An Understanding of Laboratory Testing for Lyme Disease

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 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 (14) 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 demonstrates 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 types 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 interpretations 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 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 manufacturers 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 ELISAs for Lyme disease diagnosis more sensitive.

The goal for a new generation of ELISAs 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 ELISAs 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 later 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.
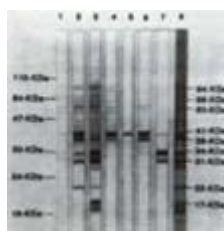
B. burgdorferi Western blotting

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.*

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.
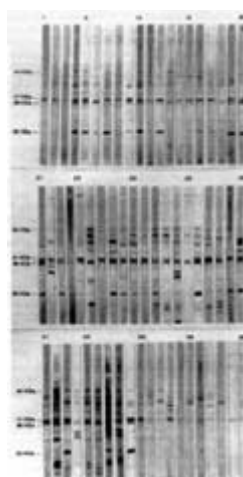

Figure 2. *Western blots to* B. burgdorferi *from various patients with Lyme disease Lanes 1-48 are IgG/IgM blots from clinically confirmed patients with various levels of antibodies. Lanes 48-57 are IgM-only blots. Reproduced by permission from Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by Western immunoblot reactivity of various significant antibodies against B. burgdorferi. J Clin Microbial 1992; 30: 370-376.*

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 IgM Western blots to *B. burgdorferi* (lanes 48-57). 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 (Osp C), 31 kDa (Osp A), 34 kDa (Osp B), 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.
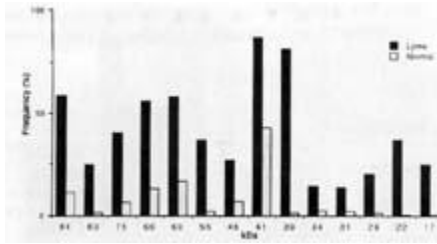
Figure 3. *Comparison of antibody reactivity to various* B. burgdorferi *antigens. The dark bars are from 186 patients with clinically confirmed Lyme disease and the light bars are from 320 normal controls. Reproduced by permission from Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by Western immunoblot: reactivity of various significant antibodies against B. burgdorferi. J Clin Microbial 1992; 30: 370-76.*

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. 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 (Osp A) and 34 kDa (Osp B), which appear later in the response. A review by Hilton et al (38) in a group of 50 patients with confirmed Lyme disease showed that they would have missed 4 patients by excluding 31 kDa (Osp A) and 34 kDa (Osp B). 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 criteria 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 a 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 (Osp C), the 39 kDa, and the 41 kDa, but exclude the 31 kDa (Osp A) and 34 kDa (Osp B). 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 (35), studies by Steere (28), and by Jam 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% specificity 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.

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 the testing of paired acute- and convalescent-phase samples. In late Lyme disease, the predominant antibody response is usually IgG. It is highly unusual that a patient with active Lyme disease has only an IgM response to *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 (Osp C), 39 kDa (Bmp A), 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 (Osp C), 28, 30, 39 (Bmp A), 41 (Fla), 45, 58 (not GroEL2), 66 and 93 kDa. Monoclonal antibodies have been developed to the Osp C, 39 (Bmp A), 41 (Fla), 66, and 93 kDa antigens and are suitable for calibrating IgG immunoblots (7).

The apparent molecular mass of Osp C 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 Osp C 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. When possible, the molecular weight of the protein should be followed by the descriptive name (eg, Osp C).

MMWR 1995; 44: 590-91

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 (Osp A) and 34 kDa (Osp B) to detect antigen in the cerebrospinal fluid (CSF) of patients with neuroborreliosis.

Harris and Stephens (50) have presented information about the development and use of antigen-capture for the detection of *B. burgdorferi* antigen in the urine of Lyme patients. The antibody used in this antigen-capture is a unique polyclonal antibody that is specific for the 31 kDa (Osp A), 34 kDa (Osp B), 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. Furthermore, 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 than a single collection (39).

