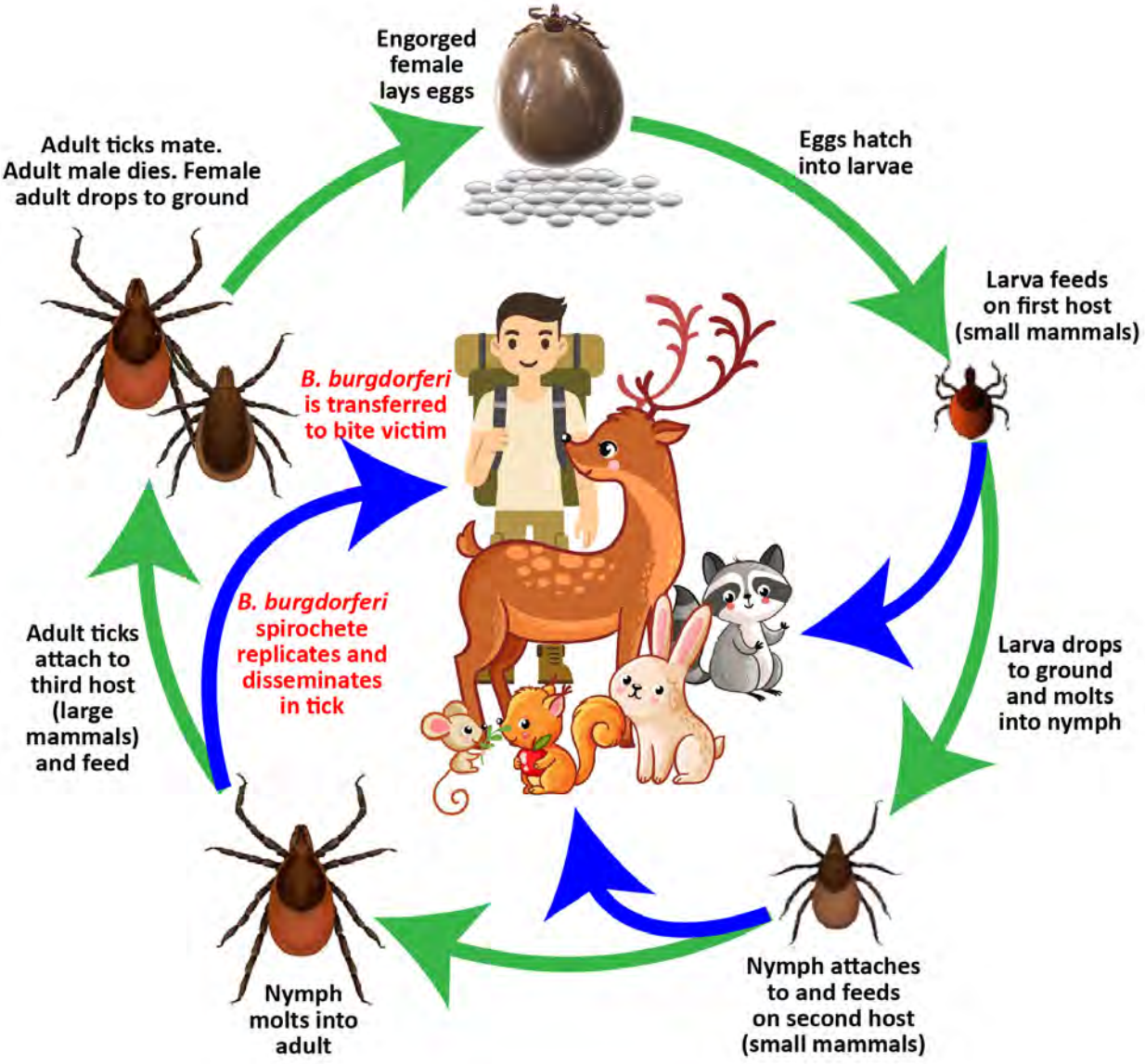
Immunoserology of Lyme Disease

Science and Results Interpretation



Immunosciences Lab., Inc. (ISL) now offers a Lyme disease panel that measures antibodies to *Borrelia* antigens grown in culture, antigens expressed *in vivo,* the borrelial subspecies *Sensu stricto, Afzelii, Garinii*, and *Miyamotoi,* and against the co-infections *Babesia, Ehrlichia* and *Bartonella*.

IMMUNOSCIENCES LAB., INC.

The advantages of the Multi-Peptide ELISA

- In the **Multi-Peptide ELISA**, we have combined the major advantages of Western Blot, which is the use of pure proteins or antigens, with the quantitative nature of the ELISA assay.
- We also measure antibodies against antigens known for their involvement with Lyme disease, including antibodies against the *Borrelia* subspecies *Sensu stricto, Afzelii, Garinii* and *Miyamotoi,* as well as the co-infections *Babesia*, *Bartonella* and *Ehrlichia*.
- In this patented methodology, we measure IgG and IgM antibodies not only against a mixture of *Borrelia* antigens but also against proteins or peptides of different molecular sizes, such as OspA, OspB, OspC, OspE, LFA, VMP, DbpA and more, each component separately.
- We use both *Borrelia* antigens grown in culture and the various proteins expressed *in vivo*, enabling us to improve the accuracy of our determinations.
- It is vital to correctly determine as early as possible whether someone is developing Lyme disease or something else. False positive test results could lead to years of incorrect treatment and unnecessary medications with their side effects, while false negative results could lead to Lyme arthritis, neuroborreliosis, and years or even a lifetime of suffering.
- The combination of the gold standard ELISA method with the 4 core principles of lab testing (antigen purity, optimization, validation, duplicate testing) has resulted in an accurate and reliable method for detecting Lyme disease.

