4 Laboratory Diagnostic Testing for *Borrelia burgdorferi* Infection[1]

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4.1 Introduction

Serology is the only standardized type of laboratory testing available to support the clinical diagnosis of Lyme borreliosis (Lyme disease) in the USA. It is also the only type of diagnostic testing approved by the US Food and Drug Administration (FDA). Of the 77 devices cleared by the FDA for *in vitro* diagnostic use for Lyme disease, all are designed to detect immune responses to antigens of *Borrelia burgdorferi* sensu stricto, particularly IgM and IgG (FDA, 2010). Serological tests do not become positive until an infected individual has had time to develop antibodies. In Lyme disease, this means that early acute disease characterized by an expanding rash (erythema migrans or EM) at the site of a tick bite cannot be reliably diagnosed by serology. After a few weeks of infection, however, immunocompetent people will have made enough antibodies that serology is useful for confirming exposure to *B. burgdorferi* in all subsequent stages of Lyme disease. Antibody levels remain elevated for months to years after the infection is cured.

A variety of direct tests for the agent of Lyme borreliosis have been developed. Direct tests include culture of *Borrelia* from skin or blood and occasionally cerebrospinal fluid (CSF), and detection of genetic material by PCR in skin, blood, synovial fluid and CSF. These tests have specialized roles in research and in academic and reference laboratories but are not available for routine use. Culture and PCR each have distinct limitations that will be noted in this chapter.

Diagnostic tests are of clinical value only if they are used appropriately. This has become particularly important in the field of diagnostic testing for Lyme disease, as both patients and doctors hear conflicting information about the risk of Lyme disease in various environments. Furthermore, patients are sometimes given laboratory diagnostic tests when they lack objective signs of Lyme disease and a history of potential exposure to infected vector ticks. A healthcare provider must estimate the pre-test likelihood that a patient has Lyme disease in order to understand the positive and negative predictive values of tests for Lyme disease. Fortunately, there are resources available to assist providers in making this judgement.

It is important to know that laboratories may offer 'in-house' testing for Lyme disease

[1] The findings and conclusions in this article are those of the author and do not necessarily represent the views of the Centers for Disease Control and Prevention.

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that does not require review and approval by the FDA. Because some in-house tests have not been rigorously developed and validated, the Centers for Disease Control and Prevention (CDC) and FDA recommend that these tests only be used when their accuracy and clinical usefulness have been documented in peer-reviewed scientific literature (CDC, 2005). Unvalidated tests as of 2010 include capture assays for antigens in urine, immunofluorescence staining or cell sorting of cell wall-deficient or cystic forms of *B. burgdorferi*, lymphocyte transformation tests, quantitative CD57 lymphocyte assays, 'reverse Western blots' (Feder *et al.*, 2007), in-house criteria for interpretation of immunoblots and measurements of antibodies in synovial fluid.

This chapter considers the diagnostic testing for *B. burgdorferi* sensu stricto infection, the only organism established to cause Lyme disease in North America. Lyme disease also results from infection by *Borrelia garinii* or *Borrelia afzelii* in Europe and Asia, as well as by the recently described *Borrelia spielmanii* in Europe (Wang *et al.*, 1999; Richter *et al.*, 2006; Fingerle *et al.*, 2008). *Borrelia valaisiana* and *Borrelia lusitaniae* have been associated anecdotally with Lyme disease in some parts of Europe (Crowder *et al.*, 2010), particularly *B. lusitaniae* in Portugal (Collares-Pereira *et al.*, 2004). *Borrelia bisettii* has been cultured from a few patients in Europe (Strle *et al.*, 1997), but has not been shown to cause human disease in North America. Diagnostic tests for *B. burgdorferi* sensu stricto will not necessarily perform well for infections by other genospecies of Lyme disease bacteria, although some do (e.g. assays based on the C6 peptide of the variable surface antigen (VlsE) or the whole VlsE protein). Guidelines for laboratory diagnosis of European Lyme borreliosis are available online in English (Health Protection Agency of the UK, 2010; German Society for Hygiene and Microbiology, 2000).

4.2 Two-tiered Serology: the Current Standard for Serodiagnosis in North America

The public health agencies of the USA and Canada advocate a two-step process for measuring antibodies in blood when Lyme disease is suspected. The CDC recommends two-tiered testing both for the evaluation of individual patients (CDC, 1995) and for epidemiological surveillance for Lyme disease (CDC, 1997). This recommendation was developed with the participation of the relevant major agencies of the USA, including the FDA, the National Institutes of Health, the Council of State and Territorial Epidemiologists, the Association of Public Health Laboratories and the Clinical Laboratory Standards Institute[2] (ASTPHLD and CDC, 1995). The Canadian Public Health Laboratory Network (2007) guidelines also recommend two-tiered testing. The Infectious Diseases Society of America (IDSA) has endorsed two-tiered serology to support the diagnosis of Lyme disease in patients who have manifestations other than acute EM (Wormser *et al.*, 2006).

A schematic summarizing the features of two-tiered serology is shown in Fig. 4.1. The first tier consists of a sensitive initial serological test or tests that detect class-specific antibodies (IgM and IgG, either together or separately). First-tier tests are enzyme immunoassays (EIAs) such as ELISAs or, rarely today, indirect immunofluorescence assays (IFAs) as they require a skilled microscopist and cannot be scored objectively. If the result of first-tier testing is negative, the serum is reported to be negative for antibodies to *B. burgdorferi* and is not tested further. If the result is positive or indeterminate (a value that is sometimes called 'equivocal' or 'borderline'), a second step should be performed. The indeterminate category is the range of test values that overlaps between Lyme disease patients and

[2] The latter two were known at the time as the Association of State and Territorial Public Health Laboratory Directors (ASTPHLD) and the National Committee for Clinical Laboratory Standards (NCCLS), respectively.

controls and is specific to each test. Further information is needed from a second test in order to call the specimen positive or negative.

The second tier consists of standardized immunoblotting, either by using Western blots or blots striped with diagnostically important purified antigens. When an IgG immunoblot is scored as positive (Dressler *et al.*, 1993; CDC, 1995), two-tiered testing is reported as positive. When an IgM immunoblot is scored as positive (Engstrom *et al.*, 1995; CDC, 1995), Lyme disease serology is reported as positive with the caveat that this finding is clinically relevant only in early disease, that is, in the first month of illness (ASTPHLD and CDC, 1995; CDC, 1995). Immunoblots and the recommended criteria for scoring them are shown in Fig. 4.2. These scoring criteria have been validated for antibodies to *B. burgdorferi* sensu stricto, the agent of Lyme disease in North America, but not for immune responses to other genospecies of *Borrelia*.

Two-tiered serology is considered positive only if the EIA (or IFA) and the immunoblot are both positive. Skipping either step increases the frequency of false-positive results (see below).