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 et al (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 (55). 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 (56) 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. garninii* and *B. afzelii* but not to *B. hermsii*. Immediately after the initiation of therapy (minocycline, 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.
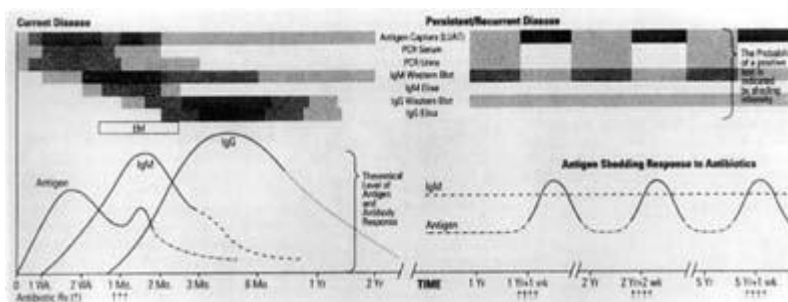
Figure 4. *A model suggesting the tests applicable in different phases of Lyme disease. The left side of the figure indicates the hypothetical patient making antibodies after infection. Less than 70% of patients are in this category. The responses shown on the right side of Figure 4 pertain to many of the patients with recurrent/persistent disease. Courtesy of IGeneX, Inc. Reference Laboratory, Palo Alto, CA.*

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 ELISAs and IFAs 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 diagnostically during persistent/recurrent disease (50). 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 that patients seropositive to *B. burgdorferi* have less antigenuria than seronegative patients (50).

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 have shown that pieces of antigen are more commonly found in urine than are whole or semi-whole *B. burgdorferi* (44). In addition to the 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* in synovial fluid was used as a diagnostic aid for patients with Lyme arthritis (54). 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 on 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 as those 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 disease.

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 Osp 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 ug/mL of immunoglobulin before doing an assay.

PCR and antigen capture assays using CSF have been useful in some patients with neurological Lyme (47,48,74), 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, and 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 Ehlichiosis (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 for HGE ([86](#)).

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 co-infections have been noted ([79](#),[81](#)).

Babesiosis is another disease transmitted by the same tick that carries *B. burgdorferi* ([76-80](#)). 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 leads 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 radio-labeled *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*.

REFERENCES

1. Lugwell P, Dennis DT, Weinstein A, et al. Laboratory evaluation in the diagnosis of Lyme disease. Ann Intern Med 1997; **127**: 1109-1123.
2. Reid MC, Schoen RT, Evans J, et al. The consequences of over diagnosis and over treatment of Lymc disease: an observational study. Ann Intern Med 1998; **128**: 355-362.

