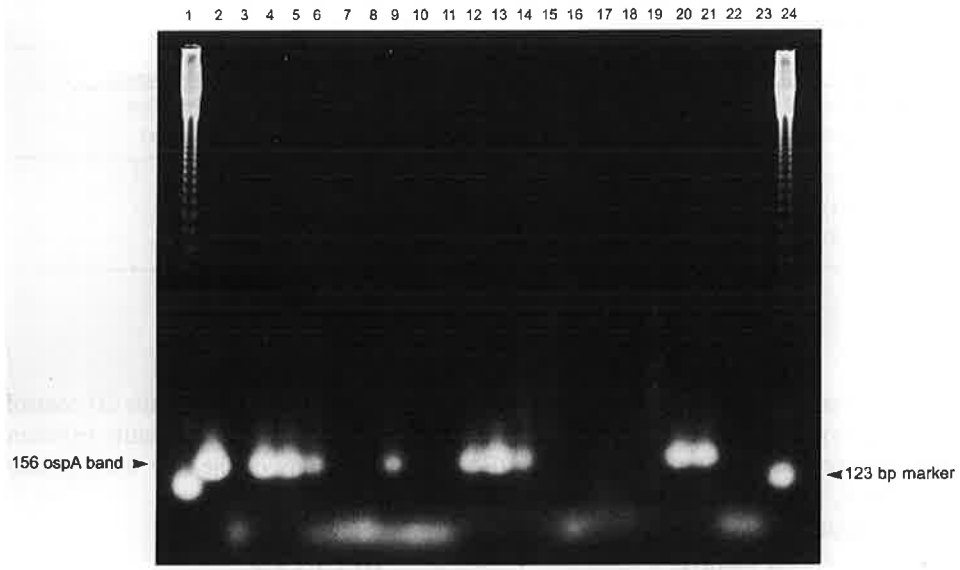

Figure. Electrophoretic analysis (1% agarose) of adult ticks collected from Watchung Reservation. Lanes: 1 and 24 DNA marker (123-bp ladder); 2, ospA-PCR positive control (pTRH43); 3, PCR negative control (H_2O). Lanes: 4, 5, 6, 9, 12, 13, 14, 20 and 21 show the 156-bp ospA band indicating a B burgdorferi*-infected tick. Bands below the 123-bp marker are most likely primer-dimers.*

infected. Of these 28 ticks tested there were four discordants. Three of the discordants were positive by PCR and negative with DFA. The fourth discordant was negative by PCR yet positive by DFA. Females tested by PCR from the 1996 series were shown to have an infection rate of 72%, while 47% of males were infected.

DISCUSSION

We have identified a public park harboring a population of *Ixodes* ticks infected with a *B burgdorferi* as determined by PCR and DFA. Although there was only a moderate concordance between the two techniques, it is believed that >50% of these adult ticks are truly infected. Females from both samplings showed a higher infection rate (65%) than male ticks (41%). This may be important from a public health standpoint because, although nymphs are implicated in transmitting most LD cases, female *I scapularis* have been found feeding on humans (male black-legged ticks do not usually feed). The most simple explanation for the difference in infection rates (by PCR or DFA) between the two samplings is probably because of the sampling size. The larger sampling size from 1994-5 is probably more representative of the actual tick population in the park. An alternative explanation for higher infection rates in females in comparison to males is based on the different feeding patterns of nymphs destined to become females. Dusbabek[16] has shown that in *I ricinus*, the vector of *B burgdoferi* in Europe, there exists a sexual dimorphism in the nymphal stage. More importantly, the feeding time and engorgement weight of female nymphs were greater when compared to male nymphs. This increased feeding time could, in theory, commensurately increase the chances of infecting nymphs as they feed on *B burgdorferi*-infected hosts, resulting in a population of females with greater infection rates than their male cohorts.

The concordance PCR and DFA of ticks sampled during 1994-96 was only 78%. In their survey of New Jersey ticks, Sun et al,[17] report a concordance of >96% between darkfield microscopy (DM) and PCR. Specifically, they tested 165 ticks and found that 29 (17.6%) were positive by PCR and 28 (17%) were positive by DM with only three discordant ticks. This high concordance may have been because their target for PCR was the 16s rDNA gene and not *ospA*. Concordance between PCR and DFA of the ticks sampled in 1996 improved slightly (86%) when compared to the 1994-1995 series. This was possibly because of the refinement of technique in addition to the use of Elongase in place of Taq polymerase.

One possible explanation for a tick that tests negative by DFA yet positive by PCR, is strain variations. Variation is evident in *Borrelia* strains from different geographic regions.[18] In addition, *B burgdorferi* isolates have been shown to alter their outer surface proteins depending on their environment.[19] The *Borrelia* isolates detected in this work are vector associated. The commercially available antibody used for this work is made to *B burgdorferi* 297, which was originally isolated from cerebral spinal fluid (host-associated), and is presumably grown in vitro when used to raise antibody. An alternative explanation for positive PCR with negative DFA is that PCR is ostensibly more sensitive than DFA and may detect ticks with very low levels of *Borrelia*, too low to detect microscopically.

DFA has shown to be slightly more sensitive than PCR in this study. This seems unusual when it is generally

accepted that PCR can detect *B burgdorferi* target sequences in living or dead spirochetes, while antibody analysis is thought only to detect live spirochetes. A possible reason for ticks providing a positive DFA result while being PCR-negative could be because of the lack of *ospA*. In fact one such isolate from New Jersey was recently isolated from *I scapularis* and characterized.[20] Even if present, *ospA* variation in the nucleotide sequence (ie, PCR primer annealing site) may explain why one specimen is positive by DFA yet negative by PCR.

Many factors determine the entomological risk for transmission of *B burgdorferi*, one such factor being, seasonal prevalence and abundance of the life stage of tick being studied. The nymphal stage is implicated in transmitting the majority of human Lyme cases because of their small size and their spring/summer emergence. The adult stage plays less of a role in human cases because of their temporal abundance (cooler weather) and their larger size, which makes them easier to detect. A second major factor in determining entomological risk for LD transmission is infection rates of *Ixodes* ticks. As mentioned, nymphs are more important in determining risk for LD, yet we chose to study the adult population, which usually have higher infection rates than nymphs because they have fed twice, while nymphs have fed only once. We have found that >50% of adult *Ixodes* ticks in this Union County park are infected with *B burgdorferi*. *Ixodes* infection rates in Union County are similar to those of Monmouth County and illustrate the need for more tick monitoring and public education. Like the situation in Perrineville, there may be a lag in the correlation between tick infection rates and incidence of human disease. The public health implications of this data are that an assumed "low-risk" county such as Union County has a tick population with the same infection rate as high-risk counties, and suggest that precautionary measures be taken when using certain outdoor recreational facilities. The focal nature of LD is such that in a hyperendemic state, a given locale should be surveyed to determine whether there is a risk of contracting LD from *B burgdorferi*-infected *Ixodes*.

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An Understanding of Laboratory Testing for Lyme Disease

Nick S. Harris, PhD, ABMLI

INTRODUCTION

While Lyme disease is a clinical diagnosis, the laboratory can provide useful and necessary information for the diagnostic process. The question is how does one use the laboratory in the most appropriate and cost effective manner? The following examples stress the need for an understanding of the tests available and when and why to use them.

HYPOTHETICAL CASE REPORT

A female patient in New Jersey presents to a university rheumatologist with symptoms of arthralgia and myalgia, fatigue and malaise, rash, photosensitivity, mild cognitive dysfunction, and nonspecific gastrointestinal complaints. After a thorough physical examination, the rheumatologist orders a WBC, multiple analyte chemistry panel, sedimentation rate, ANA with a reflux to ENA if the ANA is positive, rheumatoid factor, anticardiolipin, C3, C4, VDRL, urinalysis, and perhaps some joint x-rays if the physical diagnosis is supportive.

This same patient also noted that one of her neighbors contracted Lyme disease and believes she may have it as well. The rheumatologist then added a screening test for Lyme disease, either ELISA or an IFA quantitative (titer) test. If the ELISA or IFA were negative, the chance of a diagnosis of Lyme disease in this patient would be remote because the current dogma[1-3] is that Lyme disease is a rather rare event in most parts of the country, especially in the absence of a positive ELISA or IFA screening test.

BACKGROUND

There have been some good reviews[4-6] prior to 1994 on the laboratory aspects of diagnosis, but most of these were written before the politicizing of the diagnostic process during the CDC/ASPHLD meeting in Dearborn Michigan.[7] Prior to 1994, the CDC recognized Lyme disease from a set of clinical symptoms and a general set of laboratory findings. A certain combination of these criteria would lead to diagnosis of Lyme disease that could be reported to the CDC. The Dearborn meeting changed that.

The original clinical case definition[8] from the CDC for Public Health Surveillance and reporting of Lyme disease was:

Clinical Criteria:

A. Erythema Migrans; or

B. At least one late manifestation of musculoskeletal, nervous or cardiovascular system disorder; and laboratory confirmation.

Laboratory Criteria:

A. Isolation of *Borrelia burgdorferi* from clinical specimens; or

B. Demonstration of diagnostic levels of IgM and IgG antibodies to the spirochete in serum or CSF (Western blot, ELISA, IFA), or

C. Significant changes in IgM or IgG antibody response to *Borrelia burgdorferi* in paired acute- and convalescent-phase serum samples.

These criteria placed great emphasis on the presence of an Erythema Migrans (EM) rash. It is usually accepted that a physician's diagnosis of an EM on a patient from an endemic area is extremely useful for diagnosing Lyme disease; almost a third of the patients actually do not have an EM.[9-11] In addition, the variability of the EM rash[12,13], such as its duration, nonpruritic and nonpainful nature, and its location in obscure areas (axilla and hair regions) inhibit its use as a consistent diagnostic marker.

In 1995, the CDC added the additional recommendation from the CDC/ASPHLD meeting[7] of a two-tiered approach for reporting active disease and previous infection. That requirement means that a positive *sensitive* ELISA/IFA must be followed by a positive Western blot with a defined number of approved antibody bands. If the intention were only for public health surveillance and reporting of disease, these changes would not have caused

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a problem. Unfortunately, these recommendations became the standard in most areas and especially with insurance companies. That was unfortunate because the Dearborn meeting was not supposed to be about setting national standards for Lyme disease diagnosis; rather, it was to be a discussion regarding the Western blot during early Lyme disease. The majority of patient samples used to set the criteria were from patients being followed for four months following their diagnoses. The patients considered for entry in the study had an EM rash and either arthritis or neuroborreliosis.

Lyme disease is a problematic diagnosis because it is a complicated clinical entity. The position by the CDC makes it more complex. Some patients do not elicit an antibody response great enough to be positive by the currently available ELISA assays. Recent studies[14] by the group responsible for Lyme disease proficiency testing for the College of American Pathologists (CAP) came to the conclusion that the currently available ELISA assays for Lyme disease do not have adequate sensitivity to meet the two-tiered approach recommended by the CDC/ASPHLD group.[7] In addition, Bakken et al stated that a screening test must have sensitivity >95% to adequately screen for Lyme disease and that the currently available ELISA tests do not meet this criteria. Furthermore, if patients are treated early with antibiotics, their antibody response may be reduced or curtailed.[15] The initial mild flu-like symptoms may be overlooked. Later, if the symptoms return, most of the antibody markers have disappeared. The picture is not entirely bleak if Lyme disease is approached for what it is: *a complicated clinical entity, which requires multiple laboratory tests to assist in the diagnosis.* Thus, if clinicians use multiple tests (ie, both screening and confirmatory Western blot assays, antigen-capture and PCR), as they do in other disease entities there will be fewer problems with the diagnosis and fewer patients will be missed.