IMMUNOLOGY OF
Lyme Disease

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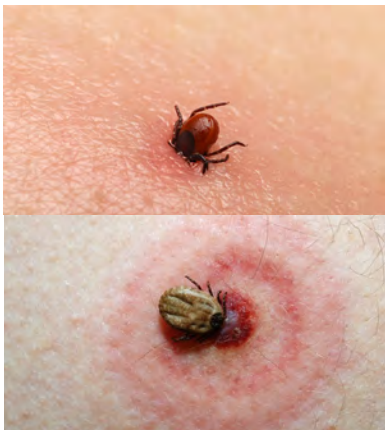
Lyme disease is the most prevalent vector-borne disease of humans in the United States. Originally thought to be just a form of arthritis, it is now known that its symptoms and effects include neurologic, cardiac and dermatologic elements as well. The agent of the disease is the spirochete *Borrelia burgdorferi*, which is transferred to humans through the bite of a tick, with the deer tick *Ixodes scapularis* being the prime vector in North America.

In 1975, in the town of Lyme, Connecticut, it was discovered that a cluster of patients thought to be suffering from juvenile rheumatoid arthritis were, in fact, suffering from a major new clinical entity, which was initially identified as Lyme arthritis. However, as greater understanding about the condition revealed that its clinical features included neurologic, cardiac, and dermatologic elements in addition to arthritis, the name was subsequently changed to Lyme disease.

In 1981 the scientist Willy Burgdorfer identified a highly motile spirochete with 7-11 flagella as the etiological agent of Lyme disease, earning the bacterium the name *Borrelia burgdorferi*. The spirochete is transferred to humans by the bite of different species of tick. In eastern North America the deer tick, *Ixodes scapularis*, is the primary vector for the disease. Spirochetes with similar morphology, antigenic determinants and protein profiles have been detected in different parts of the world, including Europe, Russia, Asia, and even Africa. Genotyping studies established that in Europe Lyme disease was caused not only by *B. b. sensu stricto*, but also by two distinct other genotypes called *B. afzelii* and *B. garinii*. These and many other genomic species with pathogenic potential are now classified under the *Borrelia burgdorferi sensu lato* complex.



The *Borrelia burgdorferi* spirochete is transmitted into humans by the bite of different species of tick.

Spirochetes usually reside in the midgut of ticks. Upon feeding and the entry of blood into the midgut, the spirochetes begin to replicate and disseminate to the tick's salivary glands. About 24-72 hours after the attachment of the tick to human skin and the transfer of considerable numbers of spirochetes, infection may occur, depending on the immune status of the host. The life cycle of the *Ixodes scapularis* tick and how it transmits *B. burgdorferi* into humans is shown in Figure 1.

After the tick bite, Lyme disease often begins with a rash called erythema migrans (EM). About 20% of patients experience no further signs or symptoms, but in addition to EM, 40-60% eventually develop arthritis, about 11% develop neurological disorders, 4-8% cardiac complaints, and a very small percentage present dermatologic and ocular abnormalities.

All these multi-organ symptomatologies bear a striking similarity to the signs and symptoms of human autoimmune disease, where breakdowns in peripheral and central tolerance play a role; thus, if *Borrelia* hadn't already been identified as its etiologic agent, Lyme disease would be considered a classic autoimmune disease.

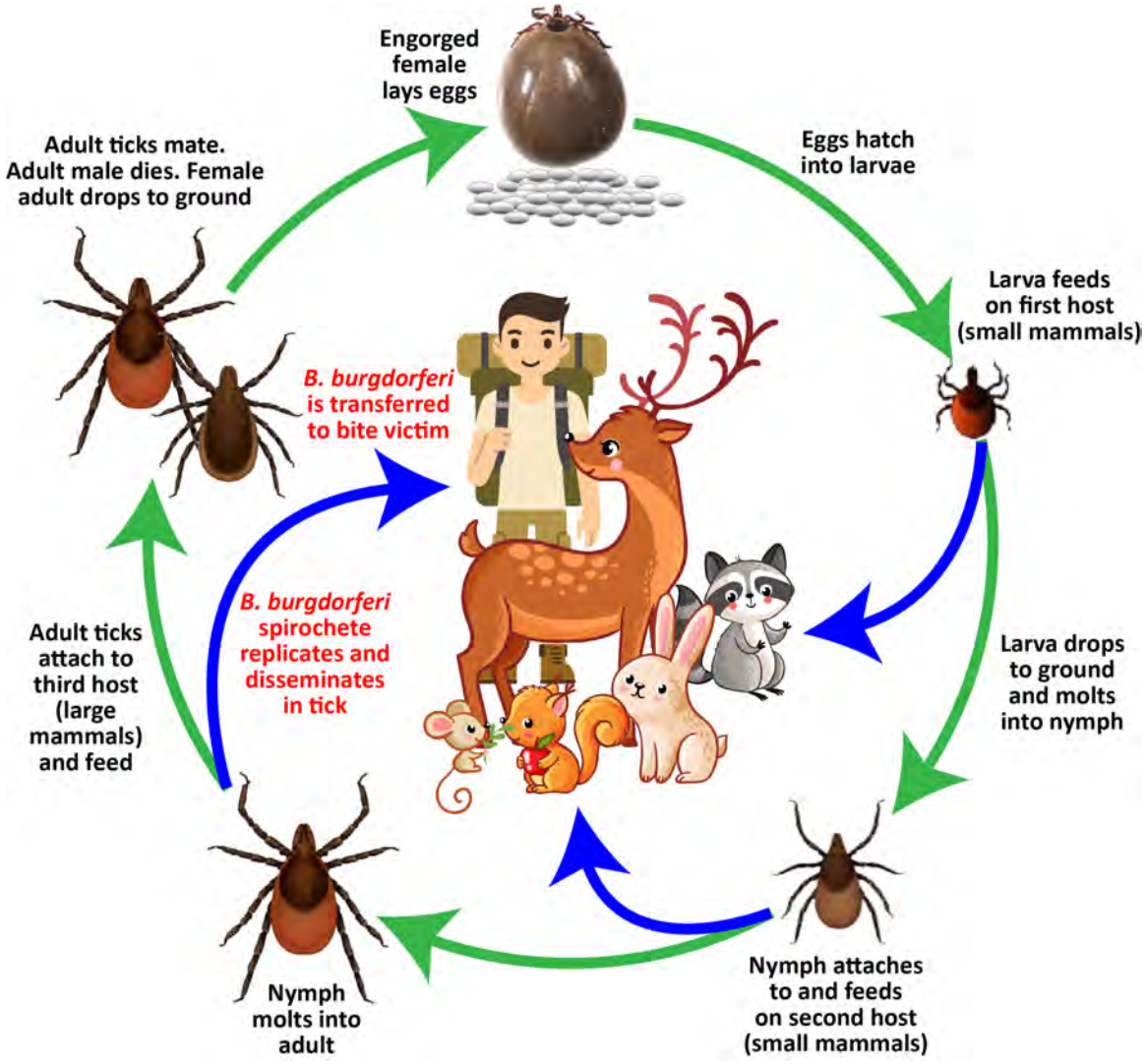


Figure 1. Life cycle of the *Ixodes scapularis* tick and transmission of *Borrelia burgdorferi*