First-tier tests commonly use whole-cell antigens of *B. burgdorferi* grown *in vitro*. The immunodominant antigen VlsE also has been

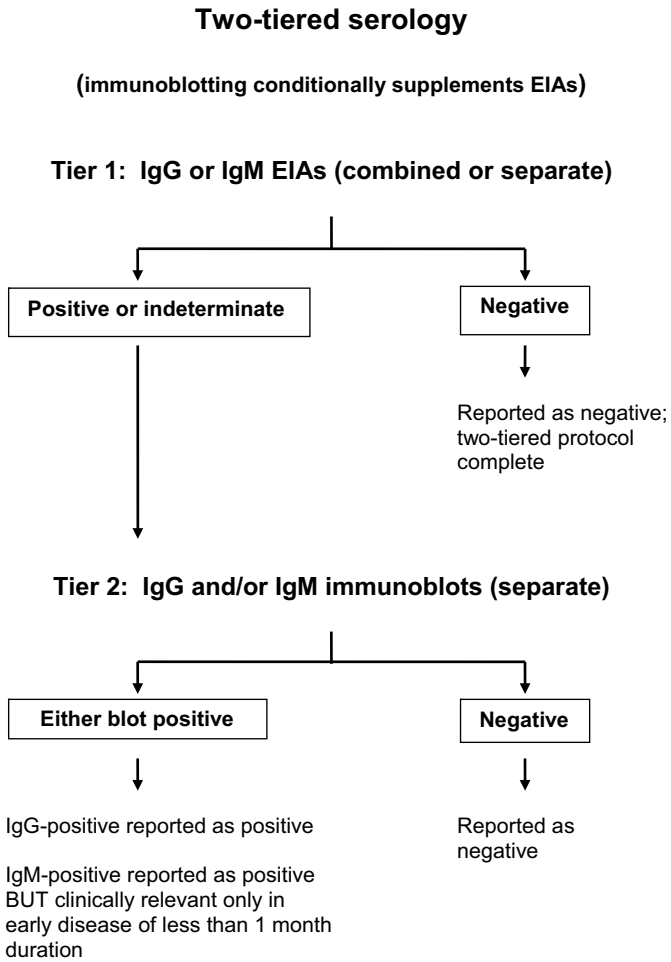

Fig. 4.1. Two-tiered serology for Lyme disease.

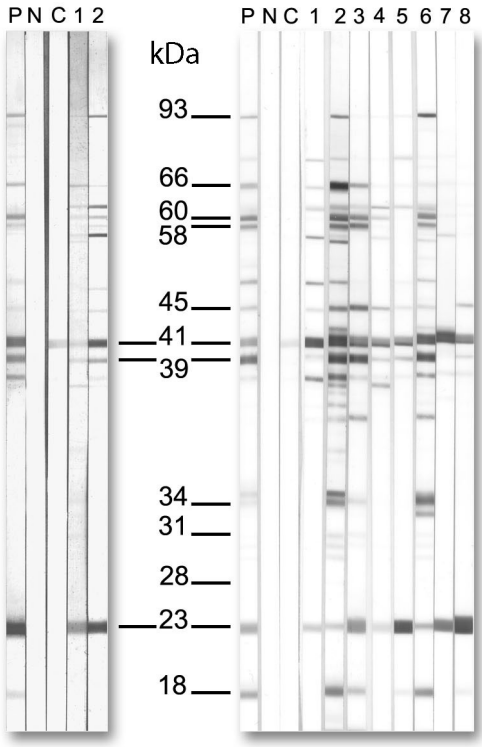

Fig. 4.2. Examples of conventional IgM (left panel) and IgG (right panel) immunoblots. Bands that are recommended for scoring are labelled. Two additional bands in the IgG blot are also labelled (OspA and OspB at 31 and 34 kDa, respectively; see text). Blots are considered to be positive if two of the three indicated IgM bands or five of the ten indicated IgG bands (excluding OspA and OspB) are present at an intensity equal to or greater than the calibration control. Left panel: IgM blot profiles for a patient with acute EM (lane 1) and for the same patient at convalescence (lane 2). Note the increase in the number and intensity of the bands at convalescence. Right panel: IgG blot profiles for eight patients with later manifestations of Lyme borreliosis (lanes 1–8). P, Positive-control serum; N, negative-control serum; C, calibration control (weak positive control). The molecular mass is indicated (kDa). The calibration controls (weak positive controls) have been digitally enhanced for greater clarity in reproduction.

approved by the FDA. One small portion of VlsE, a 26 amino acid peptide called C6 that reproduces the sixth constant region of the protein, was authorized by the FDA for commercial use (Immunetics) as a first-tier test in 2001 (Liang *et al.*, 1999; FDA, 2010). A diagnostic assay containing entire VlsE molecules expressed as recombinant proteins from both *B. burgdorferi* and *B. garinii* (Diasorin) became available as a first-tier test in 2007 (Ledue *et al.*, 2008; FDA, 2010). C6- and VlsE-based assays have the additional feature of detecting antibodies to Eurasian genospecies of *Borrelia* (i.e. *B. garinii* and *B. azfelii*) as well as *B. burgdorferi* sensu stricto.

An extensive peer-reviewed scientific literature supports the rationale for and performance of two-tiered serological testing. This algorithm has been validated in both retrospective and prospective studies. The specificity of two-tiered testing is high – 99% or greater in diagnostic reference centres. The sensitivity is also high after the acute phase of EM. Patients with Lyme arthritis or late neuroborreliosis are nearly always seropositive (97–100%). The rate of seropositivity is lower in patients with acute-phase early neurological disease (80–100%, depending on the population studied). This stage of Lyme disease in particular is the subject of research to improve the sensitivity of serodiagnosis (Dressler *et al.*, 1993; Engstrom *et al.*, 1995; Johnson *et al.*, 1996; Bacon *et al.*, 2003; Peltomaa *et al.*, 2004; Aguero-Rosenfeld *et al.*, 2005; Steere *et al.*, 2008; Branda *et al.*, 2010; Wormser *et al.*, 2011; and others).

Patients often inform themselves about diagnostic testing for Lyme disease before visiting a physician. Unfortunately, the quality of information available on the Internet varies widely and some is not evidence-based (Cooper and Feder, 2004). Here are some questions that are commonly asked:

1. Aren't ELISAs insensitive and therefore unsuitable as first-tier tests?
2. Aren't immunoblots more sensitive than ELISAs? Shouldn't they be used instead of two-tier testing?
3. Why do the recommended blot scoring criteria ignore outer-surface protein A (OspA) and OspB? OspA was used as a vaccine, so why isn't it scored in serology?
4. Why are you disregarding my IgM test result just because I have had this illness for years?

5. How sensitive is serology in late Lyme disease? How can you be sure, as seropositivity is part of the case definition for Lyme disease, except in patients with EM?

Each of these questions will be addressed with reference to the scientific literature.

4.2.1 How sensitive are ELISAs?

The sensitivity of first-tier tests varies by stage of Lyme disease. The antibody response to *B. burgdorferi* develops over the first few weeks after the spirochaete is introduced into the body, in a fashion similar to other bacterial infections. Patients with EM are often seronegative at the time of presentation, as EM can precede the development of a measurable antibody response. The probability of seroreactivity increases with duration of EM and with the development of signs of disseminated disease (Aguero-Rosenfeld *et al.*, 1993, 1996; Johnson, 2006). Although 60% or less of EM patients test positive by ELISA during acute disease, by convalescence 80–90% of treated EM patients are seropositive (Aguero-Rosenfeld *et al.*, 1993, 1996; Engstrom *et al.*, 1995; Bacon *et al.*, 2003; Johnson *et al.*, 2004; Johnson, 2006). The well-known insensitivity of ELISAs in acute EM is the reason that the CDC and IDSA do not advocate serological testing of these patients. It is appropriate to treat patients who have rashes compatible with EM with antibiotics based on clinical presentation alone.