The presence of detectable spirochetes in infected tissue is rare. The characteristic sparsity of organisms contributes to the difficulty of getting blood or tissue to grow the Lyme bacterium.[15] A positive culture may not be a predictor of an antibody response. Rawlings[16] followed a group of 14 patients in which she was able to culture *B burgdorferi,* but only 3 of those patients had positive antibody titers. Aguero-Rosenfeld et al[12,13] showed that only 70% of the documented Lyme patients in their study had a significant antibody response. They suggested that the degree of antibody response might be related to the length of time the EM rash persists. They also saw only a 64% rate of IgM to IgG seroconversion.

Early reports suggested that considerable interlaboratory and intralaboratory variability exist in Lyme disease testing.[17-19] However, a review of the 1996 Lyme proficiency results by CAP (College of American Pathologists) and those by New York State demonstrate comparable agreement between the laboratories, similar to other bacterial infections and autoimmune conditions.

Table 1. Assays for Lyme disease.

Direct
Biopsy
Culture
Antibody Assays
IFA
ELISA
Western blot
Antigen Assays
Antigen-Capture
PCR

RESULTS AND DISCUSSION

Table 1 presents the type of tests that are most commonly available for Lyme disease. To provide adequate support for the clinical evaluation, multiple tests should be used. Not only is a correct diagnosis advantageous for the patient, but also ultimately is the most cost effective.

Indirect fluorescent antibody (IFA)

B burgdorferi spirochetes are affixed to glass slides and usually a fluorescent-conjugated goat antihuman immunoglobulin of either IgM or IgG specificity is used.[20] Tests for Lyme disease using IFA have received mixed reviews and some authors believe that the interpretation of IFA assays are overly subjective and that the tests are either functionally insensitive for Lyme-specific antibodies or display considerable cross-reactions with antibodies to other spirochetal organisms.[21,22] Magnarelli et al[23,24] and Mitchell et al[20] supported IFA if used in conjunction with a clinical evaluation. Mitchell's study, with the IgM-IFA showed excellent specificity and no observed cross-reactivity with infectious mononucleosis (n=20), rheumatoid arthritis (n=19), systemic lupus (n=22), syphilis (n=13), streptococcal sequelae (n=20) or healthy subjects. Mitchell related the success of this test to the quality of the substrate slides and the level of experience of the technologists, and concludes that IFA microscopy becomes less subjective with experience.

Enzyme-linked immunosorbant assay

ELISA for *B burgdorferi* has been available since 1984.[25] Most commercial assays use a whole cell sonicate of *B burgdorferi.* Complete descriptions of methods for a Lyme ELISA can be found in the publications by Craft et al[25] Magnarelli et al[23] and Russell et al.[21] Standard ELISA

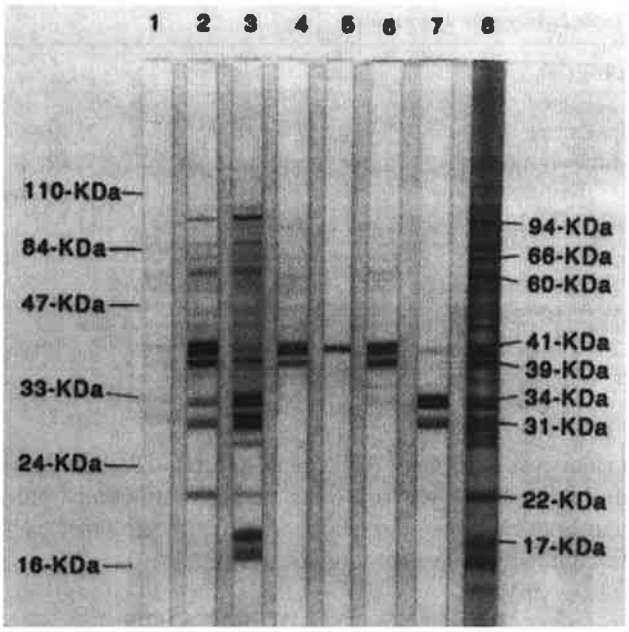

Figure 1. Western blots of B burgdorferi *B31 strain: The blots were: (1) stained with amino black, (2) reacted with rabbit antisera, (3) with goat antisera, (4-7) with various monoclonal antibodies, and (8) with pooled patient sera. Reproduced by permission from Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by Western immunoblot: reactivity of various significant antibodies against* B burgdorferi. *J Clin Microbiol 1992; 30:370-76.*

techniques have been employed[26] in all these assays.

There are a large number of commercial ELISA tests available. A review of past proficiency events by CAP and the NYS Health Department show the relationship between the various tests. Most commercial ELISA tests have comparable sensitivity and specificity because they were made to compare to one another for the FDA 510K process. However, most are inadequate as a screening test because they were not designed by the manufactures to be sensitive at the 95% level which is required for screening.[14] A substantial change in the 510K approval process would be required to make the ELISA's for Lyme disease diagnosis more sensitive.

The goal for a new generation of ELISA's should be sensitivity for the more unique and specific *B burgdorferi* antigens that are visualized in the Western blot (Figure 1). They are Osp A (31 kDa), Osp B (34 kDa), Osp C (23-25 kDa), 39 kDa, and 93 kDa.[27-32] Initially, some investigators identified 93 kDa as 94 kDa and Osp C as 22 kDa. While most ELISA's do have reactivity to these antigens, because they are prepared with a sonicate of *B burgdorferi,* they also have reactivity against 41 kDa, 58 kDa, 66 kDa, and 73 kDa. While the latter antigens are components of *B burgdorferi,* they also have considerable cross-reactivity to other spirochetes, heat-shock proteins, and some viruses.[33]

All borderline and positive ELISA assays (polyvalent, IgG only, and IgM only) for Lyme disease must be confirmed by a high quality Western blot for *B burgdorferi.* A 56% false-negative rate, depending upon the commercial kit, was found by Luger and Krause,[18] as compared to their own clinical diagnoses. Golightly et al[34] saw a lack of sensitivity with a 70% false-negative rate in early Lyme disease and from 4% to 46% with late manifestations of Lyme disease. These results support the necessity of Western blot confirmation for both positive and negative Lyme ELISA.

B burgdorferi Western blotting

The immunoblot or Western blot (Figure 1) for *B burgdorferi* is the most useful antibody test available when performed in a quality laboratory by experienced testing personnel. It is necessary to evaluate separately both the IgM and IgG antibodies of *B burgdorferi.* The study by Ma, et al[35] gives an excellent overview of the technique and provides comprehensive information about the antibodies seen in Lyme disease patients versus the normal and non-Lyme disease groups.

Figure 2 illustrates a group of IgG-IgM Western blots (lanes 1-48) from clinically confirmed Lyme patients with various levels of antibodies to *B Burgdorferi.* In this figure are (lanes 48-57) IgM Western Blots to *B burgdorferi.* While some of these patients have different patterns of antibody reactivity, all were confirmed, clinically positive Lyme patients with physician-diagnosed EM. The variability in the Western blot is characteristic of the variability observed in the immune response of other diseases (eg, Hashimoto's thyroiditis, SLE, Sjogren's syndrome, scleroderma). Our own clinical study of 186 defined patients and 320 negative controls (Figure 3) demonstrated excellent sensitivity and specificity for IgM using any two of the following bands: 23-25 kDa (OspC), 31 kDa (OspA), 34 kDa (OspB), 39 kDa and 41 kDa.[35] This study also demonstrated good specificity and sensitivity for IgG using any two of the above bands. The 83/93 kDa antibody could also be included as one of two IgG bands.

It is difficult for each laboratory to perform clinical studies and establish its own ranges for normal and disease populations. For this reason, the CDC assembled a group of academic scientists with the assistance of the FDA and the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) to reach a consensus on certain criteria for the Western blot. After several meetings they arrived at the CDC/ASPHLD consensus criteria presented in Table 2.[7,36] These criteria were based in large part on the work of Dressler et al,[37] using well-defined patients with active Lyme arthritis, or neuroborreliosis. Interestingly, in their publication none of the three CDC/ASPHLD recommended strains of *B.*

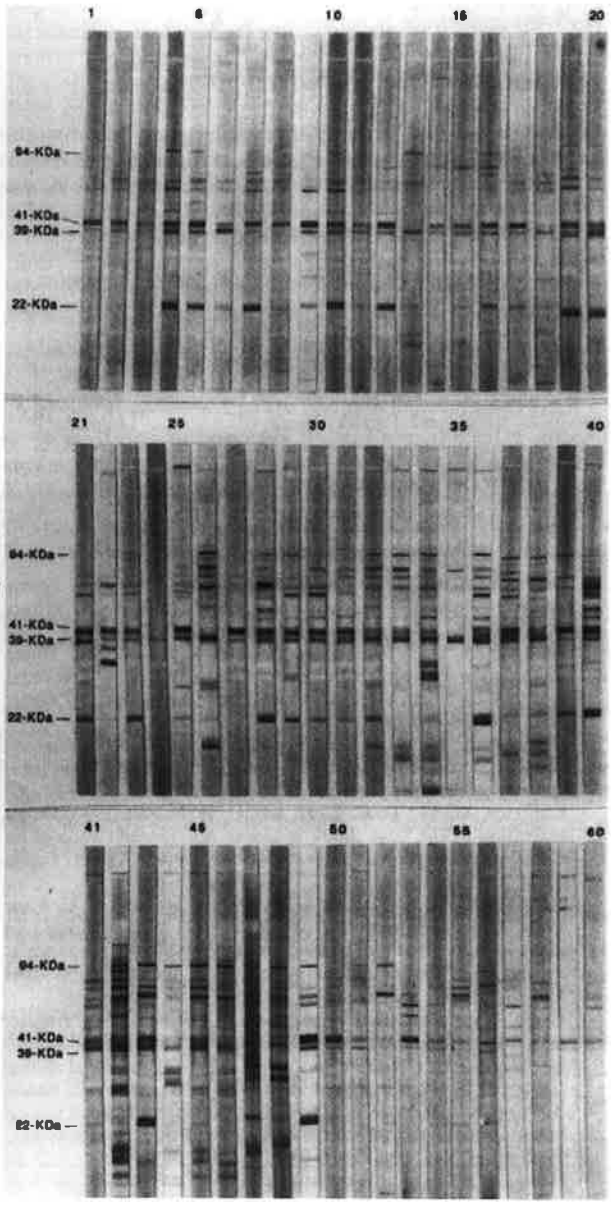

Figure 2. Western blots to B burgdorferi *from various patients with Lyme disease Lanes 1-48 are IgG/IgM blots from clinically confirmed patients with various levels of antibodies. Lanes 48-57 are IgM-only blots. Reproduced by permission from Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by Western immunoblot: reactivity of various significant antibodies against* B burgdorferi. *J Clin Microbiol 1992;30:370-76.*

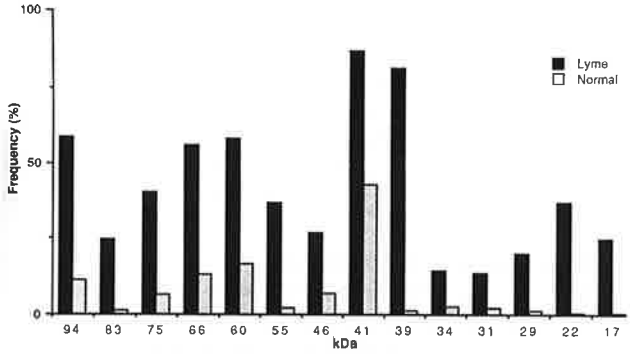

Figure 3. Comparison of antibody reactivity to various B burgdorferi *antigens. The dark bars are from 186 patients with clinically confirmed Lyme disease and the light bars are from 320 normal controls. Reproduced by permission from Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by Western immunoblot: reactivity of various significant antibodies against* B burgdorferi. *J Clin Microbiol 1992;30:370-76.*

burgdorferi (B31, 297 and 2591) were used. Rather, they used G39/40 with a 10% acrylamide gel, although a gel with less than 11% of acrylamide does not have enough resolution nor definition of all the important antigens of *B burgdorferi*.