The diagnosis of Lyme disease is based on clinical findings and laboratory blood testing such as IgG and IgM against various antigenic components of the spirochetes. Currently, however, accurate assessment of the disease can be problematic due not only to under- or over-diagnosis but also due to inter-laboratory discrepancies.

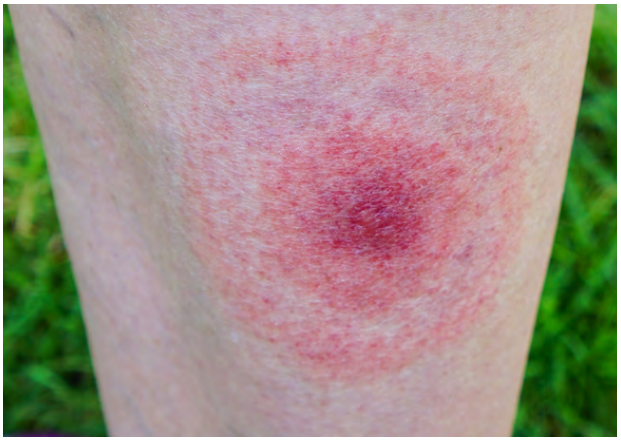
Although IgM appears in human blood 10-30 days after the introduction of the spirochete by the tick, and IgG 30-60 days after introduction, both IgM and IgG *Borrelia*-specific antibodies may persist for years in many patients. Thus, in the case of Lyme disease, it is very difficult to distinguish between past and newly-acquired infections based on blood levels of IgM and IgG antibodies.

1. Stages of Lyme Disease

Lyme disease is divided into three stages:

A. Primary lesion
B. Secondary lesion
C. Tertiary skin lesion

A. **Primary lesion or the early localized stage** occurs at the site where the tick injected the *Borrelia*. From 24-72 hours after this injection, the infection manifests as migrating redness or EM, a skin lesion that typically begins as a red macule or papule and expands over a period of days or weeks to form a large round lesion, often with a partial central clearing.



The initial symptom of Lyme disease is usually *Erythema migrans*, a red macule or papule that spreads out to form a large round lesion, often with a partial central clearing.

B. **Secondary lesion or the early disseminated stage** includes multiple EM and the wide spread of plaques that are similar to the initial plaques, but smaller. During this stage the spirochetes may spread further from the blood into different body tissues, which may induce the patient to develop Lyme arthritis, neuroborreliosis, and/or Lyme carditis.

The CDC definition of Lyme arthritis, which affects the musculoskeletal system, is recurrent brief attacks of objective swelling in one or a few joints, sometimes followed by chronic arthritis.

Neuroborreliosis is any of the following, alone or in combination: lymphocytic meningitis; cranial neuritis, particularly facial palsy (may be bilateral); radiculoneuropathy; encephalomyelitis (encephalomyelitis must be confirmed by *Bb*-specific antibody production in cerebrospinal fluid).

Lyme disease that affects the cardiovascular system comes as the acute onset of high-grade (2^{nd} or 3^{rd} degree) atrioventricular conduction defects that resolve in days or weeks and are sometimes associated with myocarditis. European patients, due to exposure to a different spirochete, may also develop borrelial lymphocytoma, or skin lesion.

C. **Tertiary lesion or the late stage** is characterized by acrodermatitis chronica atrophicans (ACA), a dermatological and progressive condition usually involving extremities that finally leads to a widespread atrophy of the skin. This stage may also include late neurological and rheumatological manifestations.

2. Lyme as an Autoimmune Disease

Lyme borreliosis in humans is an inflammatory disease affecting multiple organ systems, including the nervous system, cardiovascular system, joints and muscles (Figure 2).