The controversies about serological testing do not generally concern test performance in patients with EM, of course. Fortunately, after the first weeks of illness, the sensitivity of first-tier serology is excellent. Numerous published studies indicate that the sensitivity of whole-cell-lysate ELISAs is essentially 100% after the EM stage of illness (e.g. Dressler *et al.*, 1993; Bacon *et al.*, 2003; Johnson *et al.*, 2004; Johnson, 2006). Antibody levels remain elevated for months to years following antibiotic therapy (Engstrom *et al.*, 1995; Aguero-Rosenfeld *et al.*, 1996; Kalish *et al.*, 2001). Because IgM antibody levels may remain elevated after treatment, a single positive IgM ELISA test does not necessarily support the diagnosis of a new *B. burgdorferi* infection.

How did the misconception arise that ELISAs are insensitive in stages of Lyme disease other than EM? Firstly, studies are often cited that describe tests that are obsolete and no longer used. For example, a study conducted in 1992–1994, before two-tiered testing was recommended as a national standard, is commonly quoted (Bakken *et al.*, 1997). Many early ELISAs were designed to be stand-alone tests. Some tests were insensitive in order to achieve better specificity using whole-cell lysates. Despite this, false-positive results with some serum samples from healthy donors approached 55% (Bakken *et al.*, 1997). Of the 29 ELISAs approved by the FDA before 1993 (FDA, 2010), only three were used recently by a few laboratories (20/417) that participated in a College of American Pathologists (CAP) proficiency testing programme (CAP, 2009). Most ELISAs in current commercial use are sufficiently sensitive to perform well in a two-tiered testing scheme after the EM stage of illness (Aguero-Rosenfeld *et al.*, 1993; Bacon *et al.*, 2003; Johnson *et al.*, 2004; Johnson, 2006). The excellent performance of ELISAs in proficiency tests can be reviewed by subscribers to the surveys carried out by CAP (2009), although it must be kept in mind that only a small number of samples were used in each evaluation.

Secondly, the misconception that ELISAs are insensitive in later Lyme disease is supported by inappropriately applying data from EM patients to people with later manifestations of this illness. Online statements such as 'The test misses 35% of culture-proven Lyme disease (only 65% sensitivity)' (ILADS, 2010) fail to note that *B. burgdorferi* can be consistently cultured only from patients with acute EM, and not from patients with later disease (Aguero-Rosenfeld *et al.*, 2005). It is incorrect to cite the performance of a serological test with samples from patients with EM, for whom serological testing is not recommended, and then claim that ELISAs are poor in diagnosing infections of longer duration.

4.2.2 Why not skip the ELISA and go directly to immunoblots?

It is important to appreciate that first- and second-tier tests are not independent indicators of exposure to *B. burgdorferi* (Wormser *et al.*, 2000). ELISAs and immunoblots are usually constructed with the same antigens – whole-cell antigens of bacteria grown in culture – but they are processed differently. There is no a priori reason for immunoblots to be more sensitive than ELISAs. ELISAs provide an estimate of the magnitude of the IgG/IgM humoral antibody response to all of the antigens that are expressed under the culture conditions used to produce the whole-cell antigen or to the recombinant or peptide antigens used. ELISA results are objective and quantitative. They can be correlated with antibody titres.

Immunoblotting techniques, in contrast, separate the many bacterial antigens spatially on a solid support so that the specificity and complexity of the antibody responses are revealed. Immunoblots are qualitative or, at best, semi-quantitative tests (Fig. 4.2).

The rationale for determining IgM and IgG antibody profiles by immunoblotting is to learn whether a patient's antibodies recognize proteins of *B. burgdorferi* that have been established to be more predictive of Lyme disease than other components of the bacteria (Dressler *et al.*, 1993; Engstrom *et al.*, 1995). Many antigens have similarities to those of other organisms, such as proteins involved in motility (e.g. flagellin) and responses to stress (e.g. 'heat-shock' proteins). Recognition of one or more antigens from this set by serum antibodies is not necessarily indicative of exposure to *B. burgdorferi*, although these reactions contribute to the signal strength measured in an ELISA. ELISAs for Lyme disease commonly may give false-positive results (up to ~55%) in patients with other spirochaetal diseases such as tick-borne relapsing fever, syphilis or leptospirosis (Johnson *et al.*, 2004: Johnson, 2006), and cross-reactivity with *Treponema denticola* in patients with periodontal disease has been reported anecdotally. False-positive results also may occur in granulocytic anaplasmosis, although the frequency is unclear because coinfection with *B. burgdorferi* may be present (Wormser *et al.*, 1997). Non-specific reactions due to polyclonal B-cell activation may occur in conditions such as Epstein–Barr virus infection or malaria (Magnarelli, 1995; Burkot *et al.*, 1997). There are reports of false-positive reactions in *Helicobacter pylori* infections and bacterial endocarditis, although this has not been well studied (Kaell *et al.*, 1993). In addition, non-infectious conditions within the differential diagnosis of Lyme disease yield false-positive rates of around 10%, depending on the patients studied. Cross-reactions are sometimes seen in serum from patients with anti-nuclear antibodies, rheumatoid factor, clinical rheumatoid arthritis or multiple sclerosis (Johnson *et al.*, 2004; Johnson, 2006).

Omitting an ELISA as a first-tier test and using immunoblot results alone decreases the specificity of serological testing. Decreased specificity has been observed both with serum samples from healthy blood donors from non-endemic areas and with samples from patients with other illnesses within the differential diagnosis of Lyme disease. For donors, the decrease in specificity was from 100% for two-tiered testing to 92% for blotting alone in a study by Engstrom *et al.* (1995) and from 100 to 98.5% in work by Johnson *et al.* (1996). In patients with other illnesses, there was a 4% decrease from 100% specificity for two-tiered testing to 96% for blotting alone (Johnson *et al.*, 1996).

Seemingly small changes in specificity have large public health impacts. The volume of laboratory diagnostic testing for Lyme disease has recently been evaluated. In 2008, more than 3.4 million tests for Lyme disease were performed in the USA (A. Hinckley, CDC, 2010, personal communication). Each 1% decrease in testing specificity would generate about 34,000 false-positive results per year. To put this number in context, 38,468 cases of Lyme disease (confirmed plus probable) were reported to the CDC as part of the US national system for surveillance of notifiable diseases in 2009 (Bacon *et al.*, 2008; CDC, 2011).

Why does specificity decrease if immunoblotting alone is used? The Clinical Laboratory Standards Institute identifies one

reason: 'The erroneous scoring of a faint band is a common reason for false-positive readings…' (NCCLS, 2000). IgM results are more affected by this problem than IgG blots. In general, IgM antibodies are more non-specifically 'sticky' than IgG antibodies, in part because of their pentameric structure in serum compared with monomeric IgG. In addition, only two of three specified bands are required for an IgM blot to be reported as positive, whereas five of ten bands are necessary for an IgG blot to be positive by the recommended blot interpretation criteria (CDC, 1995; Fig. 4.2). Consequently, a single erroneously scored faint band will affect IgM results more readily than it will affect IgG results.

Faint bands, particularly in IgM blots, may not be diagnostically significant even for so-called 'specific' antigens. If healthcare providers adhere to the recommendation to demonstrate that antibodies are present at a positive or indeterminate level by a first-tier test before ordering an immunoblot, the risk of an erroneously positive serology based on scoring faint bands is reduced but not eliminated.