The criteria for a positive Western blot to *B burgdorferi* developed by the CDC/ASPHLD are very conservative and require 5 of 10 antibody bands for IgG positivity; the original recommendations do not even recognize equivocal or borderline results if less than five bands are detected. Their cut-off assumes that all Lyme patients have similar immune systems. They ignore the diversity of the immune response seen in other diseases. Their studies were problematic in that they primarily focused on patients with early (usually within four months of an EM) Lyme disease. They drew blood in most patients every two weeks during this four-month period and any positive event (five out of ten bands) was counted as a positive patient, even if they were negative at a different time of the study. In addition, the criteria include antibodies to 41 kDa, a common antigen of most flagella-bearing organisms, and exclude two of the most important and specific antigens, 31 kDa (OspA) and 34 kDa (Osp B), which appear later in the response. A review by Hilton et al[38] on a group of 50 patients with confirmed Lyme disease showed that they would have missed 4 patients by excluding 31 kDa (OspA) and 34 kDa (OspB). The author's own laboratory would have missed 2 of 18 proficiency samples by excluding antibodies to these two antigens.

Engstrom et al[11] and Aguero-Rosenfeld et al[12,13] confirmed that almost one-third of all Lyme patients are IgG seronegative during the first year. Two years after physician-diagnosed EMs, 45% of the patients were negative by ELISA. In another study, Aguero-Rosenfeld et al[13] showed that the ELISA response declined much more rapidly than the Western blot response. Their study also demonstrated that the two-step protocol of the CDC/ASPHLD criteria would fail to confirm infection in some patients with culture-proven EM. Furthermore, although a majority (89%) of patients with EM rash developed IgG antibodies detected by Western blot sometime during disease, only 22% were positive by the crite-

Table 2. CDC/ASPHLD criteria for the serologic diagnosis of Lyme disease.

Test Performance and Interpretation

Recommendation 1.1. Two-Test Protocol

All serum specimens submitted for Lyme disease testing should be evaluated in a two-step process, in which the first step is a sensitive serological test, such as an enzyme immunoassay (EIA) or immunofluorescent assay (IFA). All specimens found to be positive or equivocal by a sensitive EIA or IFA should be tested by a standardized Western Blot (WB) procedure. Specimens found to be negative by a sensitive EIA or IFA need not be tested further.

Recommendation 1.2. WB Controls

Immunoblotting should be performed using a negative control, a weakly reactive positive control, and a high-titered positive control. The weakly reactive positive control should be used to judge whether a sample band has sufficient intensity to be scored. Monoclonal or polyclonal antibodies to antigens of diagnostic importance should be used to calibrate the blots.

Recommendation 1.3. Testing and Stage of Disease

When Western immunoblot is used in the first four weeks after disease onset (early Lyme disease), both IgM and IgG procedures should be performed. Most Lyme disease patients will seroconvert within this four week period. In the event that a patient with suspected early Lyme disease has a negative serology, serologic evidence of infection is best obtained by testing of paired acute- and convalescent-phase samples. In late Lyme disease, the predominant antibody response is usually IgG. It is highly unusually that a patient with active Lyme disease has only an IgM response *Borrelia burgdorferi* after one month of infection. A positive IgM result alone is not recommended for use in determining active disease in persons with illness of longer than one month duration, because the likelihood of a false-positive tests result is high for these individuals.

Recommendation 1.4. WB Criteria

Use of the criteria of Engstrom et al [11] are recommended for interpretation of IgM immunoblots. An IgM blot is considered positive if two of the following three bands are present: 24 kDa (OspC), 39 kDa(BmpA), and 41 kDa(Fla).

Monoclonal antibodies to these three proteins have been developed and are suitable for calibrating immunoblots.[7]

Once antibodies are developed to the 37 kDa antigen, this protein could be used as an additional band for IgM criteria (> 2 of 4 bands).

Interim use of the criteria of Dressler et al[37] are recommended for interpretation of IgG immunoblots. An IgG blot is considered positive if five of the following ten bands are present: 18, 21 (OspC), 28, 30,m 39, (BmpA), 41 (Fla), 45, 58 (not GroEL2), 66 and 93 kDa. Monoclonal antibodies have been developed to the OspC, 39 (BmpA), 41 (Fla), 66, and 93 kDa antigens and are suitable for calibrating IgG immunoblots.[7]

The apparent molecular mass of OspC is recorded above as it was denoted in the published literature. The protein referred to as 24 kDa or 21 kDa is the same, and should be identified in immunoblots with an appropriate calibration reagent (see 1.6).

Recommendation 1.5. Reporting of Results

An equivocal or positive EIA or IFA result followed by a negative immunoblot result should be reported as negative. An equivocal or positive EIA or IFA result followed by a positive immunoblot result should be reported as positive.

An explanation and interpretation of test results should accompany all reports.

Recommendation 1.6. Standardization of WB Nomenclature

The apparent molecular mass of some proteins of *Borrelia burgdorferi* such as OspC will vary depending on the *B burgdorferi* strain and gel electrophoresis system used. The molecular weights of proteins of diagnostic importance should be identified with monoclonal or polyclonal antibodies.[11] When possible, the molecular weight of the protein should be followed by the descriptive name (eg, Osp C).

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ria of the CDC/ASPHLD.[13] The Engstrom et al study[11] did not use the IgG blot criteria of the CDC/ASPHLD. They found that 2 of 5 bands gave them specificity of 93 to 96% and a sensitivity of 100% in the 70% of patients who made antibody. This might imply that they would have had even less sensitivity had they used the more stringent CDC/ASPHLD criteria.

The CDC/ ASPHLD criteria[7,36] for a positive IgM Western blot include the 23-25 kDa (OspC), 39 kDa and the 41 kDa, but exclude the 31 kDa (OspA) and 34 kDa (OspB). During the presentation at the Dearborn meeting,[7] the specificity of the IgM Western blot was reported to be greater than 95%, based on several hundred negative controls. Engstrom et al[11] reported specificities of their IgM Western blot to be between 92% and 94%. It has been reported that the IFA and ELISA IgM assays may show cross-reactivity with ANA, EBV, and spirochetal infections.[24] However, studies by Mitchell et al[20] and Ma et al[35] did not observe this with their IFA and Western blot assays respectively.

A major disagreement with the CDC/ASPHLD group is with its statement that the IgM Western blot should only be used during the first month after tick bite. They have overlooked their own reported excellent specificity of the IgM Western blot. The author's laboratory,[39] studies by Steere,[28] and Jain et al[40] point to the importance of the IgM Western blot in recurrent and/or persistent disease. Sivak et al[41] found that the IgM Western blot had a 96% speci-

ficity if the patients had at least a 50% probability of having Lyme disease. A study by Oksi et al,[42] using culture and PCR to confirm Lyme disease, reported that specific IgM to *B burgdorferi* is sometimes the only antibody detected in persistent disease. They felt that this data supported the idea that some Lyme patients have a restricted IgM-only response to *B burgdorferi* Lyme disease.

It is important to note that a positive IgG and/or IgM Western blot only implies exposure to *B burgdorferi*. It is only part of the test battery and is not confirmatory for Lyme disease. It does not mean the patient has Lyme disease; that is a clinical diagnosis. It must also be kept in mind that these antibody tests are not static; they change over time. A patient negative in the Western blot may seroconvert to a positive pattern with treatment. Conversely, a patient could redevelop an IgM response, suggestive of a recurrent infection.

Antigen and antigen-capture assays for Lyme disease

Several studies, using mice, rats, guinea pigs, and dogs have found *B burgdorferi* antigen in the urine of naturally occurring and experimentally induced Lyme infections.[43-46] Dorward et al[44] and others[43,47] also detected antigen in the urine of patients with Lyme disease. Dorward's study[44] indicated that pieces or blebs of *B burgdorferi* were more commonly found in urine than was the entire organism. Coyle et al[48] has successfully used antigen-capture with monoclonal antibodies to 31 kDa (OspA) and 34 kDa (OspB) to detect antigen in the cerebrospinal fluid (CSF) of patients with neuroborreliosis.

Harris and Stephens have presented information about the development[49] and use of antigen-capture for the detection of *B burgdorferi* antigen in the urine of Lyme patients.[50] The antibody used in this antigen-capture is a unique polyclonal antibody that is specific for the 31 kDa (OspA), 34 kDa (OspB), 39 kDa, and 93 kDa antigens of *B burgdorferi*. The assay appears to be very specific for these antigens of *B burgdorferi*, and in 408 controls there was less than a 1% false positive rate. Futhermore, blocking and interference studies with human RBCs, WBCs, whole blood, serum and human serum albumin showed no effect on the urine or CSF antigen-capture assay.[50]

Urine and serum from 251 patients with Lyme disease (confirmed after a physician-diagnosed EM rash) were studied for the concurrence of a positive ELISA and a positive antigen test. In Table 3 it can be seen that 30% of this group of Lyme disease patients had a positive Lyme Urine Antigen Test (LUAT), but a concurrent positive IgG/IgM ELISA was only seen 8% of the time. Other studies[51] have suggested that antigenuria may not be a constant daily occurrence. Therefore, multiple sampling days for urine may be more effective for detecting antigenuria[39] than a single collection.

Table 3. Patients with physician diagnosed EM n=251.

History of tick bite	33/251	53%
>3 other symptoms	204/251	81%
History of arthritis	177/251	71%
Positive concurrent ELISA	19/251	8%
Positive LUAT	75/251	30%

Harris NS, Stephens BG. Detection of Borrelia burgdorferi *antigen in urine from patients with Lyme borreliosis. J Spirochet Tick-Borne Dis 1995;2:37-41.*

Polymerase chain reaction(PCR)

The PCR assay for *B burgdorferi* looks for the presence of *B burgdorferi* DNA commonly in blood, CSF, urine, and synovial fluid. There are many published articles that provide background[52-56] to this topic.

As mentioned previously, Lyme disease is characterized by a sparsity of organisms.[15] Some laboratories perform the genomic assay, which requires a minimum of one recoverable bacterium, or at least the DNA from one. A plasmid PCR assay is also available from some laboratories. Dorward et al[44] using an immune electron microscopic technique, detected pieces of antigen rather than intact organisms in urine and other tissues. In an earlier study, Garon, Dorward, and Corvin[57] detected blebs or membrane vesicles shed from the surface of *B burgdorferi*. These blebs contain the same antigen as the intact organism (Dorward, personal communication). These blebs and fragments of *B burgdorferi* antigen may be the reason that the antigen capture and plasmid PCR demonstrate great practical sensitivity. Nocton et al[54] reported on the use of a plasmid PCR that had excellent sensitivity in the synovial fluid of patients with Lyme arthritis.

Studies by Goodman et al[55] found that 30% of their patients with early Lyme disease were positive by PCR. This is comparable to blood culture data by other groups.[58] However, some groups cannot find positive cultures or positive PCR from patients with acute Lyme disease.[59] This is definitely an area that is technique dependent. Manak et al[60] was able to detect 33% of early Lyme patients and 50% of late stage Lyme disease in patients not on antibiotic therapy. Most patients become PCR negative within two weeks of antibiotic therapy. They also saw that during a relapse, patients might become PCR positive for a short period of time.