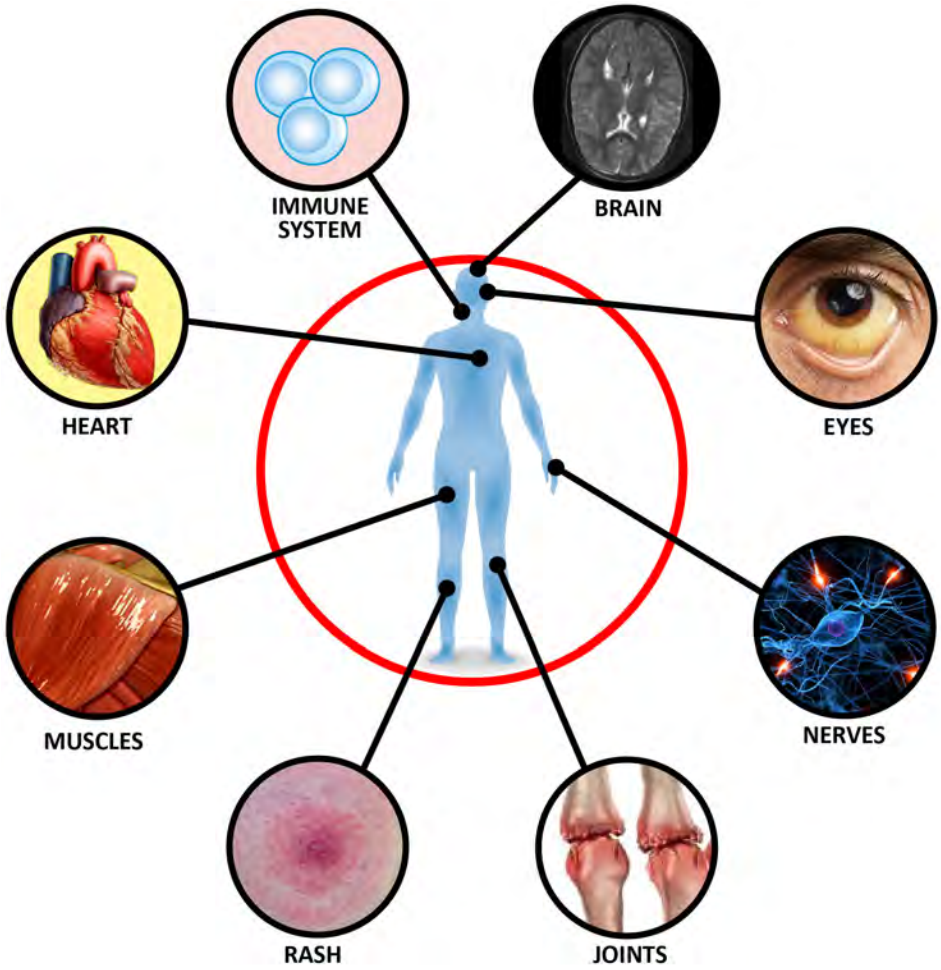


Figure 2. Symptoms of Lyme disease. Lyme disease and its symptoms are manifested in a variety of the body's organ systems:

- Immune system – swollen glands, fever/chills, weakened immune defenses;
- Heart - irregular heartbeat, inflammation;
- Muscles – weakness, pain, stiffness, fatigue;
- Rash – flat red rash, not itchy or painful, may have a central clearing;
- Brain – memory loss, inability to concentrate, headache, neck pain and stiffness, encephalitis or meningitis, psychosis;
- Eyes – blurred vision, floaters, irregular pupil;
- Nerves – Pain, often from spine down an arm or leg, pins and needles, tingling, burning, electric shock sensations, numbness, paralysis, often one side of face, twitching and spasms;
- Joints – joint pain, inflammatory arthritis in one or several joints.

2A. Lyme Arthritis (LA)

Unfortunately, about 60% of EM-positive patients develop recurrent episodes of Lyme arthritis that are often accompanied by myalgias and arthralgias. When EM-positive patients are treated with antibiotics, they very rarely develop arthritis. But when the EM goes unnoticed, undiagnosed, or for some reason doesn't actually break out, then the patient may not get the proper treatment, and the probability of developing arthritis is very high. It is possible in both children and adults for LA to develop within just days or weeks after EM, but usually LA is a late manifestation of the disease that appears months or even years after infection if left untreated. With or without EM, LA can affect both small and large joints.

Some untreated patients may develop chronic synovitis, and, in rare cases, erosions to a degree that permanent joint destruction and dysfunction resembling rheumatoid arthritis may occur. Fatigue and malaise are very common symptoms in these patients.

Patients with LA usually display high IgG antibodies against various antigens originated from *B. b. sensu lato, afzelii* and *garinii* both by multi-peptide ELISA and by Western Blot.

Because markers of inflammation are elevated in peripheral blood and synovial fluid, and radiographs of affected joints may show soft tissue swelling and other abnormalities, to date clinicians do not have the necessary predictors to differentiate between LA and septic arthritis.

2B. Lyme Neuroborreliosis (LNB)

The clinical diagnosis of LNB is very likely when a patient presents with this classic triad (lymphocytic meningitis, cranial neuritis, painful radiculoneuritis), or with one or more of these in combination with a well-documented EM. A definitive diagnosis requires laboratory confirmation using the two tiers of testing, particularly IgG and IgM ELISA in combination with Western Blot IgG and IgM.

Approximately 10-15% of patients with EM may develop neuroborreliosis within 90 days after infection if they are not treated with antibiotics. In fact, when patients were rigorously treated with antibiotics and closely monitored, the proportion of cases with LNB dropped to less than 2%. Overall, due to a lower functionality of their immune system and faster dissemination of *Borrelia* into the blood, a higher rate of LNB has been observed in both children and the elderly. Similarly, due to a stronger immune function in females, the LNB ratio of males to females is 1.5:1 among patients with Lyme disease.

The classic triad of neuroborreliosis consists of lymphocytic meningitis, cranial neuritis, and painful radiculoneuritis, either alone or in different combinations. This triad has been observed in EM-positive North American patients, in whom lymphocytic meningitis with stiff neck and headache are the most prominent. These symptoms are often accompanied by mild encephalitis. The clinical diagnosis of LNB is very likely when a patient presents with this classic triad, or with one or more of these in combination with a well-documented EM. However, a definitive diagnosis requires laboratory confirmation using the two tiers of testing, particularly IgG and IgM ELISA in combination with Western Blot IgG and IgM (see Figure 3), as recommended by both the American CDC and European guidelines.

These IgG and IgM antibodies should be measured, especially against peptides that are selected from *Borrelia burgdorferi sensu stricto, afzelii, garinii*, and *miyamotoi,* which are important for the serodiagnosis of neuroborreliosis and LA.