3. Fix AD, Strickland GT, Grant J. Tick bites and Lyme disease in an endemic setting: problematic use of serologic testing and prophylactic antibiotic therapy. JAMA 1998; **279**: 206-210.
4. Duffy J. Lyme Disease. Ann Allergy 1990; **65**: 1-13.
5. Golightly MG, Thomas JA. Lyme Borreliosis serologies in perspective. CIMNDC 1991; **11**: 113-8.
6. Tilton RC. Laboratory aids for the diagnosis of *Borrelia burgdorferi* infection. J Spirochetal Tick-Borne Inf 1994; **1**: 18-23.
7. Association of State and Territorial Public Health Laboratory Directors (ASTPHLD). Proceedings of the second national conference on the serological diagnosis of Lyme disease, October 27-29, 1994. Dearborn MI. Washington DC. ASTPHLD 1995.
8. CDC. Lyme disease: case definitions for public health surveillance. MMWR 1990; **39**: 19-21.
9. Williams CL, Strobino B, Lee A, et al. Lyme disease in childhood: clinical and epidemiologic features of ninety cases. Pediatr Infect Dis J 1990; **9**: 10-14.
10. Berger BW. Dermatologic manifestations of Lyme disease. Rev Infect Dis 1989; **11**: 1475-1481.
11. Engstrom SM, Shoop E, Johnson RC. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J Clin Microbiol 1995; **33**: 419-427.
12. Aguero-Rosenfeld ME, Nowakowski J, McKenna DF, Carbonaro CA, Wormser GP. Serodiagnosis in early Lyme disease. J Clin Microbiol 1993; **31**: 3090-3095.
13. Aguero-Rosenfeld ME, Nowakowski J, McKenna DF, Carbonaro CA, Wormser GP. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. J Clin Microbiol 1996; **34**: l-9.
14. Bakken LL, Callister SM, Wand PJ, Schell RF. Interlaboratory comparison of test results for detection of Lyme disease by 516 participants in the Wisconsin State laboratory of hygiene/College of American Pathologists proficiency testing program. J Clin Microbiol 1997; **35**: 537-543.
15. Steere AC, Grodzidki RL, Kornblatt AN, et al. The spirochetal etiology of Lyme disease. N Engl J Med 1983; **308**: 733-740.
16. Rawlings JA, Fournier PV, Teltow GA. Isolation of *Borrelia* spirochetes from patients in Texas. J Clin Microbiol 1987; **25**: 1148-1150.
17. Schwartz BS, Goldstein MD, Ribeiro JMC, Schulze TL, Shahied SI. Antibody testing in Lyme disease: a comparison of results in four laboratories. JAMA 1989; **262**: 3431-3434.
18. Luger SW, Krauss E. Serologic tests for Lyme disease: interlaboratory variability. Arch Intern Med 1990; **15**: 761-763.
19. Bakken LL, Case KL, Callister SM, Bourdeau NJ, Schell RF. Performance of 45 laboratories participating in a proficiency testing program for Lyme disease serology. JAMA 1992; **268**: 891-895.
20. Mitchell PD, Reed KD, Aspeslet TL Vandermause MF, Melski JW. Comparison of four immunoserologic assays for detection of antibodies to *Borrelia burgdorferi* in patients with culture-positive erythema migrans. J Clin Microbiol 1994; **32**: 1958-1962.
21. Russell H, Sampson JS, Schmid GP, Wilkinson HW, Pllikaytis B. Enzyme-linked immunosorbent assay for Lyme disease. J Infect Dis 1984; **149**: 465-470.
22. Hedberg CW, Osterholm MT, MacDonald KL, White KE. An interlaboratory study of antibody to *Borrelia burgdorferi*. J Infect Dis 1987; **155**: 1325-1327.
23. Magnarelli LA, Meegan JM, Anderson JF, Chappell WA. Comparison of an indirect fluorescent-antibody test with an enzyme-linked immunosorbent assay for serological studies of Lyme disease. J Clin Microbiol 1984; **20**: 181-184.
24. Magnarelli LA, Anderson JF, Johnson RC. Cross-reactivity in serological tests for Lyme disease and other spirochetal infections. J Infect Dis 1987; **156**: 183-188.
25. Craft JE, Grodzicki RL, Steere AC. Antibody response in Lyme disease: evaluation of tests. J Infect Dis 1984; **149**: 789-795.
26. Voller A, Bidwell D. Enzyme-linked immunosorbent assay. In, Manual of clinical immunology, 3rd edition. Washington, DC. ASM. 1986: 99-109.

27. Barbour AG, Tessier SL, Hayes SF. Variation in a major surface protein of Lyme disease spirochetes. Infect Immunol 1984; **45**: 94-l00.

28. Craft JE, Fischer DK, Shimamoto GT, Steere AC. Antigens of *Borrelia burgdorferi* recognized during Lyme disease: appearance of a new immunoglobulin M response and expansion of the immunoglobulin G response late in the illness. J Clin Invest 1986; **78**: 934-939.

29. Coleman JL, Benach JL. Isolation of antigenic components from the Lyme disease spirochete: their role in early diagnosis. J Infect Dis 1987; **I55**: 756-765.

30. Simpson WJ, Schrumpf ME, Schwan TG. Reactivity of human Lyme Borreliosis sera with a 39 kilodalton antigen specific to *Borrelia burgdorferi*. J Clin Microbiol 1990; **28**: 1329-1337.

31. Wilske B, Preac-Mursic, Schierz G, Kuhbeck, Barbour AG, Kramer M. Antigenic variability of *Borrelia burgdorferi*. Ann NY Head Sci 1988; **539**: 126-143.

32. Zoller L, Burkyard S, Schafer H. Validity of Western immunoblot band patterns in the serodiagnosis of Lyme Borreliosis. J Clin Microbiol 1991; **29**: 174-182.

33. Wong SJ, Brady GS, Dumler JS. Serologic responses to *Ehrlichia equi*, *Ehrlichia chaffeensis*, and *Borrelia burgdorferi* in patients from New York State. J Clin Microbiol 1997; **35**: 2198-2205.

34. Golightly MG, Thomas JA, Viciana AL. The laboratory diagnosis of Lyme Borreliosis. Lab Med 1990; **21**: 299-304.

35. Ma B, Christen B, Leung D, Vigo-Pelfry C. Serodiagnosis of Lyme Borreliosis by Western immunoblot: reactivity of various significant antibodies against *Borrelia burgdorferi*. J Clin Microbiol 1992; **30**: 370-376.