Schmidt et al[60] found that urine samples from 22 of 24 patients with an untreated EM rash were positive using a nested PCR for *B burgdorferi* sensu stricto as well as reactive to *B garinii* and *B afzelii* but not *B hermsii*. Immediately after the initiation of therapy (minocycline,

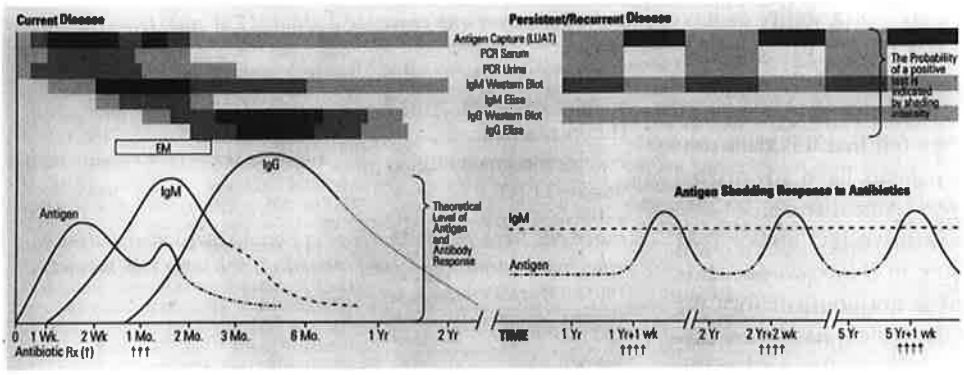

Figure 4. A model suggesting the tests applicable in different phases of Lyme disease. The left side of the figure indicates the hypothetical patient making antibodies after infection. Less than 70% of patients are in this category. The responses shown on the right side of Figure 4 pertain to many of the patients with recurrent/persistent disease. Courtesy of IGeneX, Inc. Reference Laboratory, Palo Alto, CA.

100 mg BID for 14 days) 58% were still PCR positive. Twenty weeks after therapy, none of the patients were positive. Bayer et al,[61] on the other hand, using a combination of genomic and plasmid PCR on urine samples, found that 74% of patients with chronic (persistent) Lyme disease were PCR positive. These patients had been treated between three weeks and two months continually with antibiotics, but were off antibiotics one week prior to the test.

Which tests to use?

The physician should have a logical plan for choosing the laboratory tests to be used initially, and what type of follow-up tests to order if additional information is needed to aid in the diagnosis of Lyme disease. Similar to the hepatitis model, *B burgdorferi* antigen is present early after infection. *B burgdorferi* DNA in urine has been detected by PCR within the first few weeks after infection.[56] Studies by the author using LUAT have found antigen as early as three days after a tick bite (unpublished observations).

Within two to four weeks after infection, an IgM antibody response to *B burgdorferi* may be detected in 60-70% of the patients. This is followed by a specific IgG response, which may remain detectable for a few months or in some cases, a few years. In the early period, especially during the EM, it may also be possible to detect by PCR *B burgdorferi* DNA in the urine and/or blood.[55,56]

To evaluate a new patient at any stage of disease, at least an IgM and an IgG Western blot must be performed. For completeness, an ELISA or IFA screening test may also be ordered. Contrary to popular thought,[1,2] most ELISA's and IFA's do not have enough sensitivity to be used as a screening test.[14] The Western blot is more sensitive and specific. The increased sensitivity of the Western blot is analogous to a mountain where the base is a Western blot and the summit is an ELISA. The Western blot has considerably more sensitivity because it provides detection before the peak of the response. As mentioned before, the Western blot is a qualitative assay based upon a visualization of a patient's antibody against the various unique *B burgdorferi* antigens. This type of assay should not be restricted by the same sensitivity and specificity concerns as a general screening test. An ELISA with a quantitative or semi-quantitative cut-off is usually not specific to only the unique *B burgdorferi* antigens. However, an ELISA assay developed to cloned antigens of *B burgdorferi,* would most likely have more analytical sensitivity than the Western blot.

Some of these relationships may be seen in the hypothetical model of an "idealized" *B burgdorferi* infection (Figure 4). The left side of the figure may be valid early in disease in the two-thirds of patients making antibody. In the other third of patients, or later than the first year or with persistent/recurrent disease, the right side of Figure 4 may pertain. Therefore, a specific battery of tests (as used with other diseases such as hepatitis, thyroid dysfunction, or autoimmunity) provides a more complete picture to help with the clinical diagnosis and is ultimately more economical for the patient.

Persistent/recurrent (chronic) infection is a unique diagnostic problem because the IgG response may be absent in more than 50% of the patients.[11-13] Thus, in addition to the IgG Western blot, an IgM Western blot should be used. This technique has been helpful for some patients with persistent/recurrent disease.[28,42] The physician must rule out possible cross-reactions from rheumatoid factor, other spirochetal and tick-borne diseases, and infectious mononucleosis.[18,24] This can usually be accomplished during the differential diagnostic process. In addition, a recent study has indicated that the IgM Western blot may be as high as 96% specific, with almost a 93% predictive value of disease, if the patient has at least a 50% prior probability of Lyme disease.[41]

Assays that focus on antigen detection or DNA may be particularly useful[50] diagnostically during persistent/recurrent disease. Antigen capture in urine has been a useful diagnostic tool, especially during the initiation of new antibiotics, which seems to enhance antigenuria.[39] However, antigen capture assays in urine (LUAT) should

only be used after patients have been properly evaluated by sensitive antibody assays. Studies have shown[50] that patients seropositive to *B burgdorferi* have less antigenuria than seronegative patients.

The PCR and the Lyme Urine Antigen Test (LUAT) are sometimes complementary. As mentioned, patients responding to antibiotics may have a negative PCR. While a genomic PCR requires one recoverable bacterium or at least the DNA from one, studies at the Rocky Mountain National Laboratory[44] have shown that pieces of antigen are more commonly found in urine than are whole or semi-whole *B burgdorferi.*

In addition to Western blot, PCR and antigen capture can be used for testing the synovial fluid of inflamed joints, a common occurrence in Lyme disease. The plasmid PCR for *B burgdorferi*[54] in synovial fluid was used as a diagnostic aid for patients with Lyme arthritis. This study showed that 96% of the patients with untreated Lyme disease and those treated with only a short course of antibiotics had a positive PCR assay of their synovial fluid.

Tests for neurological Lyme disease

A wide range of neurological symptoms has been reported in Lyme disease. They include Bell's palsy, meningitis, meningoencephalitis, radiculoneuritis, encephalopathy, psychiatric syndromes, fatigue, multiple sclerosis-like symptoms, and Parkinson-like symptoms.[48,62-69]

Diagnostic assays for neurological Lyme disease must evaluate the CSF.[70-72] According to Coyle, the blood of the brain is CSF, and it is impossible to make a diagnosis of neurological Lyme disease without performing a spinal tap and analyzing the CSF for antibodies and antigens to *B burgdorferi* (personal communication). One assay that has been commonly used is the CSF to serum index; it is a combination of immunological tests that measures specific antibodies to *B burgdorferi* in both serum and spinal fluid. Calculations are based on the results of quantitation of IgG in both the serum and CSF, as well as the results of the CSF and serum ELISA. An index greater than one (>1.0) of the CSF/serum ELISA suggests *in situ* synthesis of antibody in the central nervous system. The use of an index is important because if a test were only performed on the CSF, there would be no control for leakage across the blood-brain barrier. Unfortunately, this series of tests uses the same flawed ELISA assays used on serum. Therefore, sensitivity is a concern. A positive result is serological evidence of neuroborreliosis, whereas a negative result indicates only that antibody was not detected, not the absence of disese.

Because of sensitivity concerns reported with the ELISA, the IgM and IgG Western blot is the antibody test of choice for the CSF, but the two tests require 2 ml of CSF. A positive result with either the IgG or IgM Western blot is serological support of neurological Lyme disease. A recent study[73] confirmed what has been observed for some time in the author's laboratory, that is: specific *B burgdorferi* proteins such as Osp A and C may also be seen in the CSF in early neurological Lyme disease using an IgM Western blot. Since it is always necessary to control for leakage across the blood-brain barrier, CSF Western blots should be compared to those on the patient's serum. Tests in the author's laboratory suggest that the detectable level of antibody, using standard techniques, with the Western blot is 50-100 ng/mL of specific antibody. This would imply that for practical purposes the CSF should contain at least 1 µg/mL of immunoglobulin before doing an assay.

PCR and antigen capture assays using CSF have been useful in some patients[47,48,74] with neurological Lyme, especially since some patients with neurological Lyme disease are negative for Borrelia antibody in the CSF.[63,68,74] These patients may also be negative for all assays in blood and urine. A recent study by Fallon et al[75] suggested that brain imaging using a single photon emission computed tomographic (SPECT) technique is another diagnostic approach for neurological Lyme disease.

Tests for associated tick-borne diseases

There appears to be an association between Lyme disease, ehrlichiosis and babesiosis,[76-81] and the same type of tests (IFA, ELISA, Western Blot and PCR) used for Lyme disease (Table 1) can be used for these associated tick-borne diseases. Usually, however, the IFA test is more commonly available.

Human ehrlichiosis is a disease caused by rickettsial type organisms transmitted by some of the same ticks that carry Lyme disease. Human granulocytic ehrlichiosis (HGE) has been closely linked to the bites of *Ixodes scapularis* and *Ixodes pacificus.*[82,83] Human monocytic ehrlichiosis has been linked to the bites of *Amblyomma americanum* (Lone Star tick).[82,83] Currently, IFA serology is performed using *E. chaffeensis*[84] for HME, and the closely related *E equii*[85] or the newly discovered organism[86] for HGE.

Ehrlichiosis usually presents with high fever, malaise, headache, myalgia, sweats, and nausea. These patients generally have high titers (> 1:1000) during or shortly after this acute disease. Those patients diagnosed with *Ehrlichia* should also be tested for Lyme disease, since the same tick transmits the disease and coinfections have been noted.[79,81]

Babesiosis is another disease transmitted by the same tick that carries *B burgdorferi.*[76-78,80] Symptoms of babesiosis are also similar to some of the symptoms of

Lyme disease: fatigue, malaise, myalgia, arthralgia, chills and fever. Usually the fever is high. This disease is particularly life threatening in splenectomized or immune suppressed patients.

Babesiosis is caused by an intraerythrocytic parasite, *Babesia microti,*[78,87] which is similar to *Plasmodium falciparum*, the causative agent of malaria. In fact, many of the symptoms and the appearance of ring shaped intraerythrocytic parasites in red cells stained with Giemsa or Wright's, often lead to the incorrect diagnosis of malaria. Serology by IFA is done using red cells from infected Syrian hamsters. The antibody titers are usually high (>1:640) in acute babesiosis, and the piroplasm can be seen in the red blood cells of patients. Seroconversion usually occurs between two and four weeks after infection.

Lower levels of antibody to *B microti, E chaffeensis,* and *E equii* have been seen in some patients diagnosed with Lyme disease. The significance of these antibodies is not understood and it is not known if they represent a subclinical infection of Babesiosis[95] or Ehrlichiosis associated with Lyme disease, or if they are merely low levels of insignificant antibody.