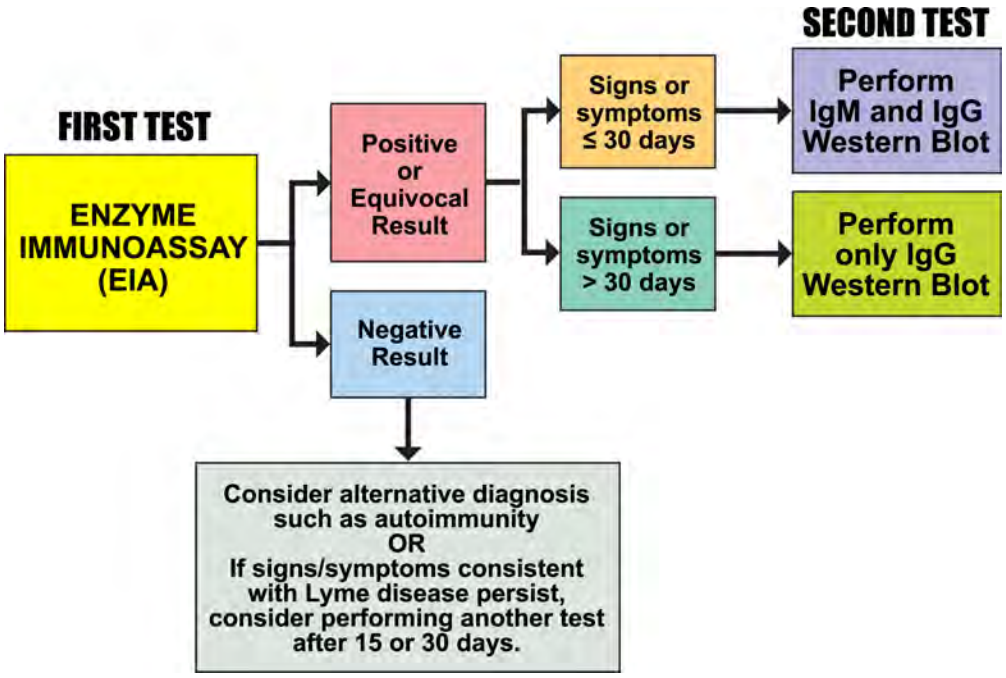


Figure 3. The Two-Tiered testing approach for Lyme disease recommended by both the American CDC and European guidelines.

2C. Lyme Cardioborreliosis

Lyme carditis or Lyme cardioborreliosis primarily affects individuals between the ages 20 to 40, with males outnumbering females by at least 3:1. Cardioborreliosis is an early manifestation of Lyme disease, presenting within days and up to three months after the appearance of EM or other early symptoms of Lyme disease. In endemic areas about 4-8% of patients with Lyme disease exhibit cardiac abnormalities. The most common manifestation of cardioborreliosis is acute onset rapidly fluctuating atrioventricular conduction disturbances. Among cases of reported cardioborreliosis, almost half progress to complete heart block or second grade atrioventricular block, which requires a temporary pacemaker in some patients. Additionally, endocarditis, myocarditis, pericarditis, pericardial effusion and even, in rare cases, congestive heart failure have been reported. Overall, patients with Lyme carditis can be asymptomatic, but patients with high-grade atrioventricular abnormalities usually show symptoms of light-headedness, shortness of breath, palpitations, chest pain or syncope. Lyme carditis should be suspected in younger individuals with symptoms of cardiac disease in the absence of the apparent risk factors. Usually, patients with cardioborreliosis are positive for IgG and IgM against various antigens of *B. burgdorferi* and its subspecies at the presentation of symptomatologies, but for unknown reasons they become antibody-negative soon thereafter. However, patients with dilated cardiomyopathy are strongly positive for antibodies against *B. burgdorferi.* Because spirochetes have been visualized in biopsies taken from endomyocardial tissue, antibiotic treatment is highly recommended for this potentially life-threatening disease that is associated with Lyme disease.

3. Methods for diagnosis of Lyme disease

The following different methodologies are used for the detection of antibodies associated with Lyme disease:

A. Enzyme-linked immunosorbent assay (ELISA)
B. Western Blot
C. Multi-Peptide ELISA (MPE)
D. Dot Blot
E. Microarray
F. Radioimmunoassay
G. Non-antibody testing methods

Each of these methods has its advantages and disadvantages.

3A. Enzyme-linked immunosorbent assay (ELISA)

The enzyme-linked immunosorbent assay (ELISA) is a colorimetric assay for accurate measurement of antibodies or antigens in blood and other clinical specimens. ELISA is an analytic biochemistry assay that involves detection of an antigen or antibody in a liquid sample by a method that continues to use liquid reagents during the "analysis" that will generate a signal which can be easily quantified and interpreted as a measure of the amount of analyte in the sample.

The ELISA method is the internationally recognized gold standard for antibody testing in the blood.

Antigens from *B. burgdorferi* or other subspecies are prepared from spirochetes grown in special media. After centrifugation and washing, the spirochetes undergo sonication to release their major antigens, such as outer surface protein A (OspA), immunodominant protein (C6), leukocyte function associated antigen (LFA), and outer surface protein C (OspC), as shown in Figure 4.

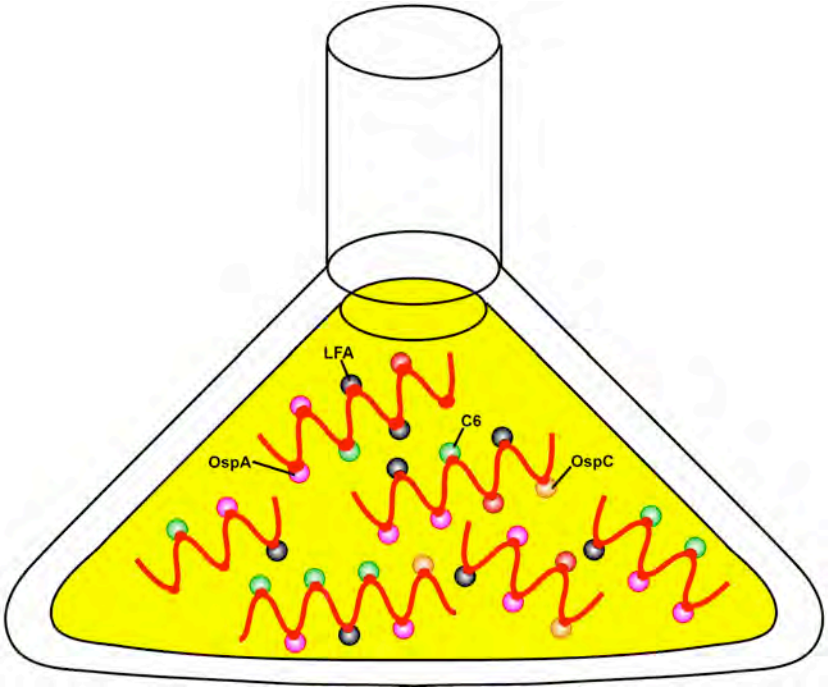
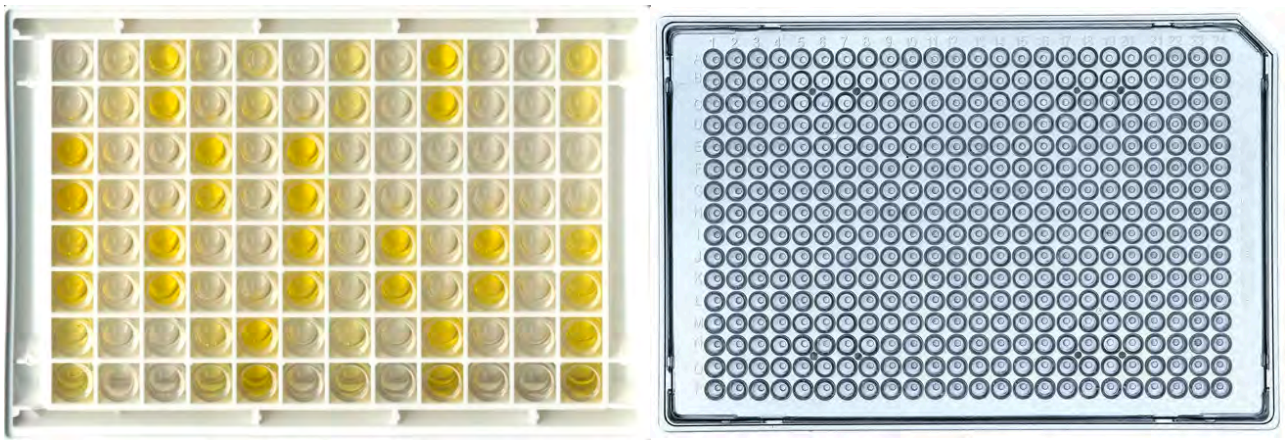