36. CDC. Recommendations for test performance and interpretation from the second national conference on serologic diagnosis of Lyme disease. MMWR 1995; **44**: 590-591.

37. Dressler F, Whalen JA, Reinhardt BN, Steere AC. Western blotting in the serodiagnosis of Lyme disease. J Infect Dis 1993; **167**: 392-400.

38. Hilton E, Devoti J, Sood S. Recommendation to include OspA and OspB in the new immunoblotting criteria for serodiagnosis of Lyme disease. J Clin Microbiol 1996; **34**: 1353-1354.

39. Harris NS, Harris SJ, Joseph JJ, Stephens BG. *Borrelia burgdorferi* antigen levels in urine and other fluids during the course of treatment for Lyme disease: a case study. Presented at the VII International Congress of Lyme Borreliosis meeting, June 16-21, 1996. San Francisco, CA.

40. Jain VK, Hilton E, Maytal J, Dorante G, Ilowite NT, Sood SK. Immunoglobulin M immunoblot for diagnosis of *Borrelia burgdorferi* infection in patients with acute facial palsy. J Clin Microbiol 1996; **34**: 2033-2035.

41. Sivak SL, Aguero-Rosenfeld ME, Nowakowski J, et al. Accuracy of IgM immunoblotting to confirm the clinical diagnosis of early Lyme disease. Arch Intem Med 1996; **156**: 2105-2109.

42. Oski J, Uksila J, Marjamaki M, Mikoskelainen J, Viljanen MK. Antibodies against whole sonicated *Borrelia burgdorferi* spirochetes, 41 kilodalton flagellin and P39 protein in patients with PCR- or culture-proven late Lyme Borreliosis. J Clin Microbiol 1995; **33**: 2260-2264.

43. Hyde EW, Johnson RC, White TJ, Shelburne CE. Detection of antigens in urine of mice and humans infected with *Borrelia burgdorferi*, etiologic agent of Lyme disease. J Clin Microbiol 1989; **27**: 58-61.

44. Dorward DW, Schwan TG, Garon CF. Immune capture and detection of extracellular *B burgdorferi* antigens in fluids or tissues of ticks, mice, dogs, and humans. J Clin Microbiol 1991; **29**: 1162-1171.

45. Goodman JL, Jurkovich P, Kodner C, Johnson RC. Persistent cardiac and urinary tract infections with *Borrelia burgdorferi* in experimentally infected Syrian hamsters. J Clin Microbiol 1991; **29**: 894-896.

46. Magnarelli LA, Anderson JF, Stafford KC. Detection of *Borrelia burgdorferi* in urine of Peromyscus leucopus by inhibition enzyme-linked immunosorbent assay. J Clin Microbiol 1994; **32**: 777-782.

47. Goodman JL, Jurkovich P, Kramber JM, Johnson RC. Molecular detection of persistent *Borrelia burgdorferi* in the urine of patients with active Lyme disease. Infect Immun 1991; **59**: 3-12.

48. Coyle PK, Deng Z, Schutzer SE, et al. Detection of antigens in cerebrospinal fluid. Neurol 1993; **43**: 1093-1097.

49. Shelburne CE. Method and composition for the diagnosis of Lyme disease. US Patent 4,888,276. 1989.

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

51. Harris N, Drulle J, Eiras E, Stephens B. Detection of *B burgdorferi* antigen and antibody in patients presenting with an erythema migrans. Presented at 6th annual Lyme disease scientific conference, May 5-6, 1993, Atlantic City, NJ.

52. Rosa PA, Schwan TG. A specific and sensitive assay for the Lyme disease spirochete *B burgdorferi* using the polymerase chain reaction. J Infect Dis 1989; **160**: 1018-1029.

53. Persing DH, Telford SR, Spielman A, Barthold SW. Detection of *Borrelia burgdorferi* infection in Ixodes dammini ticks with the polymerase chain reaction. J Clin Microbiol 1990; **28**: 566-572.

54. Nocton JJ, Dressler F, Rutledge BJ, Tys PN, Persing DH, Steere AC. Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. N Engl J Med 1994; **330**: 229-234.

55. Goodman JL, Bradley IF, Ross AE, et al. Bloodstream invasion in early Lyme disease: results from a prospective, controlled, blinded study using the polymerase chain reaction. Am J Med 1995; **9**: 6-12.