4.2.3 Why don't the scoring criteria for immunoblots include OspA and OspB?

The bands at the 31 and 34 kDa positions of immunoblots are produced by OspA and OspB, respectively (Fig. 4.2). It has been recognized since the early 1990s that antibodies to OspA and OspB are infrequently detected and when they are observed, it is usually in patients with longstanding Lyme arthritis. Ma *et al.* (1992) wrote that '… antibodies against the 31- and 34-kDa proteins were rarely detected and, consequently, became less significant when compared with other protein bands in this study'. Steere's laboratory reported in Dressler *et al.* (1993) that, although antibodies to OspA and OspB were detectable in some patients with Lyme arthritis or late neurological disease, the frequency of antibody responses to these polypeptides was not as high as to ten other antigens. Blot interpretation criteria that could best discriminate Lyme disease patients from controls therefore did not include scoring antibodies to OspA or OspB. When bands at 31 or 34 kDa are observed, they are virtually always in the context of a robust IgG response to a large number of scored antigens.

Patients may inquire specifically about why OspA is not scored when it was the basis for an effective vaccine (ILADS, 2010). People naturally think of the usual way that vaccines work, neutralizing infection in a mammalian host, and expect a vaccine antigen to be a good diagnostic antigen. They may be unaware that the OspA vaccine works by killing *B. burgdorferi* in vector ticks as they feed (de Silva *et al.*, 1996). OspA is well expressed by *B. burgdorferi* in unfed ticks and is a suitable target for antibodies that enter a tick during a blood meal from an OspA-vaccinated host. When ticks are exposed to a blood meal and the body temperature of a mammal, *B. burgdorferi* stops expressing OspA (Schwan and Piesman, 2000). Another outer-surface protein, OspC, is expressed instead. Reciprocal expression of these two Osps has been demonstrated at the level of single cells (Srivastava and de Silva, 2008). It is not surprising, therefore, that antibody responses to OspC are diagnostically useful in early Lyme disease, but responses to OspA are lacking.

In later manifestations of Lyme disease, especially Lyme arthritis, some people develop antibodies to OspA and/or OspB. OspA expression is upregulated in an inflammatory milieu such as an arthritic joint. OspA expression can be artificially up-regulated in a controlled *in vivo* environment by exposure to zymosan, a yeast cell-wall extract that induces inflammation (Kalish *et al.*, 1993; Crowley and Huber, 2003). Thus, it is no longer a paradox that *B. burgdorferi* expresses little or no OspA as it is transmitted to mammalian hosts, but that OspA can be produced late in the course of untreated Lyme disease.

Some claim that patients should be judged seropositive based on finding immunoblot bands solely at the 31 or 34 kDa positions, even when their serum is negative by an ELISA that uses whole-cell antigens. However, *B. burgdorferi* grown in culture expresses OspA and OspB abundantly (Crowley and Huber, 2003) and ELISAs made

from cultured whole cells contain these antigens. Thus, samples from patients who have diagnostically significant levels of antibodies to OspA or OspB will react in a whole-cell-lysate ELISA. When an ELISA is negative but an immunoblot of the same sample is scored positive, it is probable that faint immunoblot bands are being 'over-read'.

4.2.4 When is IgM testing clinically useful?

IgM testing should be performed only in patients with early Lyme disease, defined by the CDC (1995) as within the first month of infection. Some investigators have suggested recently that IgM responses may have diagnostic utility for an additional 2 weeks (Branda *et al.*, 2010, and personal communication). Whether the cut-off for IgM testing is best set at 4 or 6 weeks, IgM testing is appropriate only during a limited early time window. Recall also that serological testing is not useful in patients with EM, the earliest manifestation of Lyme disease, simply because antibodies have not yet had time to develop. This further restricts the clinical utility of IgM testing.

Some physicians use IgM serology to assess patients with longstanding illness (many months to years). They point to the new IgM responses to OspB that have been observed to develop late in infection in patients with prolonged disease. This new IgM response, however, occurs in the context of a robust IgG response to a large number of the antigens in the recommended IgG scoring criteria (Kalish *et al.*, 1993). The existence of a new IgM response in Lyme arthritis patients is not good evidence that IgM serology alone, and especially not IgM immunoblotting alone, can properly support the diagnosis of late Lyme disease.

During the month or so after initial infection, antibodies rise in titre, recognize an increasing number of borrelial antigens and switch class from a predominantly IgM response to IgG. The evolution of the immune responses during early infection is illustrated by the serological findings in patients with early neurological disease. In a study of patients with facial paralysis, 87% had diagnostic levels of IgM antibodies, 66% were IgG positive and all were seropositive for at least one antibody class (Peltomaa *et al.*, 2004). This profile of antibody reactivity by class (i.e. a greater frequency of positive IgM responses than IgG, with many people seropositive for both classes) also is seen in patients with other manifestations of early neurological disease, typically meningitis and/or radiculoneuritis (Roux *et al.*, 2007). In the event that a patient with a suspected early manifestation of Lyme disease is seronegative, CDC guidelines note that 'serologic evidence of infection is best obtained by testing of paired acute- and convalescent-phase samples' (ASTPHLD and CDC, 1995) obtained several weeks apart.

By the time patients develop later manifestations of Lyme disease, they are almost universally seropositive for IgG (Dressler *et al.*, 1993; Kannian *et al.*, 2007). Numerous studies with robust sample sizes have been published about the immune responses in Lyme arthritis. Patients with Lyme arthritis typically have high IgG titres, higher than those seen in any of the other various manifestations of Lyme disease, and waning IgM responses.

Late neurological Lyme disease, presenting as encephalomyelitis, peripheral neuropathy or encephalopathy, is rare (Wormser *et al.*, 2006; Halperin *et al.*, 2007). It has been speculated that late neuroborreliosis has become rarer in recent years due to earlier diagnosis and treatment, preventing progression to late-stage manifestations. Serum IgG antibodies have been found consistently in patients who have been available for study (Dressler *et al.*, 1993; Bacon *et al.*, 2003).

For these reasons, the CDC does not recommend the use of IgM responses in the absence of diagnostic levels of IgG antibodies to support the diagnosis of any manifestation of Lyme disease after 1 month of illness. Furthermore, as noted by Sivak *et al.* (1996), the predictive value of a positive IgM blot is 'poor in patients with minimal clinical evidence of Lyme disease'.

4.2.5 How can you study the sensitivity of tests for Lyme disease when seropositivity is part of the definition of a case?

The clinical signs and symptoms of Lyme disease after the first weeks of infection are not unique to this illness. Clinical findings are not specific enough to permit a confident diagnosis without laboratory testing. As noted by Steere *et al.* (2008), 'It is problematic to determine the frequency of seroreactivity in patients with neurological, cardiac, or joint manifestations of Lyme disease, because serological confirmation is a part of the case definition.' These considerations raise the important question of how to properly select serum samples for studying the performance of serological tests. To avoid circular reasoning, a previous positive serological result should not be the basis for inclusion of a specimen in such a study. However, independent assessment of infection status, for example by bacteriological culture, is routinely successful only in early disease (Aguero-Rosenfeld *et al.*, 2005) and is generally performed only in research settings.

To approach this problem, investigators look to the natural history of untreated Lyme disease. Patients with late disease frequently have a documented history of earlier signs and symptoms of Lyme disease that support the clinical diagnosis. For selection of 'gold standard' specimens for assessment of serological test performance in later Lyme disease, serum from patients with antecedent clinical findings compatible with earlier Lyme disease are used. Supplementary research tests such as PCR add additional confidence to the classification of some specimens (Bradley *et al.*, 1994; Nocton *et al.*, 1994).