Figure 4. Growth of *Borrelia burgdorferi* in culture medium and expression of different surface proteins, mainly Outer Surface Protein A (OspA), Immunodominant Protein (C6), Leukocyte Function Associated antigen (LFA), and Outer Surface Protein C (OspC).

The ELISA technique was conceptualized and developed by Swedish scientists Peter Perlmann and Eva Engvall at Stockholm University in 1971. They were honored for their invention when they received the German scientific award of the "Biochemische Analytik" in 1976.



Samples of a 96-well and 384-well ELISA plate.

There isn’t a modern clinical laboratory today that doesn't use the ELISA or one of its descendants. The different steps involved in this reliable assay are shown in Figure 5.

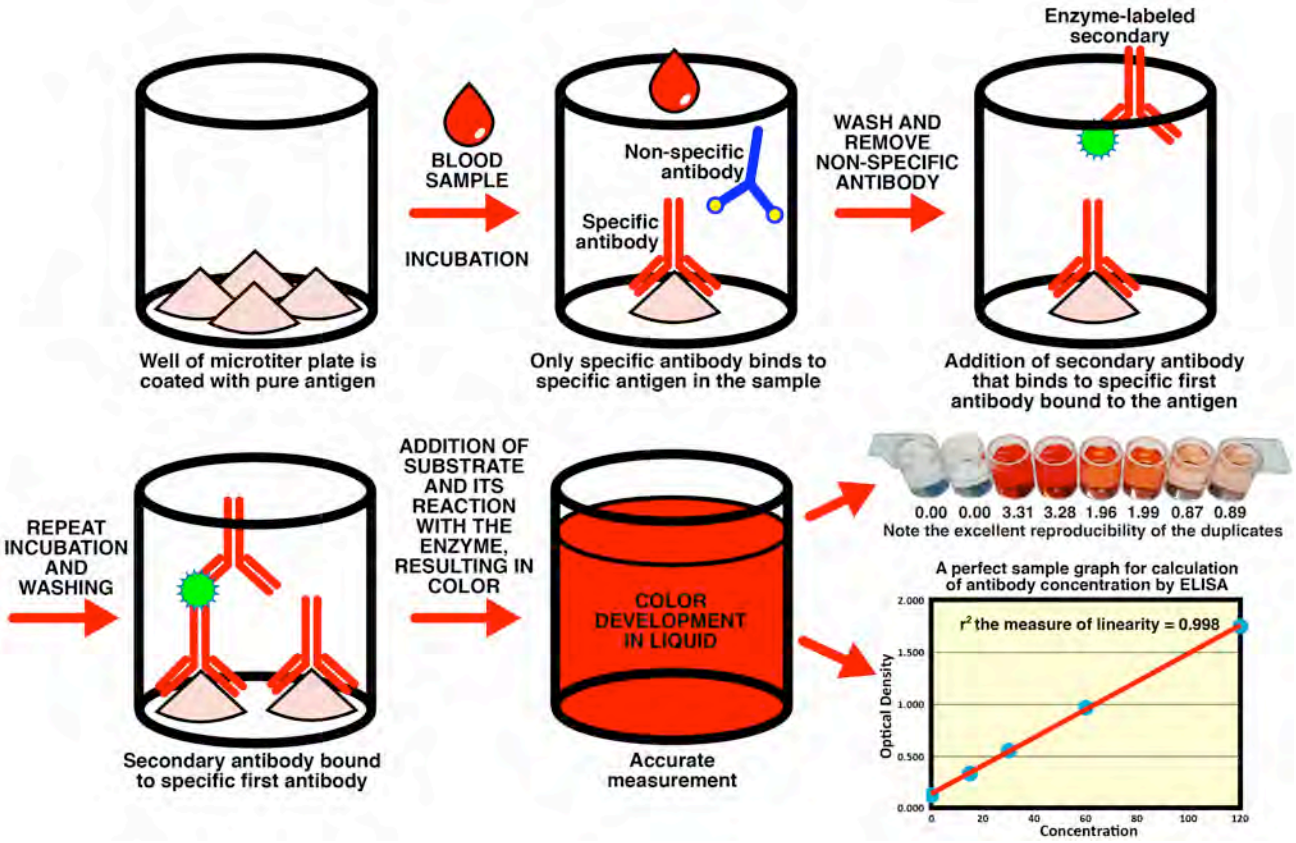


Figure 5. Steps involved in an ELISA assay that generates color in the liquid phase, creating highly reproducible duplicate test results. The wells of a microtiter plate are coated with pure antigens, followed by the addition of the patient's serum. Antibodies present in the serum would bind to the antigens on the plates and not rinse off. The enzyme-labeled secondary antibodies would in turn bind to the immobilized serum antibodies, resulting in a color reaction that can be read quantitatively by an ELISA plate reader.