56. Schmidt B, Muellegger RR, Stochenhuber C, et al. Detection of *Borrelia burgdorferi*-specific DNA in urine specimens from patients with erythema migrans before and after antibiotic therapy. J Clin Microbiol 1996; **34**: 1359-1363.

57. Garon GE, Dorward DW, Corwin MD. Structural features of *Borrelia burgdorferi*-the Lyme disease spirochete: silver staining for nucleic acids. Scanning Microsc Suppl 1989; **3**: 109-115.

58. Wormser GP, Nowakowski J, Nadelman RB, et al. Improving the yield of blood cultures for patients with early Lyme disease. J Clin Microbiol 1998; **36**: 296-298.

59. Wallach FR, Forni AL, Hariprashad J, et al. Circulating *Borrelia burgdorferi* in patients with acute Lyme disease: results of blood cultures and serum DNA analysis. J Infect Dis 1993; **168**: 1541-1543.

60. Manak MM, Gonzalez-Villasenor LI, Crush-Stanton S, Tilton RC. Use of PCR to monitor the clearance of *Borrelia burgdorferi* DNA from blood following antibiotic therapy. J Spirochet Tick-Borne Dis 1997; **4**: 11-20.

61. Mayer ME, Zhang M, Bayer MH. *Borrelia burgdorferi* DNA in the urine of treated patients with chronic Lyme disease symptoms. A PCR study of 97 cases. Infection 1996; **24**: 347-353.

62. Finkel ME. Lyme disease and its neurologic complications. Arch Neurol 1988; **45**: 99-104.

63. Pachner AR. Neurologic manifestations of Lyme disease: the new "great imitator." Rev Infect Dis 1989; **11** (6 Suppl): 1483-1486.

64. Halperin JJ. Nervous system manifestations of Lyme disease. Rheum Dis Clin North Am 1989; **15**: 635-647.

65. Pahner AR, Duray P, Steere AC. Central nervous system manifestations of Lyme disease. Arch Neurol 1989; **46**: 790-795.

66. Halperin JJ, Loft BJ, Anand AK, et al. Lyme neuroborreliosis: central nervous system manifestations. Neurology 1989; **39**: 753-759.

67. Logigian EL, Kaplan RF, Steere AC. Chronic neurologic manifestations of Lyme disease. N Engl J Med 1990; **323**: 1438-1444.

68. Fallon BA, Nields JA, Burrascano JJ, Leigner K, BelBene D, Liebowitz MR. The neuropsychiatric manifestations of Lyme borreliosis. Psychiatr Q 1992; **63**: 95-117.

69. Coyle PK, Neurologic complications of Lyme disease. Rheum Dis Clin North Am 1993; **19**: 993-1009.

70. Wilske B, Schierz G, Preac-Mursic V, et al. Intrathecal production of specific antibodies against *Borrelia burgdorferi* in patients with lymphocytic meningoradiculitis. J Infect Dis 1986; **153**: 304-314.

71. Steere AC, Berardi VP, Weeks KE, Logigian EL, Ackermann R. Evaluation of the intrathecal antibody response to *Borrelia burgdorferi* as a diagnostic test for Lyme neuroborreliosis. J Infect Dis 1990; **161**: 1203-1209.

72. Halperin JJ, Volkman DJ, Wu P. Central nervous system abnormalities in Lyme neuroborreliosis. Neurology 1991; **41**: 1571-1582.

73. Schutzer SE, Coyle PK, Krupp LB, et al. Simultaneous expression of Borrelia OspA and OspC and IgM response in cerebrospinal fluid in early neurologic Lyme disease. J Clin Invest 1997; **I00**: 763-767.

74. Keller TL, Halperin JJ, Whitman M. PCR Detection of *Borrelia burgdorferi* DNA in cerebrospinal fluid of Lyme neuroborreliosis patients. Neurology 1992; **42**: 32-42.

75. Fallon BA, Das S, Plutchok, et al. Functional brain imaging and neuropsychological testing in Lyme disease. Clin Infect Dis 1997; **25**: 557 63

76. Benach JL, Coleman JL, Habicht GS. Serologic evidence for simultaneous occurrences of Lyme disease and babesiosis. J Infect Dis 1981; **144**: 473-477.

77. Grunwaldt E, Barbour AG, Benach JL. Simultaneous occurrence of babesiosis and Lyme disease. N Engl J Med 1983; **308**: 1166.