CONCLUSION

Antibody assays for Lyme disease will improve when recombinant antigens become available to the unique antigens of *B burgdorferi.*[30, 88-94] Individual recombinant antigens could then be added, one by one, to construct a series of highly sensitive (>95%) ELISA assays that also could have acceptable specificity (>90%). At such a time, a two-tiered testing procedure would make more sense. Furthermore, new genetic markers for *B burgdorferi* are being discovered and new PCR-like assays will become easier to perform in the laboratory.

Additional progress, however, will be slow in Lyme diagnostics, until we learn more regarding the biology of *B burgdorferi.* In the course of disease, long periods of remission are followed by acute symptoms, that may last for weeks or months. Therefore, basic research studies are needed to evaluate the cyclical nature of the disease, and the idiosyncrasies of the organism, such as where it may reside in extra-vascular spaces.

Science has progressed to the point where it effectively uses techniques associated with molecular diagnostics and genetics, but some of the traditional techniques may also be appropriate to study Lyme disease. Tissue culture studies provide one level of understanding of how the organism interacts with lymphocytes. The infection of research animals, such as mice and dogs, using ticks with radiolabeled *B burgdorferi,* may provide information in a homeostatic environment, where different types of cells and tissues can be studied. Progress for better diagnosis and treatment, in this very complex disease, will come through better knowledge of the spirochete *B burgdorferi.*

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Syphilis in the Shadow of HIV and Lyme Disease: the Laboratory Diagnosis of Syphilis

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INTRODUCTION

Syphilis, caused by infection with *Treponema pallidum* ssp *pallidum (T pallidum)* once a major problem, became a clinical rarity by the mid-1950s, only to resurface as a disease of interest and concern with the appearance of the human immunodeficiency virus (HIV) and the increase in the drugs for sex trade. The number of new cases of syphilis in the USA again decreased substantially. In 1996, 4/100,000 cases were reported[1] but new outbreaks may be expected. This recent decrease in the incidence of syphilis cannot be attributed to new diagnostic or treatment strategies. New molecular technologies, except for polymerase chain reaction (PCR), have not led to significant improvements in the laboratory diagnosis of syphilis.

Syphilis is a disease with various manifestations that change with duration of the illness. Clinically, syphilis has been divided into several stages that follow each other temporally in untreated patients (primary, secondary, latent, and tertiary). These stages have implications regarding diagnosis and treatment of syphilis.

There are many helpful tests for direct and indirect laboratory diagnosis, but there is no single optimal test. Some tests may have their optimal use at different stages of the disease. *T pallidum* cannot be cultured in vitro. Therefore, diagnosis must be made using available specimens such as exudates, body fluids or tissues for direct identification of the organism or examination of serum for antibodies. The antigenic structure of *T pallidum* is very complex and shares properties with other microorganisms. The antibodies produced as a consequence of *T pallidum* infection cross-react widely with other spirochetal pathogens, nonpathogens, and human tissue antigens hindering a correct serodiagnostic interpretation.

There have been a number of reviews describing techniques used in serodiagnosis of syphilis.[2-4] Our focus in this writing is on particular problems of diagnosis of early primary syphilis, neurosyphilis, congenital syphilis, coinfection with HIV, or infection with other spirochetes such as *Borrelia burgdorferi*, and the problems associated with Veneral Disease Research Laboratory (VDRL) antigen used for serodiagnosis of syphilis.

LABORATORY TESTS

Diagnosis of syphilis is based on clinical evaluation, detection of the organism, and confirmation of the disease by serodiagnosis. The variability of syphilitic lesions and the frequent atypical manifestations, make the detection of the organisms and serodiagnosis an essential service.

Direct detection of *T pallidum Darkfield (DF)*. One of the oldest, simplest, and still reliable methods for identification of *T pallidum* is darkfield microscopy. However, this technique has limitations in sensitivity; 10^3 to 10^4/mL organisms are required. Furthermore, not all lesions are visible (rectum) or appropriate (mouth) for reliable examination. Failure to detect *T pallidum* by darkfield examination does not indicate that the patient is free of syphilis. Detailed descriptions and critical evaluation of the technique have been published.[2-5]

Direct fluorescent antibody test (DFA). Fluorescent isothiocyanate (FITC)-labeled human or animal anti-*T pallidum* antibody, rendered treponema-pathogen specific, is used for detection of intact treponemes in body fluids and tissue sections. The sensitivity is similar to that of DF. A detailed description of this method is in references 2-5.

Polymerase chain reaction (PCR). The PCR technique has been used for identification of *T pallidum* in body fluids and tissues by a few investigators.[6-10] For the diagnosis of syphilis, in certain situations PCR is the most practical, sensitive, and specific technique available. PCR has

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*Table 1. Reactivity of standard tests used for serodiagnosis of untreated syphilis.**

Disease Stage	Percentage of Patients Reactive By VDRL	FTA-ABS	MHA-TP
Primary	78	84	76
Secondary	100	100	100
Latent	95	100	97
Late	71	96	94

** Percentages given are estimated averages inferred from numerous reports. With permission, from Reference 2.*

not been standardized. Interlaboratory differences are found in the source of primers and in the method of DNA extraction. In spite of differing methods, the sensitivity of PCR has been reported from several laboratories to be approximately 1-5 organisms per specimen.[6-10] PCR also has been successfully applied for detection of *T pallidum* in paraffin embedded tissues,[11] blood, cerebrospinal fluid (CSF), and amniotic fluid.[6-9,12] Application of PCR in experimental models of syphilis has yielded new information on persistence of infection,[13] and systemic dissemination of *T pallidum.*[14] The shortcoming of PCR is that it can not differentiate between live and dead organisms. The recently described RTPCR, targeting 16S rRNA for detection of *T pallidum*, may help to eliminate this problem.[15] However, PCR correlates well with the rabbit infectivity test (RIT), which detects only virulent organisms.[9,12,13] PCR is not a laboratory procedure to replace serodiagnosis, but it is extremely valuable in diagnosis of very early syphilis (seronegative with doubtful chancre or location of chancre prohibiting the use of DF or DFA), in congenital syphilis, in neurosyphilis where serodiagnosis is still unsatisfactory, and in examination of paraffin embedded tissues.

Silver staining. This staining, widely used by pathologists, is not specific for *T pallidum*. The silver nitrate will impregnate a number of various organisms and allows only for identification of the morphology of the organisms. Tissue artifacts also are potential hazards for misidentification.

STANDARD TESTS FOR SYPHILIS

Indirect confirmation of syphilis: antibody detection

In the course of infection with treponemes, two types of antibodies are produced, cardiolipin antibodies, frequently referred to as nontreponemal, and treponemal antibodies. They are detected by various methods.

Cardiolipin antibodies. Cardiolipin antibodies react with an antigen consisting of cardiolipin-lecithin-cholesterol (VDRL antigen). The level of antibodies parallels the pathological process of the infected host. They do not bear any relationship to development of immunity. The cardiolipin antibodies are detected by flocculation tests. The principle of the flocculation reaction is similar to that of agglutinations; antibody reacts with antigen, forming visible aggregates. The antigen is a colloidal suspension of molecules that remain in suspension until antibodies attach to them, at which time, they precipitate in the form of aggregates. The CDC-approved standard tests are: the VDRL slide test, the Rapid Plasma Reagin card test (RPR), the Unheated Serum Reagin Test (USR), and the Toluidin Red unheated serum test (TRUST). Most laboratories use RPR and VDRL is now primarily used for CSF. The Automated Reagin test (ART) is still used in large commercial or public health laboratories. These flocculation tests can be used as qualitative tests for screening, or as quantitative tests for treatment evaluation. The VDRL test is the only one that is used for examination of CSF. False negative reactions may be encountered with all of these tests due to improper technique, or because of the prozone effect (excess of antibodies). The prozone effect appears very infrequently (1% to 2%) and may be corrected by diluting the examined serum. All of the tests using cardiolipin antigen have approximately the same sensitivity and specificity (Table 1). The techniques and interpretations of those tests are described in detail in references 2-5.

Unfortunately, antilipoidal antibodies are produced, although in lower incidence, in other conditions besides treponemal infection. They may be present in sera of patients with infection because of various microorganisms, in connective tissue disorders, in sera of pregnant women, and even in sera of apparently healthy individuals, especially the elderly. They may persist in the serum for various lengths of time. The reaction of anticardiolipin antibodies in sera from individuals without syphilis are known as biological false-positive reactions (BFP). Table 2 lists causes of antilipidal antibody production. All tests using cardiolipin antigen may react with sera from BFP reactors. The development of a sensitive solid-phase immunoassay, using various phospholipids as antigens, has permitted the differentiation between anticardiolipin antibodies found in syphilis and those appearing in sera from patients with connective tissue disorders and in patients with anticardiolipin syndrome. More details on this topic may be found in reference 16.

Treponemal Antibodies. T pallidum was identified in 1906. However, the first test identifying antitreponemal antibodies, the *T pallidum* immobilization test (TPI), was not introduced until 1949.[17] This test is rarely used for diagnostic purposes because of its complexity. Presently, there are three CDC approved standard tests for treponemal antibody detection: fluorescent treponemal absorption

*Table 2. Some common causes of BFP reactions.**

Long Term Persistence	Short Term Persistence
Systemic lupus erythematosus	Infection with various microorganisms
Rheumatoid arthritis	Vaccination against smallpox
Idiopathic thrombocytopenic purpura	Infectious mononucleosis
Malignancy	Infective hepatitis
Scleroderma	Viral pneumonia
Hemolytic anemia	Chickenpox
Thyroiditis	Measles
Sjögren's syndrome	Viral encephalitis
Various neuropathies	Tuberculosis
Hepatic cirrhosis	Malaria
Ulcerative colitis	Narcotic addiction (injections)
Erythema nodosa	Pregnancy
Vasculitis	Apparently healthy with undetermined causes
Atopic dermatitis	
Waldenström's macroglobulinemia	
Systemic sclerosis	
Leprosy	
Narcotic addiction (injections)	
Apparently healthy (increase with age)	

**Reprinted with permission from reference 2.*

test (FTA-ABS), FTA-ABS double staining (FTA-ABS DS), and the microhemagglutination assay for *T pallidum* (MHA-TP). All of these tests are qualitative tests, detecting antibodies to pathogenic treponemes. They may be used for quantitative purposes. However, because of long persistence of the treponemal antibodies in circulation, quantitation has little practical value. The treponemal tests are primarily used to confirm reactivity with a flocculation screening test and to confirm a clinical impression of syphilis.

Fluorescent techniques FTA-ABS. The principle of the fluorescent antibody technique is the reaction of antibodies with a *T pallidum* preparation fixed on a microscope slide. In the FTA-ABS test, sera are diluted 1:5 with sorbent before they are applied to slides. The sorbent is a heated, concentrated culture filtrate of *T phagedenis* Reiter that reacts with antibodies to common or group-specific treponemal antigens. Since the FTA-ABS requires the participation of two antibodies (patients serum and conjugate), of which only the second is under control by laboratory staff, the quality of the conjugate is critical. The antibodies reacting in the FTA-ABS are the first to be detected after infection. Whether treponemal antibodies appear before cardiolipin antibodies is not known. Their early detection may reflect the difference in sensitivity of the tests. During the primary stage of untreated syphilis, particularly in patients seronegative for cardiolipin antibodies, most (70%) of the antibodies are IgM and approximately 25% are IgG. The IgM antibodies disappear when the disease is allowed to run its course untreated, generally during the latent period. Persistence of IgM and IgG cardiolipin antibodies and IgG antitreponemal antibodies may indicate reactivated syphilis and potential development of late symptomatic disease.