3A1. Measurement by ELISA of IgG or IgM antibody against a mixture of B. burgdorferi antigens prepared in culture (Lyme ELISA).

The Lyme ELISA test is intended for the quantitative detection of IgG and IgM antibodies to *Borrelia burgdorferi* in human serum. In the ELISA method, pure antigens without any chemical modifications are bound to the surface of a microtiter plate. Then, the patient serum is diluted with a special diluent and added to *B. burgdorferi* antigens bound to the ELISA plate wells. If antibodies to the microbe are present, they bind to the antigen in the coated wells and do not rinse off in the washing process. Subsequently, when enzyme-labeled anti-human IgG/IgM is added, it in turn binds to the immobilized antibodies that have bound with the antigens. After the washing and addition of a chromogenic substrate and stopping solution, samples containing IgG/IgM antibodies to *B. burgdorferi* produce a color endpoint reaction that will be read with an ELISA plate reader, and the results are expressed quantitatively.

Interpretation of Results. Titers of IgG are generally low during the first weeks of illness. They peak 3 months to a year after infection and may remain elevated for years. Titers of IgM peak within 3 to 6 weeks after onset but are often not detectable in asymptomatic patients.

Because antigens prepared from *B. burgdorferi* grown in culture contain many impurities or cross-reactive antigens or peptides, the following may occur, as is noted in the manufacturer's insert from the MarDx ELISA kit:

1. **Sera from patients with other pathogenic spirochetal diseases such as syphilis, yaws, pinta, leptospirosis, and relapsing fever may give false positive results. Sera from patients with mononucleosis or lupus erythematosus may also give positive results.** In cases where false positive results occur, clinical, epidemiologic and laboratory workups should be carried out. Although the clinical picture is quite different between active syphilis and Lyme disease, an easy means of differentiating these two diseases is by the use of the Veneral Disease Research Laboratory (VDRL) or Rapid Plasma Reagin (RPR) tests. In active syphilis, the VDRL or RPR are positive. In Lyme disease, the VDRL and RPR are negative.
2. Patients with early stages of Lyme disease may not test positive with this test. The IgG antibodies may not have reached detectable levels in early Lyme during the EM clinical phase of the disease. Negative results in early stages of the disease have low predictive value.
3. The ELISA IgG or IgM against *Borrelia* grown in culture is not capable of aiding in the diagnosis of Lyme arthritis and neuroborreliosis.

3B. Lyme Western Blot Assay

The advantage of the Western Blot assay is that cross-reactive antibodies are excluded and antigen-specific antibodies are observed against proteins of different molecular sizes. For IgG, 5 bands out of 10 is considered by the CDC to be positive, while for IgM 2 bands out of 3 is considered positive.

The Western Blot (WB) assay has been widely used to detect the presence of antibodies in human serum and plasma to various infectious disease agents. In this procedure, component proteins of purified, inactivated organisms are electrophoretically separated by SDS-polyacrylamide electrophoresis followed by electrotransfer to nitrocellulose sheets. Each strip serves as the solid-phase antigen for an ELISA test. Specific antibodies present in human serum/plasma will bind to several of the separated polypeptides upon incubation with the strip. These antibodies are then treated with an enzyme-antibody conjugate which binds to the human immunoglobulins, if present. The final product of membrane antigen, human antibody and conjugate is visualized upon incubation with a chromogenic enzyme substrate. This will result in a blue-colored "band" at the polypeptide location on the membrane strip if the specific human antibody is present. This assay, which detects *Borrelia*-specific peptide antibodies in human serum, was refined by different investigators and was recommended for better diagnosis of Lyme disease.

Interpretation of Results. According to the CDC/ASTPHLD working group, an IgG blot is considered positive (reactive) if 5 of the following 10 peptide bands are present: p18, p23, p28, p30, p39, p41, p45, p58, p66, p93. An IgM blot is considered positive (reactive) if 2 of the following 3 peptide bands are present: p23, p39, p41. If the IgG or IgM antibodies against the above peptides are negative, the patient is considered non-reactive (see Figure 6).

However, even by applying the Western Blot assay, since these antibodies may not be present in the blood during early or chronic stages of the disease, false negative results will be obtained.

The advantage of Western Blot over the ELISA method is that while ELISA uses a mixture of proteins for testing, in WB the antigens are separated based on their molecular size, for example, 18, 23, 28, 30, 39, 41, 45, 58, 66, and 93 kDa, prior to completion of the ELISA steps. The disadvantage is that the antibody reaction with each pure antigen can only be observed qualitatively, or simply a positive or negative, yes or no result.

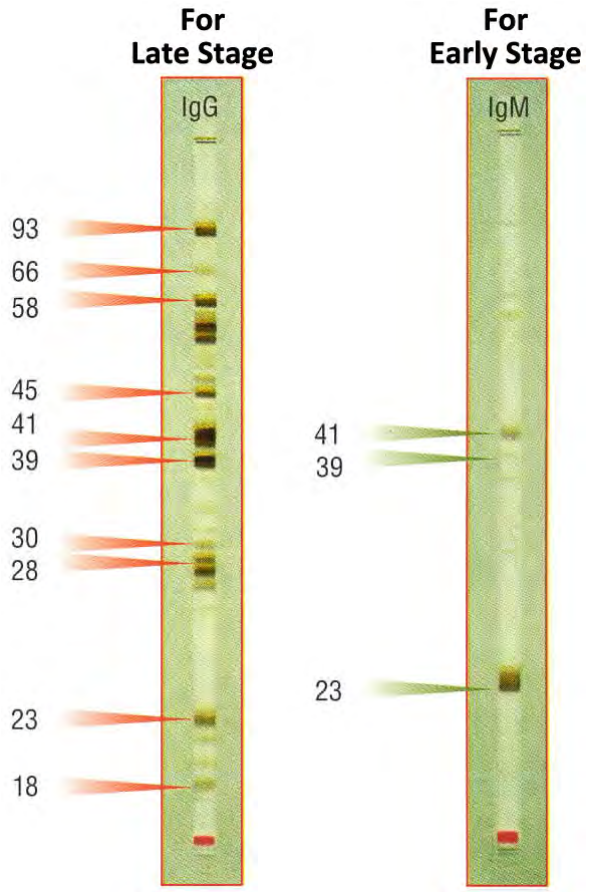


Figure 6. IgG and IgM antibody patterns with different peptides of *Borrelia burgdorferi* in patient confirmed with Lyme disease.