78. Anderson JF, Mintz ED, Gadbaw JJ, Magnarelli LA. *Babesia microti*, human babesiosis, and *Borrelia burgdorferi* in Connecticut. J Clin Microbiol 1991; **29**: 2779-2783.

79. Magnarelli LA, Dumler SJ, Anderson JF, Johnson RC, Fikrig E. Coexistence of antibodies to tick-borne pathogens of babesiosis, ehrlichiosis, and Lyme borreliosis in human sera. J Clin Microbiol 1995; **33**: 3054-3057.

80. Krause PJ, Telford SR, Spielman A, et al. Concurrent Lyme disease and babesiosis: evidence for increased severity and duration of illness. JAMA 1996; **275**: 1657-1660.

81. Mitchell PD, Reed KD, Hofkes JM. Immunoserologic evidence of coinfection with *Borrelia burgdorferi*, *Babesia microti*, and human granulocytic *Ehrlichia* species in residents of Wisconsin and Minnesota. J Clin Microbiol 1996; **34**: 724-727.

82. Fishbein DB, Dawson JE, Robinson LE. Human ehrlichiosis in the United States, 1985 to 1990. Ann Intern Med 1994; **120**: 736-743.

83. Dumler JS, Bakken JS. Ehrlichial diseases of humans: emerging tick-borne infections. Clin Infect Dis 1995; **20**: 1102-1110.

84. Anderson BE, Dawson JE, Jones DC, Wilson KH. *Ehrlichia chaffeensis*, a new species associated with human ehrlichiosis. J Clin Microbiol 1991; **29**: 2838-2842.

85. Walker DH, Barbour AG, Oliver JH, et al. Emerging bacterial zoonotic and vector-borne diseases: ecological and epidemiological factors. JAMA 1996; **275**: 463-469.

86. Goodman JL, Nelson C, Vitale B, et al. Direct cultivation of the causative agent of human granulocytic ehrlichiosis. N Engl J Med 1996; **334**: 209-215.

87. Persing DH, Mathiesen D, Marshall WE, et al. Detection of *Babesia microti* by polymerase chain reaction. J Clin Microbiol 1992; **30**: 2097-2103.

88. Luft BJ, Mudri S, Jiang W, et al. The 93-kilodalton protein of *Borrelia burgdorferi*: an immunodominant protoplasmic cylinder antigen. Infect Immun 1992; **60**: 4309-4321.

89. Comstock LE, Fikrig E, Shoberg RJ, Flavell RA, Thomas DD. A monoclonal antibody to OspA inhibits association of *Borrelia burgdorferi* with human endothelial cells. Infect Immun 1993; **61**: 423-431.

90. Padula SJ, Sampieri A, Dias F, Szczepanski A, Ryan RW. Molecular characterization and expression of p23 (OspC) from a North American Strain of *Borrelia burgdorferi*. Infect Immun 1993; **61**: 5097-5105.

91. Schwan TG, Schrumpf ME, Karstens RH, et al. Distribution and molecular analysis of Lyme disease spirochetes, *Borrelia burgdorferi*, isolated from ticks throughout California. J Clin Microbiol 1993; **31**: 3096-3108.

92. LeFebvre RB, Perog GC, Johnson RC. The 83-kilodalton antigen of *Borrelia burgdorferi* which stimulates Immunoglobulin (Ig) M and IgG responses in infected hosts is expressed by a chromosomal gene. J Clin Microbiol 1990; **28**: 1673-1675.

93. Magnarelli LA, Fikrig E, Berland R, Anderson JF, Flavell RA. Comparison of whole-cell antibodies and an antigenic flagellar epitope of Borrelia burgorferi in serologic tests for diagnosis of Lyme borreliosis. J Clin Microbiol 1992; **30**: 3158-62.

94. Probert WS, Allsup KM, LeFebvre RB. Identification and characterization of a surface-exposed 66-kilodalton protein from *Borrelia burgdorferi*. Infect Immun 1995; **63**: 1933.

95. Krause PJ, Spielman A, Telford SR, et al. Persistent parasitemia after acute babesiosis. N Engl J Med 1998; **339**: 161-165.

IGeneX, Inc.
795 San Antonio Rd., Palo Alto, CA USA 94303
Tel. 650.424.1191 / 800.832.3200 Fax. 650.424.1196