Fluorescent technique-double staining (FTA-ABS DS). Negative FTA-ABS results are difficult to evaluate. One cannot be sure whether the negative results are because of the lack of antibodies, or lack of treponemes on the slide. This may be obviated by using the FTA-ABS DS, which applies two conjugates to the slide. The first, tetramethylrhodamine isothiocyanate-conjugated antihuman immunoglobulin (μ or γ specific) detects the presence and isotype of antitreponemal antibodies. The second, FITC conjugated anti-*T pallidum* globulin, stains all treponemes. A 2-filter system is needed for reading reactions.

The specificity of the fluorescent tests is very high. However, false positive reactions may occur in ca 1% of normal individuals and 3% of pregnant women.[4] The frequency of false positive reactions is higher in sera from patients with various immunological disorders such as systemic lupus erythematosus (SLE), drug induced lupus, rheumatoid arthritis, scleroderma, and others (Table 2). Sera of patients with SLE may show an atypical, uneven granular staining of organisms. However, some SLE sera may present homogenous staining that does not differ from a true positive FTA-ABS test. The false positive reaction is presumably because of anti-DNA antibodies or

other antibodies to nuclear proteins reacting with the proteins on the surface of the fixed *T pallidum*.

FTA-ABS 19S IgM test. The predecessor of this test was the FTA-ABS IgM test, which had unsatisfactory sensitivity and specificity. In the latter, the conjugated antibody reacted not only with infants' IgM, but also with rheumatoid factor (RF) consisting of neonates' IgM and placentally transmitted maternal IgG antibodies. In congenital syphilis, RF may be found in 80% of sera of newborns.[18] To eliminate this interference, the new FTA-ABS 19S IgM requires the removal of all IgG free or bound to IgM (RF) in the infant serum. This is achieved by passing the neonatal serum through a small column containing r-protein G (Quick-Sep IgM separation kit. Isolab Inc. Akron, Ohio). The serum IgM rich fraction is then examined with the conjugated antibody specific for IgM. This test has a sensitivity of 92% and a specificity of 93% compared to 90% and 75% for the FTA-ABS IgM test using unfractionated serum for the diagnosis of congenital syphilis. This is a confirmatory test, not a screening technique. Technical details are noted in references 2-5.

Microhemagglutination test for T pallidum antibodies (MHA-TP). The principle of the MHA-TP is passive hemaglutination. Erythrocytes stabilized by gluturaldehyde and coated with *T pallidum* antigen are added to patient serum properly diluted with sorbent. The MHA-TP was initially developed to be performed as an automated test. Today the test is carried out primarily as a manual qualitative test. The sensitivity in early primary syphilis is lower than that of VDRL or FTA-ABS tests (Table 1). Quantitation of treponemal antibody is possible, but not practical. The treponemal antibody levels, when examined by the fluorescent or MHA-TP tests, do not reflect the effect of treatment. The technique and interpretation of results are described in detail in references 2-5.

NONSTANDARD TESTS FOR SYPHILIS

There are a number of tests that are still provisional or under investigation. These tests are usually done in academic institutions or Public Health Laboratories in parallel with standard tests.

Enzyme immunoassay for cardiolipin antibodies (EIA-VDRL). The EIA or enzyme-linked-immunosorbent assay (ELISA), introduced in the mid-seventies, has become a widely used immunological assay for the diagnosis of infectious and noninfectious diseases. The principle of this technique is based on a reaction between antigen fixed onto a solid phase (most frequently wells of a microhemagglutination plate) and antibodies in tested serum. Antibodies binding to the antigen are identified by enzyme-labeled antiglobulin serum and a substrate that reacts with the enzyme. The enzyme-substrate causes a color reaction. The intensity of the color, as measured by spectrophotometry, is directly related to the concentration of the antibodies in the tested serum. An EIA for detection of cardiolipin antibodies has been developed.[19] The antigen fixed onto the plates is the VDRL reagent. Both IgM and IgG antibodies may be detected by this test. Evaluation of indirect EIA for the diagnosis of primary syphilis has demonstrated a specificity and sensitivity below that of the treponemal FTA-ABS test. In untreated secondary syphilis, the EIA-VDRL gave similar results to the FTA-ABS.[4]

Enzyme Immunoassay for T pallidum antibodies (EIA-TP). The antigen used for the assay is sonicated or solubilized *T pallidum*.[20-23] The EIA-TP was found to have a sensitivity and specificity equal to those of the standard treponemal tests. The EIA is economical only when large number of specimens are examined. A number of variations of the EIA-TP have been developed.

EIA-TmpA. Among recombinant DNA antigens attempted for the serodiagnosis of syphilis, only the membrane localized protein, the TmpA has shown promise for further diagnostic development.[24] Evaluation of the EIA-TmpA has demonstrated similar sensitivity and specificity as the FTA-ABS. Moreover, the levels of the antibodies to TmpA antigen decrease sharply within 1 year after antibiotic treatment of patients with syphilis, suggesting that the EIA-TmpA may be suitable for monitoring treatment of syphilis.

CAPTIA| syphilis G. A commercially available kit under the name CAPTIA| syphilis G is being used more frequently,[25] particularly in Europe. This EIA uses microtitration plates coated with sonicated *T pallidum* antigen. The reacting human IgG antibodies in the tested sera are detected by antihuman IgG monoclonal antibodies labeled with biotin and enzyme-labeled streptavidin. Biotin has a high labeling efficiency and high affinity for streptavidin. Therefore, a strong signal is produced for each molecule of bound antibody. In some laboratories, the test has performed as well as combined VDRL and MHA-TP tests.[26-28]

CAPTIA™ syphilis M. This EIA is specifically designed for detection of IgM treponemal antibody in sera of congenitally infected babies. The principle is similar to that of an EIA. However, instead of treponemal antigen, antihuman μ chain specific antibodies are fixed onto a well of a microplate. These fixed antibodies indiscriminately bind IgM present in serum. The addition of purified *T pallidum* antigen will react only with the IgM having combining sites for treponemal antigen. A monoclonal antibody to the *T pallidum* antigen, conjugated with horseradish peroxidase, is used to detect the IgM-antigen complex. A substrate for the enzyme provides a color reaction. The CAPTIA™ syphilis M assay is as good as,

or even slightly better than, the FTA-ABS IgM 19S.[25,29,30] A detail description of the test is provided in reference 31 and in commercial literature provided by Centocor, Inc., Malvern, PA.

Western blot (Immunoblot, IB): This assay provides a characterization of the humoral immune response to *T pallidum*[32-37] based on detection of antibodies to a variety of *T pallidum* epitopes. The technique is based on gel-electrophoretic separation of the solubilized *T pallidum* proteins according to their molecular size. The separated proteins are transferred onto a nitrocellulose membrane and exposed to the tested serum. The antibody-antigen complex is visualized by adding antihuman globulin-enzyme-substrate causing a color reaction. The IB is a very helpful adjunct in the laboratory diagnosis of congenital syphilis. It has been demonstrated that IgM antibodies in congenital syphilis always react with certain *T pallidum* peptides. However, because of cross-reactivity between spirochetes including *Borrelia* and other organisms such as *E coli*, IB results must be interpreted critically, especially in Lyme disease endemic areas.

LABORATORY DIAGNOSIS IN VARIOUS CLINICAL CONDITIONS

The VDRL or RPR, FTA-ABS, and MHA-TP, are proven standard tests in confirming syphilis. As indicated earlier, problems occur in the diagnosis of very early syphilis, neurosyphilis, and asymptomatic congenital syphilis. Problems also may arise in diagnosis of patients co-infected with *T pallidum* and HIV, in intravenous drug users (IDU), and in patients infected with cross-reactive microorganisms.

Primary syphilis. In the very early stage of syphilis infection, when the lesion is still not well formed or appears in an unusual location of the body (mouth, anorectal region), or escapes the patient's attention, the diagnosis of syphilis is extremely difficult to make. Such cases are rarely seen. In some instances, however, a patient seeks medical help for knowingly or, *post factum* having learned of sexual contact with an untreated person with syphilis. In the very early stage (first 2-3 weeks) after infection, serology is positive in only 30% of cases (VDRL) or 50% with the FTA-ABS test. If regional lymph nodes are enlarged and tender, needle biopsy material may be examined for presence of *T pallidum* by DF or DFA tests. It would be preferable to examine lymph node tissue by PCR, because of PCR's high sensitivity and specificity. However, PCR testing for syphilis is done in only a few institutions in the USA, generally for research purposes. When a suspicious lesion is visible and characteristic, it may be examined by any of the methods available and listed for the direct detection of *T pallidum*. By this time, usually serologic tests are positive (Table 1). Should direct method of detection of *T pallidum* be negative, serologic markers are helpful parameters for the diagnosis of syphilis.

Not every lesion in the urogenital region indicates syphilis. The differential diagnosis should include: balanitis, zipper accident, self-induced ulcer, erysipelas, herpes progenitilis, and chancroid.

Secondary syphilis. The secondary stage of syphilis commences at 6-8 weeks after the onset of the disease. It is characterized by spirochaetemia and wide spread dissemination of *T pallidum*. Early nonspecific symptoms may resemble an influenza-like syndrome. Generalized lymphadenopathy and a generalized or local maculopapular rash involving the skin (including palms and soles) and mucosa membranes lesions also occur frequently. Lesions are teeming with treponemes. When serology is positive, there is no need to exam the lesions for presence of *T pallidum*. At this stage, in untreated patients, nontreponemal (VDRL or RPR) and treponemal antibody tests (FTA-ABS or MHA-TP) are positive nearly 100% of the time (Table 1). However, among immunocompromised patients, results of these tests may vary. The rash and symptoms of secondary syphilis, especially when not typical, may mimic a variety of nonsyphilitic diseases including: pityriasis rosea, rosacea, erythema multiforme, psoriasis, condyloma acuminatum, and traumatic traction alopecia.

Latent syphilis. The natural course of untreated disseminated syphilis is to resolve spontaneously. The patient is then free of symptoms. In the prepenicillin era, relapses with recurrent fulminant secondary syphilis occurred in 20% of patients. Such relapses generally occurred within the first two years of primary infection. Relapses after two years were rare. As a rule, when untreated patients have fulminant secondary syphilis, they develop a degree of immunity that helps contain the infection and prevents further relapses. Between early latency and the onset of tertiary syphilis, the untreated individual enters a late latent period in which the patient is both noninfectious and immune to reinfection with *T pallidum*. An exception is the pregnant woman who can transmit infection to the fetus at any time during untreated disease, even when she is no longer infectious to her sexual partner.

VDRL antibodies are the expression of the dynamics of the pathologic process. These antibody levels diminish, ultimately becoming negative. However, there is a general opinion that treponemal antibodies persist almost for life. In early latency, the VDRL or other tests detecting cardiolipin antibodies are positive in approximately 70% of cases with a diminishing frequency as the time from infection increases. The FTA-ABS or MHA-TP are positive in >90% in early latency (Table 1).