Patients with early neurological Lyme disease commonly have a history of recent EM. Lyme facial paralysis, for example, was associated with EM in 72–87% of patients, depending upon the study (Peltomaa *et al.*, 2004). Patients with carditis, an uncommon presentation of Lyme disease that occurs in the early weeks of infection and manifests primarily as atrioventricular block, also typically have either previous or concurrent EM (>80%) or sometimes early neurological Lyme disease (Wormser *et al.*, 2006). Patients with late neurological Lyme disease, a rare condition, generally have a history of other clinical manifestations of Lyme disease such as EM or Lyme arthritis. In a report by Bacon *et al.* (2003), 100% of 11 late neurological Lyme disease patients were seropositive. All of these patients had antecedent other clinical manifestations of Lyme disease that were the basis for including the serum samples in the study.

4.3 Newer Serological Tests

Two-tiered serology has good performance characteristics, that is, high sensitivity and specificity after the first weeks of *B. burgdorferi* infection. Experienced laboratories with good-quality control and quality assurance programmes obtain consistent results (e.g. Bacon *et al.*, 2003; Kannian *et al.*, 2007; CAP, 2009). Nevertheless, there are limitations to two-tiered testing that are being addressed by newer testing methods. As noted, two-tiered testing is insensitive in acute EM and may be negative in early neuroborreliosis. Other drawbacks are that the two-step procedure is complex, technically demanding and costly. Immunoblots are only semi-quantitative. Traditional blots are hard to standardize, as reading them involves judgement about the significance of weak bands. Other difficulties with two-tiered serology are the need to know the date of disease onset to appropriately request IgM testing and the inconvenience of sometimes having to draw a second blood sample. The latter may occur if the second test is indicated and the first test was performed by a laboratory that does not offer immunoblotting.

The research community is actively addressing these limitations, and a number of new testing approaches have been developed. The Public Health Service agencies have established the standard that new tests should meet or exceed the performance of two-tiered testing in order to be deemed suitable for clinical use (ASTPHLD and CDC, 1995). New approaches are either improvements in one of the steps of the two-step

testing regime or a potential alternative to two-tiered testing.

Striped blots with defined, purified antigens were FDA-approved in 2009 and are now commercially available (FDA, 2010). Viramed offers these immunoblots (Virablots) as an improvement over Western blots. Bands are striped at pre-defined positions so that calibration problems are avoided. They are read with a scanning densitometer to provide an objective measure of whether each band has sufficient colour density to be scored as a diagnostically significant reaction. Branda *et al.* (2010) have devised a two-tiered procedure consisting of whole-cell ELISAs and IgG Virablots that include a new band of VlsE. Only a VlsE band would be required for a positive reaction in early Lyme disease and five or more of 11 bands in the late disease (the bands in Fig. 4.2 plus VlsE). This approach provides sensitivity comparable to or higher than standard two-tiered testing in each stage of Lyme disease, while maintaining high specificity. If adopted, it would render IgM blots obsolete. The problems of false-positive IgM blots due to over-reading of faint bands and the difficulty of knowing how long a patient has been infected would be avoided.

A second approach, developed by Zeus Scientific, seeks to avoid immunoblotting altogether by using defined peptides in a multiplex microsphere assay on the Luminex diagnostic platform. This assay, called the AtheNA Multi-Lyte test system, has been FDA-approved as a first-tier test and also evaluated with favourable results as an alternative to immunoblotting when other approved assays are used as the first-tier test (FDA, 2010; Porwancher *et al.*, 2011).

Both the C6 peptide and whole VlsE assays have been approved as alternatives to whole-cell ELISAs as first-tier tests. In addition, the Immunetics C6 assay has recently been evaluated as an assay that could be used in place of both steps of two-tiered testing, that is, as a simple 'stand-alone' test. The C6 ELISA as a single step is significantly more sensitive in patients with EM than two-tiered testing (66.5 versus 35.2%, $P<0.001$; Wormser *et al.*, 2011). Furthermore, the C6 assay performed comparably to two-tiered testing in sera from patients with early neuroborreliosis or Lyme arthritis. The specificity of the C6 assay was slightly less than two-tiered testing (98.9 versus 99.5%, $P<0.05$), however, which will be a key consideration when the assay is reviewed for approval as a stand-alone test.

Various diagnostic testing approaches will offer value to clinicians. The general practitioner may prefer a simple, objective, less-costly one-step test. The specialist may prefer the added information that immunoblots provide to diagnose atypical cases. The type and number of reactive bands offer insights about the stage of Lyme disease. Expanding profiles of reactivity with paired samples may support suspicion of ongoing infection.

4.4 Direct Assays

Two types of direct assay have been important in Lyme disease research and are useful in the laboratory diagnosis of some patients. These assays are culture of *B. burgdorferi* and detection of DNA by molecular methods (PCR or quantitative real-time PCR). Neither culture nor PCR are components of the routine evaluation of patients with suspected Lyme disease and no nationally standardized or FDA-approved tests are available. Both techniques have played important roles in understanding the pathogenesis of *B. burgdorferi* infections, however, and have assisted investigators in establishing serum banks from authenticated Lyme disease patients.

Direct detection methods have been reviewed in detail by Aguero-Rosenfeld *et al.* (2005) and have not changed significantly since this work was published. *B. burgdorferi* can be recovered from skin biopsy samples of EM patients with >50% efficiency. Efficiency of recovery is inversely correlated to the duration of EM, indicating that spirochaetes are rapidly cleared from the region of skin inoculated by tick bite. In acute EM, spirochaetes also can be grown from blood, especially high-volume plasma cultures, with recovery rates of >40%. The period of haematogenous dissemination of borreliae, however, is brief (several weeks). In later stages of the disease, blood cultures are

generally negative. There are only anecdotal reports of *B. burgdorferi* cultured from synovial fluid, an apparently hostile environment, and CSF. The low sensitivity of culture after the EM stage of illness (which can be treated based on the appearance of the rash) and the length of time necessary to monitor cultures (3 weeks or longer, depending on the protocol) greatly limit the clinical usefulness of bacteriological culture.

PCR is a sensitive method to detect *B. burgdoferi* DNA in skin biopsy and synovial fluid specimens (Dumler, 2001). Aguero-Rosenfeld *et al.* (2005) calculated median PCR sensitivities of 64% in skin biopsy samples from EM patients (four studies, range 59–67%) and 83% in synovial fluid specimens (four studies, range 76–100%). PCR has been particularly useful diagnostically in evaluating patients with treatment-resistant Lyme arthritis (Nocton *et al.*, 1994). DNA detection methods have been less helpful in evaluating patients with neurological signs. Reported PCR sensitivities in CSF have been low and highly variable. PCR tests were positive in 38% of early and 25% of late US neuroborreliosis patients (n = 60; Nocton *et al.*, 1996). Urine is not a suitable sample for PCR testing (Rauter *et al.*, 2005).

4.5 Appropriate Use of Diagnostic Tests

Laboratory testing of patients without objective signs of Lyme disease or a history of potential exposure to infected vector ticks is not clinically useful. Laboratory diagnostic tests with excellent sensitivity and specificity will not have helpful predictive values if they are used inappropriately (Sackett *et al.*, 1991). Predictive value is determined both by test characteristics (sensitivity and specificity) and, importantly, by the population in which it is used. The practice of testing patients with a low likelihood of Lyme disease can generate more false-positive results than true-positive results, resulting in mis-diagnosis and thereby harming ill people (Seltzer and Shapiro, 1996; Tugwell *et al.*, 1997).

The positive predictive value is the probability that a patient who has a positive test result truly has Lyme borreliosis. Negative predictive value is the probability that a patient who has a negative test result does not have Lyme borreliosis. An assay with high diagnostic sensitivity improves negative predictive value; one with high diagnostic specificity improves positive predictive value.

Serological testing is recommended only for patients who have appropriate pre-test probabilities of Lyme disease in order for the results to have useful predictive values. A position paper published by the American College of Physicians (ACP) concluded that laboratory testing should be requested only for patients who have an estimated pre-test probability of Lyme disease between 0.20 and 0.80 (Tugwell *et al.*, 1997). The ACP panel members pointed out that patients who have only non-specific signs and symptoms of illness such as headache, fatigue and muscle or joint pains, even when they reside in a geographical area endemic for Lyme disease, have a pre-test probability of Lyme disease of less than 0.20, usually much less. Patients with non-specific findings and no risk of exposure to infected ticks will have an extremely low pre-test probability.

When the pre-test probability of Lyme disease is greater than 0.80, laboratory evaluation adds little useful information (Tugwell *et al.*, 1997). This situation only occurs in patients presenting with EM in an endemic area, as all of the other clinical manifestations of Lyme disease can be found in other conditions.

The risk of Lyme disease is geographically focal. Of more than 300,000 cases reported to the CDC over the last 15 years, most occurred in ten states of the northeast and upper midwest. Maps of reported cases of Lyme disease by county and tables of incidence by state are updated annually by the CDC and published online (CDC, 2011)c. The mapped density of host-seeking *Ixodes scapularis* nymphs in the USA is consistent with the pattern of reported human cases (Diuk-Wasser *et al.*, 2006). A 'Lyme disease tick map' has recently become available as an iPhone application through the Apple iTunes store (American Lyme Disease Foundation, 2010).

The concepts of positive and negative predictive value are well established and

Table 4.1. Effect of disease prevalence on predictive values of diagnostic tests [a]

Prevalence = 1%			
	Test positive	Test negative	Total
Disease	10	0	10
No disease	20	970	990
Total	30	970	1000
Predictive value of a negative result = 970/970 = 100%			
Predictive value of a positive result = 10/30 = 33% (67% false positives)			
Prevalence = 40%			
	Test positive	Test negative	Total
Disease	392	8	400
No disease	12	588	600
Total	404	596	1000
Predictive value of a negative result = 588/596 = 99%			
Predictive value of a positive result = 392/404 = 97% (3% false positives)			

[a]Illustration assumes that test sensitivity and specificity are each 98%.

have been described carefully elsewhere (e.g. Sackett *et al.*, 1991; Seltzer and Shapiro, 1996; Tugwell *et al.*, 1997). They are briefly illustrated in Table 4.1 for two different clinical situations. In both cases, diagnostic tests with good performance characteristics are assumed: 98% sensitivity and 98% specificity. In the first situation, the true frequency of disease in the population to be tested (prevalence) is only 1%. This represents the pre-test likelihood of Lyme disease in a patient with non-specific symptoms and no objective physical signs of this illness who resides in an endemic area (CDC, 2011). For patients with no history of residence in or travel to an endemic area, the prevalence of Lyme disease is much less than 1%. In the second situation, the true frequency of Lyme disease is 40%. This prevalence (or higher) is the approximate pre-test likelihood of Lyme arthritis in patients with pronounced knee swelling who reside in an endemic area (Tugwell *et al.*, 1997).

Good tests have markedly different predictive values depending on the setting of use (Table 4.1). When the pre-test probability is 40%, the predictive values of both negative and positive results are very high (99% and 97%, respectively). However, when the pre-test probability is low, most positive test results are false positives (67%). Clinicians are currently ordering an extraordinary number of diagnostic tests for Lyme disease – more than 3.4 million tests annually, as noted above. It is critically important to the well-being of patients that tests only be used when the predictive value of a positive result is high (Fig. 4.3).

Where disease is rare

Positives mostly deceive

Even with good tests

Paul Mead

Fig. 4.3. Haiku to diagnostic testing.

References

Aguero-Rosenfeld, M.E., Nowakowski, J., McKenna, D.F., Carbonaro, C.A. and Wormser, G.P. (1993) Serodiagnosis in early Lyme disease. *Journal of Clinical Microbiology* 31, 3090–3095.

Aguero-Rosenfeld, M.E., Nowakowski, J., Bittker, S., Cooper, D., Nadelman, R.B. and Wormser, G.P. (1996) Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. *Journal of Clinical Microbiology* 34, 1–9.

Aguero-Rosenfeld, M.E., Wang, G., Schwartz, I. and Wormser, G.P. (2005) Diagnosis of Lyme borreliosis. *Clinical Microbiology Reviews* 18, 484–509.

American Lyme Disease Foundation (2010) Lyme disease tick map iPhone app <http://www.aldf.com/ALDF_iPhone_Application.shtml>.

ASTPHLD and CDC (1995) *Proceedings of the Second National Conference on Serologic Diagnosis of Lyme Disease*, Dearborn, Michigan, 27–29 October 1994. Association of State and Territorial Public Health Laboratory Directors and the Centers for Disease Control and Prevention, Washington, DC.

Bacon, R.M., Biggerstaff, B.J., Schriefer, M.E., Gilmore, R.D. Jr, Philipp, M.T., Steere, A.C., Wormser, G.P., Marques, A.R. and Johnson, B.J. (2003) Serodiagnosis of Lyme disease by kinetic enzyme-linked immunosorbent assay using recombinant VlsE1 or peptide antigens of *Borrelia burgdorferi* compared with 2-tiered testing using whole-cell lysates. *Journal of Infectious Diseases* 187, 1187–1199.

Bacon, R.M., Kugeler, K.J. and Mead, P.S. (2008) Surveillance for Lyme disease – United States, 1992–2006. *MMWR Surveillance Summaries* 57, 1–9.

Bakken, L.L., Callister, S.M., Wand, P.J. and Schell, R.F. (1997) 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. *Journal of Clinical Microbiology* 35, 537–543.

Bradley, J.F., Johnson, R.C. and Goodman, J.L. (1994) The persistence of spirochetal nucleic acids in active Lyme arthritis. *Annals of Internal Medicine* 120, 487–489.

Branda, J.A., Aguero-Rosenfeld, M.E., Ferraro, M.J., Johnson, B.J., Wormser, G.P. and Steere, A.C. (2010) 2-Tiered antibody testing for early and late Lyme disease using only an immunoglobulin G blot with the addition of a VlsE band as the second-tier test. *Clinical Infectious Diseases* 50, 20–26.

Burkot, T.R., Schriefer, M.E. and Larsen, S.A. (1997) Cross-reactivity to *Borrelia burgdorferi* proteins in serum samples from residents of a tropical country nonendemic for Lyme disease. *Journal of Infectious Diseases* 175, 466–469.

CAP (2009) *Tick-transmitted Disease Survey TTD-B*. College of American Pathologists, Northfield, IL.

Canadian Public Health Laboratory Network (2007) The laboratory diagnosis of Lyme borreliosis: guidelines from the Canadian Public Health Laboratory Network. *Canadian Journal of Infectious Diseases and Medical Microbiology* 18, 145–148.

CDC (1995) Recommendations for test performance and interpretation from the second national conference on serologic diagnosis of Lyme disease. *Morbidity and Mortality Weekly Report* 44, 590–591.