Tertiary syphilis. Clinical manifestations of tertiary

syphilis develop after a highly variable period of time. In studies conducted between 1890 and 1910, which were still being analyzed in the 1950s, it was observed that 60% of untreated patients with syphilis undergo self-cure while only 40% showed one or another form of active disease.[38] Benign syphilis, which involves nonvital structures such as skin, soft tissues, bones, cartilages or certain organs, became clinically apparent in approximately 15%, cardiovascular syphilis in 10-25%, and neurosyphilis in approximately 10% of untreated individuals.[38]

Activation of syphilis is noticeable not only by clinical symptoms, but also by production of anticardiolipin antibodies (approximately 80% positive) and treponemal antibodies (FTA-ABS, MHA-TP positive in >90% of the patients).[4] The lesions of tertiary syphilis are not suitable for direct examination by DF or DFA because they may contain undetectable number of treponemes. The presence of *T pallidum* in the lesions of tertiary syphilis was disputed for decades. We have only recently documented by PCR the presence of *T pallidum* in a brain gumma of an HIV-syphilis coinfected subject.[11]

Neurosyphilis. The protean neurologic and psychologic clinical manifestations, the decreasing number of classic symptoms, and the lack of a single laboratory criterion as being absolutely diagnostic of neurosyphilis, make the diagnosis of neurosyphilis frequently difficult. Early neurosyphilis ($\leq$ 2 years after primary infection) was uncommon in the prepenicillin era. In contrast, early neurosyphilis is now more frequently encountered, especially in HIV-infected patients.[39,40] Some time ago U.J. Wile, discussing the report of Moore and Hopkins, proposed to distinguish between invasion and involvement of the central nervous system by *T pallidum*.[41] Invasion was a common phenomenon, indicating presence of *T pallidum* in the CNS. On the other hand, involvement suggesting pathological changes in the CNS, was less common. This concept seems to have been confirmed. In recent studies, Lukehart et al using RIT found *T pallidum* in CSF in 12 of 40 (30%) patients with primary or secondary syphilis.[42] In this study, four patients whose CSF were negative by RIT had a reactive CSF-VDRL, strongly suggesting neurological involvement. To further determine brain infection, the authors examined five additional parameters in the CSF including: leukocyte count, protein concentration, CSF-VDRL, CSF-FTA-ABS, and HIV antibodies. Although *T pallidum* was not isolated in CSF from patients with early latent and late latent syphilis, reactive CSF serology, elevated leukocytes count, and elevated protein concentration were detected in three cases of early latent syphilis and 15 patients with late latent syphilis. It is believed that if any of the five CSF parameters examined is abnormal and serum antibodies to *T pallidum* are present, CNS involvement must be considered and treated. The high affinity of *T pallidum* for brain tissue has also been demonstrated by PCR in the guinea pig experimental model.[14] *T pallidum*-DNA was detected in brain specimens of 7 of 14 (50%) intradermally infected adults and in 12 of 33 (36%) congenitally infected animals.

The low percentage of patients with positive CSF-VDRL or CSF-FTA does not encourage the use of these tests for proving asymptomatic cases of neurosyphilis. The IB technique in the laboratories of some investigators has demonstrated encouraging results. Lewis et al found CSF IgM reactivity by IB(47-,17-,15.5 kDa) in 17 of 18 (94%) patients with symptomatic congenital syphilis.[36] Only 71% were CSF-VDRL positive. Conflicting results were obtained by Sanchez et al who evaluated *T pallidum* invasion of CNS by PCR, RIT and for the presence of IgM antibodies to *T pallidum* by IB.[35] Six of seven (86%) symptomatic infants had RIT-positive CSF; five of six (83%) infants who were RIT-positive were also positive by PCR, and only two of six (33%) were positive for IgM by IB. CNS invasion by *T pallidum* was uncommon among asymptomatic infants; one of 12 (8%), was RIT positive.

When neurosyphilis is suspected in patients, the results of syphilis testing in both sera and CSF, as well as CSF abnormalities must be considered when making treatment decisions. The vast majority of patients with neurosyphilis should have positive serum treponemal assays. However, a negative CSF-FTA-ABS in patients with serum FTA-ABS reactivity generally excludes CNS syphilis. A positive CSF-VDRL is diagnostic of CNS syphilis. Caution must be used in patients with a negative CSF-VDRL but positive FTA-ABS because of the exquisite sensitivity of the FTA-ABS and potential contamination by blood during lumbar puncture. Unfortunately, sometimes even these parameters are not specific and the physician's intuition and clinical experience must be used to decide about treatment needs.

Congenital syphilis. The best prevention of congenital syphilis (CS) is prenatal serologic screening of pregnant women in high risk groups. However, as Dorfman and Glaser,[43] and Sanchez et al[44] reported, some women are seronegative in prenatal screening but gave birth to congenitally infected babies. Repeat examination of mothers and newborns immediately after birth is advisable in areas where syphilis is common. The serodiagnosis of congenital syphilis is difficult because of the transmission of treponemal and nontreponemal IgG antibodies from mother to fetus. The fetus produces IgM antibodies against maternal IgG forming IgM RF. This IgM-RF reacts in the FTA-ABS IgM test and in the IB. On average, 80% of symptomatic infants produce RF reacting with fixed *T pallidum* in the FTA-ABS test.[18] Elimination of the 22S IgM-IgG complexes by passing the sera to be tested through an

Isolab column (or eliminating it in other ways) improves the sensitivity and specificity of the FTA-ABS 19S IgM assay.[4] The CAPTIA syphilis M test or solid phase hemadsorption assay were as sensitive and specific for making the diagnosis of CS as the FTA-ABS 19S IgM.[45] Symptomatic CS is generally not a diagnostic problem; clinical symptoms are characteristic, *T pallidum* are present in lesions or secretions, and the serodiagnostic tools for confirmation, such as FTA-ABS 19S IgM or CAPTIA syphilis M, are positive in >90% of cases.

The diagnosis of congenital neurosyphilis and the diagnosis of asymptomatic CS are more complex. The previously mentioned treponemal IgM antibody tests are very helpful tools.

The most reliable diagnostic modality for syphilis is identification of *T pallidum*. Since *T pallidum* cannot be cultured in vitro, PCR is a suitable substitute. A useful comparison of PCR with the "gold standard" for syphilis (RIT) was done using materials from CS patients.[9,12] Amniotic fluid from 11 serologically reactive pregnant women with untreated syphilis, and sera and CSF from 7 symptomatic and 12 asymptomatic infants with probable infection were examined by PCR, RIT, and IB for IgM antibodies. Amniotic fluids from babies of seropositive mothers were positive by PCR in 9 of 11 (82%) and by RIT in 7 of 9 (78%) specimens.[9] PCR correlated well with RIT in serum and CSF in the symptomatic group in which IgM antibodies were found in almost all cases and no additional tests were needed. However, in the asymptomatic group, neither test was positive, except in one case. In asymptomatic CS a comprehensive approach using IgM antibodies and *T pallidum* DNA technology is advisable. Adequate assays are still lacking for diagnosis of asymptomatic CS, which accounts for >60% of cases of CS. A stringent case definition is the only guideline for management.[46] One should not rely on results of one test alone, especially when it is negative in suspicious cases.

SYPHILIS AND HIV

HIV infection and syphilis have become remarkably intertwined in the 1980s and 1990s. Studies done in the USA and Africa have demonstrated that ulcerative genital lesions such as chancres enhance the transmission of HIV.[47] The clinical course of syphilis may be accelerated and more severe in HIV-infected individuals. For instance, manifestations of neurosyphilis that usually appear after many years or decades, develop within a few months of *T pallidum* infection in HIV co-infected individuals.[39,40] However, it has been demonstrated that penetration of *T pallidum* into the CNS is not greater in HIV co-infected patients compared to patients with syphilis alone.[42] The response to syphilis treatment and serological reactions to treponemal and nontreponemal antigens may be different in HIV co-infected patients, thereby introducing a degree of uncertainty into the interpretation of laboratory tests used for diagnosis of syphilis. Furthermore, the diagnosis of neurosyphilis in HIV-infected patients may be complicated by the fact that HIV itself can cause lymphocytosis and elevated protein level in the CSF.

There have been a number of reports describing altered humoral response in patients with HIV-syphilis co-infection.[47-49,52] Hicks et al[48] reported a case in which an HIV positive patient with secondary syphilis had a negative VDRL on two different occasions but became seropositive later during treatment. Hass et al[50] evaluated the sera of 109 HIV patients with prior syphilis and found that 7% of HIV-asymptomatic and 38% of HIV-symptomatic individuals demonstrated negative reactions to treponemal antigens. On the other hand, control sera from 98 HIV-negative syphilitic patients were all serologically positive for treponemal antigens. Loss of reactivity to treponemal antigen was related to the degree of immune disfunction caused by HIV infection. Johnson et al reported that 10% of AIDS patients with a past history of syphilis lacked detectable treponemal antibodies. Control groups without AIDS did not show such a high rate of seronegativity.[51] Radolf and Kaplan reported an interesting case of an abnormal humoral response to *T pallidum* in an HIV-infected patient demonstrating unusual symptoms of secondary syphilis.[52] Although the patient's serum was positive by VDRL and FTA-ABS testing, it reacted only with three *T pallidum* peptides by IB. The strongest reaction was with the 47kDa protein. (Sera from secondary syphilis in normal conditions react with multiple *T pallium* proteins.)

Biological false positive reactions are found among HIV-infected patients and intravenous drug users. Glatt et al found that in 8 HIV-positive patients or IDU examined for cardiolipin antibodies, the RPR titers ranged from 1:16 to 1:28 although no obvious syphilitic symptoms were present and patients were FTA-ABS negative.[53] While the incidence of BFP reactions is high in IDU, varying from 13% to 95%.[53-55] Rompalo et al found that the incidence of BFP reaction is low (0.8 to 4%) in HIV nonsyphilitic patients (56%). The possibility of a dichotomy in the production of cardiolipin antibodies and treponemal antibodies in syphilitic patients has also been suggested. Erbelding et al evaluated IDU for the incidence of HIV and syphilis by serological analysis.[57] They found that in 35 of 112 (31%) BFP reactors, at least one RPR test had a titer $\geq$ 1:8 when the FTA-ABS test was negative. Moreover, they found antibodies to *T pallidum* membrane proteins (47-and 17kDa) in three of five patients sera analyzed by IB. The authors suggested that some BFP reactions may represent FTA-ABS negative syphilis. More data are needed to confirm this observation.

It is important to reiterate that the information provided above represents isolated cases. The majority of patients co-infected with HIV and syphilis have normal or even elevated antibodies to *T pallidum* and cardiolipin.[49, 58-60]

SYPHILIS AND LYME DISEASE

Borrelia burgdorferi the causative agent of Lyme disease, belongs to a different genus of the family *Spirochaetaceae* than *T pallidum. B burgdorferi* can be cultivated in vitro, is transmitted by *Ixodes* sp. ticks, and has a number of hosts. Some similarities exist between syphilis and Lyme disease. *B burgdorferi* and *T pallidum* share antigenic properties,[61] are motile, adhere to cells, can invade various internal organs such as heart, and cross the blood-brain barrier causing neurologic disease. When treated with antibiotics in an early stage of the disease, both can be eliminated from the host. However, when untreated, late stage disease can cause many problems. Late stage disease due to either organism is not easily treatable.

Antigenic cross reactivity between *Treponema*, *Borrelia* and *Leptospira* has been demonstrated.[62] The *B burgdorferi* 41kDa flagellar protein is similar to the flagellar antigens of other spirochetes.[63-65] It must therefore be expected that antibodies in Lyme disease patients may cross-react with a variety of microorganisms, especially with *T pallidum* and other treponemes that cause oral infection.[66] EIA and IB are the most commonly used assays in which antigenic cross-reactivity may interfere with interpretation of results.

Russel et al examined the sensitivity and specificity of an indirect immunofluorescent assay (IFA) and ELISA for Lyme disease using sera from patients with Lyme disease and other infections. Significant cross-reactivity occurred only with sera from patients with syphilis, yaws, and pinta.[67] Ma et al used an IB, with *B burgdorferi* as antigen, to evaluate sera from Lyme disease, syphilis, and normal individuals.[68] The sera from patients with Lyme disease reacted to 14 proteins ranging from 94-to 17 kDa . However, sera from patients with syphilis also reacted with 12 proteins including those of 94-, 66-, 60-, and 41 kDa, which were most frequently found in sera of patients with Lyme disease sera. Even normal human serum reacted with 94-,66-, 60- and 41kDa proteins. Aguero-Rosenfeld et al using an IB assay, found that IgM antibodies from syphilis patients sera usually reacted only with a single *B burgdorferi* protein (25kDa).[69] However, IgG antibodies reacted with several proteins between 41- and 60kDa. Dressler et al examined 25 VDRL and FTA-ABS-positive sera by EIA and IB using *B burgdorferi* as antigen.[70] By EIA, 13 of 25 (52%) VDRL positive patients sera had low level or indeterminate IgM antibodies and 16 of 25 (64%) patients had low level or indeterminate IgG reactivity to *B burgdorferi* antigen. By IB, one of 25 (4%) had two bands in the required location for a positive Lyme disease-IgM blot while 8 of 25 (32%) had more than 2 IgG bands. Of note is the fact that the IgG reactivity was not diagnostic for Lyme disease. Johnson et al employed a two–step protocol for examination of sera from various patients cohorts.[71] For EIA, *B burgdorferi* flagellar antigen was used. For IB, the investigators used commercial Lyme disease blots (MarDx Diagnostic). By EIA, 10 of 11 (91%) sera from syphilis patients were positive. However, when tested by IB only one of 11 (9%) was reactive.

In summary, although sera from patients with syphilis may react positively by EIA for Lyme disease, when tested by IB, they generally do not meet the established criteria for Lyme disease positivity.[72] There is generally not a problem with the false diagnosis of syphilis in the setting of Lyme disease. Sera of these patients may cross-react in treponemal assays, but are generally negative by the VDRL test.

SEROLOGIC RESPONSE TO ANTIBIOTIC TREATMENT

Effectiveness of cure in syphilis may be evaluated by remission of symptoms, seroreversion or in extreme or experimental conditions by using RIT or PCR. Clinical improvements may not always represent cure of syphilis. Seroreversion, may suggest cure, only if monitored for long periods of time. The most certain test of cure is the one that shows lack of *T pallidum* in organs. Traditionally, RIT was the only test to prove elimination of *T pallidum.* In view of the fact that PCR correlates very well with RIT, it may replace RIT, which is impractical because of the length of time required to obtain results (several months) and the difficulty in getting RIT performed.

The only reliable tests evaluating seroreversion are those detecting cardiolipin antibodies (eg, VDRL/RPR). However, it must be stressed that even without treatment, a patient may become serologically negative after several years.[38] Seroreversion also may depend on various conditions, eg, length of time of infection, severity of disease, titer of cardiolipin antibodies, status of reactivation of the disease process, and HIV status. Retrospective studies have demonstrated that cardiolipin antibodies in patients treated with standard doses of penicillin (2.4 million units, for early syphilis) decline 4-fold after 3 months and 8-fold after 6 months.[73] To follow seroreversion, serum must be examined on average every three to six months for at least two years. Seroreversion, will also depend on the antibiotic regimen utilized. Fiumara treated patients with double the recommended dose of antibiotic and observed com-

plete seronegativity for cardiolipin antibodies in 100% of primary syphilis within 1 year.[74] All patients with secondary syphilis were seronegative for cardiolipin antibodies after two years.[75] Ninety three percent of patients treated for early latent syphilis become seronegative after two years.[76] However, in the late latent group only 44% of 128 patients become seronegative within five years, and 56% were VDRL-antibody fast.[77] Less optimistic results were obtained by Romanowski et al, who retrospectively evaluated patients treated with standard doses of antibiotics.[78] Based on 800 patients, the cumulative proportion showing seroreversion by RPR was 63%. Of note is the fact that patients with primary, secondary and early latent disease seroreverted by three years 72%, 56% and 26% of the time, respectively. However, patients with primary syphilis who had repeated episodes of infection become seronegative only 34% of the time after 3 years. A cohort of 13 patients with repeat infections in the secondary stage and two patients with early latent syphilis never seroreverted during five years of observation.

Romanowski et al also made another interesting observation.[78] There is a general notion that treponemal antibodies persist for years, if not for life, after treated infection. These authors observed seroreversion in the FTA-ABS and MHA-TP testing in patients treated when experiencing first episodes of primary syphilis. Those treated during early latent syphilis never seroreverted. The FTA-ABS test was more likely to become negative than the MHA-TP. This confirms the observations made by Ijsselmuiden et al[24] that seroreversion occurred in treated patients whose sera were examined by EIA-TmpA. It seems that when the host is not exposed to *T pallidum* for a long enough period of time and the organisms have been eliminated rapidly after treatment, long memory B cell clones are unable to produce lifelong treponemal antibodies.

It is of note that HIV-infected patients with primary,[79] secondary,[79,80] and late[79] syphilis appear to have slower, fourfold decreases in nontreponemal tests by 6 to 12 months than non-HIV infected patients. Furthermore, as noted by Haas et al,[50] FTA-ABS and MHA-TP tests do not reliably identify prior syphilis in HIV-infected patients.

A major problem for physicians is how to evaluate patients who do not serorevert after treatment. Patients in the primary, secondary, and early latent syphilis generally serorevert within one, two, and five years respectively. Failure to serorevert may indicate reinfection, reactivation, persistence of infection or biologically false positive reactions. If material is available for biopsy, PCR may be used to demonstrate persistence of infection. However, the decision for retreatment will frequently be based upon the level of persistent nontreponemal antibodies and the exclusion of false positive reaction. Patients with other treponemal infection such as *T pallidum* ssp. *endemicum* or *T pallidum* ssp *pertenue* frequently do not serorevert after treatment. Age and geographical origin of the patient may lead the physician to a correct diagnosis.

CONCLUSION

Despite the significant advances made in microbiologic and molecular techniques, *T pallidum* remains noncultivable in vitro and the diagnosis of syphilis relies upon the standard, tests that have been with us for years. For most patients, a nontreponemal screening test is generally followed when positive by a specific treponemal test (FTA-ABS or MHA-TP). During primary syphilis, the darkfield examination remains the most rapid and direct method of making a diagnosis. During secondary and latent syphilis, antibody testing is nearly always positive. In some instances of late latent and tertiary syphilis, the utilization of immunofluorescent antibody staining or PCR of tissue may be of benefit. For the diagnosis of congenital syphilis, the FTA-ABS 19S IgM and CAPTIA syphilis M should prove useful in improving the diagnosis in symptomatic cases. Asymptomatic congenital syphilis remains a diagnostic problem. Although serology may be difficult to interpret in HIV-infected patients, they are generally positive in the face of active disease. The VDRL test remains the best assay for response to treatment when there are no lesions to biopsy for PCR testing.

Because of the problems associated with current diagnostic modalities for syphilis and the potentially devastating sequelae of tertiary syphilis, many patients are treated and retreated with antibiotic therapy. Unfortunately, others are missed entirely. With hope, with more widespread availability of immunoblotting for *T pallidum* and PCR testing, some of the issues currently confronting physicians can be improved.

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INFORMATION FOR CONTRIBUTORS

Journal of Spirochetal and Tick-borne Diseases

Dedicated to science and art in spirochetal and tick-borne diseases

INFORMATION FOR AUTHORS AND EDITORIAL POLICY

The following guidelines are in accordance with the "Uniform Requirements for Manuscripts Submitted to Biomedical Journals" and the International Committee of Medical Journal Editors (the "Vancouver Group") statement, agreed at the January 1993 Meeting.

The *Journal of Spirochetal and Tick-borne Diseases* publishes quarterly reviews and original work studies about any aspect of spirochetal and tick-borne diseases. The primary purpose is to broaden our understanding of spirochetal and tick-borne diseases. Special focus is given to Lyme borreliosis (also known as Lyme disease), as the most prevalent spirochetal and tick-borne disease. The clinical topics may involve all medical disciplines, nursing, and pharmacy, as well as the social, ethical, and biological features of spirochetal and tick-borne diseases.

The Journal is composed of two major sections. One section is devoted to the review of a specific topic that is established by the Associate Editors, and a special guest editor is invited to coordinate the development of up to six manuscripts relating to the specific topic. The second section of original works is composed of unsolicited manuscripts that are subsequently reviewed by the Review Board, as well as external reviewers, depending on the potential for conflict of interest within the editorial panel and the potential interest by the readership.

Expeditious review of all manuscripts is carried out with a projected response time of not more than 6 weeks. Rejected manuscripts usually are returned to authors within 6 weeks. Decisions about potentially acceptable manuscripts may take somewhat longer.

The Journal will publish material defined within the categories described in the following.

Reviews

Each issue includes a series of state-of-the-art articles on a topic related to spirochetal and tick-borne diseases. The articles represent invited presentation by authorities in the field on topics related to spirochetal and tick-borne diseases, with an emphasis on Lyme borreliosis.

Submissions to this category should present a comprehensive state-of-the-art analysis and should be accompanied by an abstract of 300 words or less summarizing major points.

Peer Review Articles

Original articles of 5000 words or less may be submitted to the editorial office. Each article should be accompanied by an abstract of 300 words or less describing the findings of the original research. All articles will be peer reviewed within a 3-week period with subsequent notification to the authors within 5 weeks of submission.

Case Reports

Specific clinical case reports describing a unique approach to Lyme disease and other related disorders in the area of diagnosis or treatment may be submitted for review. An abstract of 250 words or less should accompany the text.

Correspondence

Letters to the Editor in the form of correspondence related to material published in the Journal or some aspect of Lyme borreliosis and other spirochetal and tick-borne diseases may be submitted. Such letters, if related to work previously published in the Journal, will be referred to the author of the original work for a response. Letters to the Editor should be submitted in duplicate, typewritten and double-spaced, not exceeding 400 words of text and a maximum of five references. Letters should have no more than three authors, and should be signed by all of the authors. Please include a word count.

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Editorials may be published, usually at the solicitation of the Associate Editors, but unsolicited submissions that relate to an unusual topic of interest exceeding the usual designation of correspondence, i.e., 1000 words or less, will be considered.

Photographic Section

The topical photographic section will be a regular feature. Photographs pertinent to articles presented in the Journal, as well as other photographs related to any aspect of spirochetal or tick-borne diseases, will be considered for the publication. The guidelines for the submission are designated in **Illustrations**.

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An original and three copies of the manuscript should be submitted to:

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With the manuscript, provide a page giving the title of the article; titles should be concise and descriptive (not declarative). Also include a running head of fewer than 40 letter spaces; the name(s) of the author(s), including the first name(s) and academic degree(s); the name of the department and institution in which the work was done; the institutional affiliation of each author; and the name and address of the author to whom reprint requests should be addressed. Any grant support that requires acknowledgment should be mentioned on this page.

Abstract

Provide on a separate page an abstract of not more than 300 words (original and review articles) or 250 words (case report). This abstract should consist of four paragraphs, labeled Background, Methods, Results, and Conclusion. They should briefly describe the problem being addressed in the study, how the study was performed, the results, and what the authors conclude from the results.

Text

All material should be typed and

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Tables

Submit tables typed and double-spaced and provide a heading for all columns with a comprehensive title on separate sheets. A disk copy with a separate file for each table should be on the disk containing the text.

Illustrations

Photographs and figures should be submitted as glossy prints 5×7 in., with one copy of each print for each copy of the manuscript. Figure legends should be provided on a separate sheet with identification of the figure. The back of the glossy print should indicate the number of the figure.

References

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