CDC (1997) Case definitions for infectious conditions under public health surveillance. *Morbidity and Mortality Weekly Report. Recommendations and Reports* 46, 1–55.

CDC (2005) Notice to readers: caution regarding testing for Lyme disease. *Morbidity and Mortality Weekly Report* 54, 125–126.

CDC (2011) Lyme disease data and statistics <http://www.cdc.gov/lyme/stats/index.html>.

Collares-Pereira, M., Couceiro, S., Franca, I., Kurtenbach, K., Schafer, S.M., Vitorino, L., Goncalves, L., Baptista, S., Vieira, M.L. and Cunha, C. (2004) First isolation of *Borrelia lusitaniae* from a human patient. *Journal of Clinical Microbiology* 42, 1316–1318.

Cooper, J.D. and Feder, H.M. Jr (2004) Inaccurate information about Lyme disease on the internet. *Pediatric Infectious Disease Journal* 23, 1105–1108.

Crowder, C.D., Matthews, H.E., Schutzer, S., Rounds, M.A., Luft, B.J., Nolte, O., Campbell, S.R., Phillipson, C.A., Li, F., Sampath, R., Ecker, D.J. and Eshoo, M.W. (2010) Genotypic variation and mixtures of Lyme *Borrelia* in *Ixodes* ticks from North America and Europe. *PLoS One* 5, e10650.

Crowley, H. and Huber, B.T. (2003) Host-adapted *Borrelia burgdorferi* in mice expresses OspA during inflammation. *Infection and Immunity* 71, 4003–4010.

de Silva, A.M., Telford, S.R. III, Brunet, L.R., Barthold, S.W. and Fikrig, E. (1996) *Borrelia burgdorferi* OspA is an arthropod-specific transmission-blocking Lyme disease vaccine. *Journal of Experimental Medicine* 183, 271–275.

Diuk-Wasser, M.A., Gatewood, A.G., Cortinas, M.R., Yaremych-Hamer, S., Tsao, J., Kitron, U., Hickling, G., Brownstein, J.S., Walker, E., Piesman, J. and Fish, D. (2006) Spatiotemporal patterns of host-seeking *Ixodes scapularis* nymphs (Acari: Ixodidae) in the United States. *Journal of Medical Entomology* 43, 166–176.

Dressler, F., Whalen, J.A., Reinhardt, B.N. and Steere, A.C. (1993) Western blotting in the serodiagnosis of Lyme disease. *Journal of Infectious Diseases* 167, 392–400.

Dumler, J.S. (2001) Molecular diagnosis of Lyme disease: review and meta-analysis. *Molecular Diagnosis* 6, 1–11.

Engstrom, S.M., Shoop, E. and Johnson, R.C. (1995) Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. *Journal of Clinical Microbiology* 33, 419–427.

FDA (2010) Medical devices: in vitro diagnostics/510(k) premarket notification <http://www.accessdata.fda.gov/scripts/cdrh/cfdocs/cfpmn/pmn.cfm>; product code LSR.

Feder, H.M. Jr, Johnson, B.J., O'Connell, S., Shapiro, E.D., Steere, A.C., Wormser, G.P., Agger, W.A., Artsob, H., Auwaerter, P., Dumler, J.S., Bakken, J.S., Bockenstedt, L.K., Green, J., Dattwyler, R.J., Munoz, J., Nadelman, R.B., Schwartz, I., Draper, T., Mcsweegan, E., Halperin, J.J., Klempner, M.S., Krause, P.J., Mead, P., Morshed, M., Porwancher, R., Radolf, J.D., Smith, R.P. Jr, Sood, S., Weinstein, A., Wong, S.J. and Zemel, L. (2007) A critical appraisal of "chronic Lyme disease". *New England Journal of Medicine* 357, 1422–1430.

Fingerle, V., Schulte-Spechtel, U.C., Ruzic-Sabljic, E., Leonhard, S., Hofmann, H., Weber, K., Pfister, K., Strle, F. and Wilske, B. (2008) Epidemiological aspects and molecular characterization of *Borrelia burgdorferi* s.l. from southern Germany with special respect to the new species *Borrelia spielmanii* sp. nov. *International Journal of Medical Microbiology* 298, 279–290.

German Society for Hygiene and Microbiology (2000) Quality standards for the microbiological diagnosis of infectious diseases: Lyme borreliosis <http://nrz-borrelien.lmu.de/miq-lyme/>.

Halperin, J.J., Shapiro, E.D., Logigian, E., Belman, A.L., Dotevall, L., Wormser, G.P., Krupp, L., Gronseth, G. and Bever, C.T. Jr (2007) Practice parameter: treatment of nervous system Lyme disease (an evidence-based review): Report of the Quality Standards Subcommittee of the American Academy of Neurology. *Neurology* 69, 91–102.

Health Protection Agency of the UK (2010) Diagnosis and treatment of Lyme borreliosis <http://www.hpa.org.uk/Topics/InfectiousDiseases/InfectionsAZ/LymeDisease/Guidelines/lymDiagnosisofLymeborreliosis/>.

ILADS (2010) Basic information about Lyme disease. International Lyme and Associated Diseases Society, Bethesda, MD <http://www.ilads.org/lyme_disease/about_lyme.html>.

Johnson, B. (2006) Lyme disease: serologic assays for antibodies to *Borrelia burgdorferi*. In: Detrick, B., Hamilton, R. and Folds, J. (eds) *Manual of Molecular and Clinical Laboratory Immunology*, 7th edn. ASM Press, Washington, DC.

Johnson, B.J., Robbins, K.E., Bailey, R.E., Cao, B.L., Sviat, S.L., Craven, R.B., Mayer, L.W. and Dennis, D.T. (1996) Serodiagnosis of Lyme disease: accuracy of a two-step approach using a flagella-based ELISA and immunoblotting. *Journal of Infectious Diseases* 174, 346–353.

Johnson, B.J.B., Bacon, R.M. and Schriefer, M.E. (2004) Correspondence. *Journal of Infectious Diseases* 189, 1962–1964.

Kaell, A.T., Redecha, P.R., Elkon, K.B., Golightly, M.G., Schulman, P.E., Dattwyler, R.J., Kaell, D.L., Inman, R.D., Christian, C.L. and Volkman, D.J. (1993) Occurrence of antibodies to *Borrelia burgdorferi* in patients with nonspirochetal subacute bacterial endocarditis. *Annals of Internal Medicine* 119, 1079–1083.

Kalish, R.A., Leong, J.M. and Steere, A.C. (1993) Association of treatment-resistant chronic Lyme arthritis with HLA-DR4 and antibody reactivity to OspA and OspB of *Borrelia burgdorferi*. *Infection and Immunity* 61, 2774–2779.

Kalish, R.A., Mchugh, G., Granquist, J., Shea, B., Ruthazer, R. and Steere, A.C. (2001) Persistence of immunoglobulin M or immunoglobulin G antibody responses to *Borrelia burgdorferi* 10–20 years after active Lyme disease. *Clinical Infectious Diseases* 33, 780–785.

Kannian, P., McHugh, G., Johnson, B.J., Bacon, R.M., Glickstein, L.J. and Steere, A.C. (2007) Antibody responses to *Borrelia burgdorferi* in patients with antibiotic-refractory, antibiotic responsive, or non-antibiotic-treated Lyme arthritis. *Arthritis and Rheumatism* 56, 4216–25.

Ledue, T.B., Collins, M.F., Young, J. and Schriefer, M.E. (2008) Evaluation of the recombinant VlsE-based Liaison chemiluminescence immunoassay for detection of *Borrelia burgdorferi* and diagnosis of Lyme disease. *Clinical and Vaccine Immunology* 15, 1796–1804.

Liang, F.T., Steere, A.C., Marques, A.R., Johnson, B.J., Miller, J.N. and Philipp, M.T. (1999) Sensitive and specific serodiagnosis of Lyme disease by enzyme-linked immunosorbent assay with a peptide based on an

immunodominant conserved region of *Borrelia burgdorferi* VlsE. *Journal of Clinical Microbiology* 37, 3990–3996.

Ma, B., Christen, B., Leung, D. and Vigo-Pelfrey, C. (1992) Serodiagnosis of Lyme borreliosis by Western immunoblot: reactivity of various significant antibodies against *Borrelia burgdorferi*. *Journal of Clinical Microbiology* 30, 370–376.

Magnarelli, L.A. (1995) Current status of laboratory diagnosis for Lyme disease. *American Journal of Medicine* 98, S10–S12; discussion S12–S14.

NCCLS (2000) Western blot assay for antibodies to *Borrelia burgdorferi*; approved guideline. National Committee for Clinical Laboratory Standards document M34-A, Vol. 20, No. 20.

Nocton, J.J., Dressler, F., Rutledge, B.J., Rys, P.N., Persing, D.H. and Steere, A.C. (1994) Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in synovial fluid from patients with Lyme arthritis. *New England Journal of Medicine* 330, 229–234.

Nocton, J.J., Bloom, B.J., Rutledge, B.J., Persing, D.H., Logigian, E.L., Schmid, C.H. and Steere, A.C. (1996) Detection of *Borrelia burgdorferi* DNA by polymerase chain reaction in cerebrospinal fluid in Lyme neuroborreliosis. *Journal of Infectious Diseases* 174, 623–627.

Peltomaa, M., Mchugh, G. and Steere, A.C. (2004) The VlsE (IR6) peptide ELISA in the serodiagnosis of Lyme facial paralysis. *Otology and Neurotology* 25, 838–841.

Porwancher, R.H., Hagerty, C.G., Fan, J., Landsberg, L., Johnson, B.J.B., Kopnitsky, M., Kulas, K., and Wong, S.J. (2011) Multiplex immunoassay for Lyme disease using VlsE1-IgG and pepC10-IgM antibodies: improving test performance through bioinformatics. *Clinical and Vaccine Immunology* 18, 851–859.

Rauter, C., Mueller, M., Diterich, I., Zeller, S., Hassler, D., Meergans, T. and Hartung, T. (2005) Critical evaluation of urine-based PCR assay for diagnosis of Lyme borreliosis. *Clinical and Diagnostic Laboratory Immunology* 12, 910–917.

Richter, D., Postic, D., Sertour, N., Livey, I., Matuschka, F.R. and Baranton, G. (2006) Delineation of *Borrelia burgdorferi* sensu lato species by multilocus sequence analysis and confirmation of the delineation of *Borrelia spielmanii* sp. nov. *International Journal of Systematic and Evolutionary Microbiology* 56, 873–881.

Roux, F., Boyer, E., Jaulhac, B., Dernis, E., Closs-Prophette, F. and Puechal, X. (2007) Lyme meningoradiculitis: prospective evaluation of biological diagnosis methods. *European Journal of Clinical Microbiology and Infectious Diseases* 26, 685–693.

Sackett, D.H., Haynes, R.B., Guyatt, G.H., and Tugwell, P. (1991) *Clinical Epidemiology: a Basic Science for Clinical Medicine*. Little, Brown and Co., Boston, MA.

Schwan, T.G. and Piesman, J. (2000) Temporal changes in outer surface proteins A and C of the Lyme disease-associated spirochete, *Borrelia burgdorferi*, during the chain of infection in ticks and mice. *Journal of Clinical Microbiology* 38, 382–388.

Seltzer, E.G. and Shapiro, E.D. (1996) Misdiagnosis of Lyme disease: when not to order serologic tests. *Pediatric Infectious Disease Journal* 15, 762–763.

Sivak, S.L., Aguero-Rosenfeld, M.E., Nowakowski, J., Nadelman, R.B. and Wormser, G.P. (1996) Accuracy of IgM immunoblotting to confirm the clinical diagnosis of early Lyme disease. *Archives of Internal Medicine* 156, 2105–2109.

Srivastava, S.Y. and de Silva, A.M. (2008) Reciprocal expression of OspA and OspC in single cells of *Borrelia burgdorferi*. *Journal of Bacteriology* 190, 3429–3433.

Steere, A.C., McHugh, G., Damle, N. and Sikand, V.K. (2008) Prospective study of serologic tests for Lyme disease. *Clinical Infectious Diseases* 47, 188–195.

Strle, F., Picken, R.N., Cheng, Y., Cimperman, J., Maraspin, V., Lotric-Furlan, S., Ruzic-Sablijic, E. and Picken, M.M. (1997) Clinical findings for patients with Lyme borreliosis caused by *Borrelia burgdorferi* sensu lato with genomic and phenotypic similarities of strain 25015. *Clinical Infectious Diseases* 25, 273–280.

Tugwell, P., Dennis, D.T., Weinstein, A., Wells, G., Shea, B., Nichol, G., Hayward, R., Lightfoot, R., Baker, P. and Steere, A.C. (1997) Laboratory evaluation in the diagnosis of Lyme disease. *Annals of Internal Medicine* 127, 1109–1123.

Wang, G., Van Dam, A.P., Schwartz, I. and Dankert, J. (1999) Molecular typing of *Borrelia burgdorferi* sensu lato: taxonomic, epidemiological, and clinical implications. *Clinical Microbiology Reviews* 12, 633–653.

Wormser, G.P., Horowitz, H.W., Nowakowski, J., Mckenna, D., Dumler, J.S., Varde, S., Schwartz, I., Carbonaro, C. and Aguero-Rosenfeld, M. (1997) Positive Lyme disease serology in patients with clinical and laboratory evidence of human granulocytic ehrlichiosis. *American Journal of Clinical Pathology* 107, 142–147.

Wormser, G.P., Carbonaro, C., Miller, S., Nowakowski, J., Nadelman, R.B., Sivak, S. and Aguero-Rosenfeld, M.E. (2000) A limitation of 2-stage serological testing for Lyme disease:

enzyme immunoassay and immunoblot assay are not independent tests. *Clinical Infectious Diseases* 30, 545–548.

Wormser, G.P., Dattwyler, R.J., Shapiro, E.D., Halperin, J.J., Steere, A.C., Klempner, M.S., Krause, P.J., Bakken, J.S., Strle, F., Stanek, G., Bockenstedt, L., Fish, D., Dumler, J.S. and Nadelman, R.B. (2006) The clinical assessment, treatment, and prevention of Lyme disease, human granulocytic anaplasmosis, and babesiosis: clinical practice guidelines by the Infectious Diseases Society of America. *Clinical Infectious Diseases* 43, 1089–1134.

Wormser, G., Schriefer, M., Aguero-Rosenfeld, M., Levin, A., Steere, A., Nadelman, R., Nowakowski, M., Marques, A., Johnson, B. and Dumler, J. (2011) Single-tier testing with the C6 peptide ELISA kit compared with two-tiered testing for Lyme disease. *Journal of the American Medical Association* (in press).