Because the Western Blot is performed using the same *Borrelia* antigen grown in culture but separated by electrophoresis into different-sized proteins, it still suffers from many limitations, as is noted in the manufacturer's insert from the MarDx Western Blot kit:

1. Sera from patients with other pathogenic spirochetal diseases such as syphilis, yaws, pinta, leptospirosis, relapsing fever and periodontal disease may give false positive results. **Individuals with connective tissue autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus, and individuals with anti-nuclear antibody may also give false positive results. Individuals with other bacterial and viral infections such as Rocky Mountain Spotted Fever, Epstein-Barr Virus, and cytomegalovirus may also have antibodies which cross-react with *B. burgdorferi*.**
2. A positive *B. burgdorferi* IgM Western Blot result only indicates probable immunologic exposure. However, the presence of an immunologic response has not been correlated with active infection. When testing specimens from patients during early *B. burgdorferi* infection, IgM tests are generally sensitive within the first 2 months after onset of symptoms. A suitable IgG Western Blot test can be used at any time after onset but is most sensitive during the later stages of the disease.

3. Individuals with positive Western Blot for antibodies to *B. burgdorferi* should be referred for medical evaluation which may include additional testing. The diagnosis of Lyme disease must include careful clinical evaluation and should not be based on detection of antibodies to *B. burgdorferi*.

Because antigens prepared from cultured *Borrelia* are not actually representative of the spirochete antigens expressed in the human body, this test may miss the detection of IgG and IgM antibodies against antigens such as variable major protein. Western Blot also does not measure antibodies against the decorin binding protein of *B. burgdorferi sensu stricto, B. garinii, B. afzelii*, and *B. miyamotoi*, and thus is not capable of detecting Lyme arthritis, neuroborreliosis, or hard tick-borne relapsing fever.

Both the American CDC and European guidelines strongly recommend the two-tier approach of a sensitive ELISA IgG, IgM, followed by Western Blotting of the samples that were found to be indeterminate (borderline positive) or positive in the first step. However, **for patients infected in Europe, the testing should be performed with at least three pathogenic species, such as *B. b. sensu stricto, B. b. garinii,* and *B. burgdorferi*, all of which are part of the MPE performed by Immunosciences Lab.**

3C. Multi-Peptide ELISA (MPE) – The most sensitive method for the detection of Lyme disease and other tick-borne diseases (*Babesia, Ehrlichia, Bartonella*)

Multi-Peptide ELISA or MPE uses not only antigens from *Borrelia* grown in culture but also advanced *in vivo* induced antigen technology. This technique identifies *Borrelia* antigens that are immunogenic and are expressed *in vivo* during human infection. Utilization of this technique increases the accuracy of the diagnostic process and abridges the time of treatment, resulting in improved quality of care.

Prompt diagnosis and treatment of LD is the key to avoiding chronic Lyme borreliosis and its serious effects on the human system. Diagnosis can be difficult because symptoms of LD share commonalities with amyotrophic lateral sclerosis (ALS), Alzheimer's, autism, chronic fatigue, fibromyalgia, lupus, Parkinson's and RA. Therefore, it is crucial to combine clinical symptomatology with the most sensitive technique available to diagnose Lyme disease.

The antigenic diversity of *Borrelia burgdorferi* in the host suggests that antigenic variation plays an important role in immune invasion. This antigenic variation is detected by a very new technique called *in vivo* induced antigen technology. This technique identifies pathogen antigens that are immunogenic and expressed *in vivo* during human infection.

The **Multi-Peptide ELISA** or **MPE** (US Patent 7,390,626 B2) measures antibodies to antigens of *Borrelia* grown in culture (the traditional method), as well as antibodies against antigens expressed *in vivo* which *Borrelia* uses to invade the immune system during the process of human infection.

Based on this novel technique, ISL uses particular peptides from various components of Borrelia during different cycles, including peptides from outer surface proteins A, C and E, leukocyte function associated (LFA) antigens, immunodominant antigens, variable major proteins, and peptides from decorin-binding proteins of Borrelial subspecies (*B. b. sensu stricto, B. afzelii, B. garinii, B. miyamotoi*).

In addition to the measurement of antibodies against antigens expressed *in vivo* and *in vitro*, ISL's Lyme profiles also assess antibodies against antigens of *Borrelia* subspecies and its co-infections, such as *Babesia, Ehrlichia* and *Bartonella;* the clinical manifestations of these infections may be similar to Lyme, but their treatments differ from that of Lyme disease.

In structuring the MPE, ISL tested 103 different specimens from patients with Lyme disease symptoms from two different clinics, one on the East Coast and the other on the West Coast. Altogether, with this population of patients, the sensitivity of Western Blot was 44.6%, while with MPE sensitivity was 71%. Since *Borrelia, Babesia, Ehrlichia* and *Bartonella* are transmitted by the same tick species, serum samples were tested for the simultaneous elevation in IgG and IgM antibodies against multiple organisms. Between 35-64% of specimens, in addition to *Borrelia* antibodies, were also positive for *Babesia, Ehrlichia* or *Bartonella*.

The Multi-Peptide ELISA methodology has been tested on over 10,000 clinical specimens; while correlation between Western Blot and Multi-Peptide ELISA is good, the Multi-Peptide ELISA assay has greater advantages over the Western Blot method. These advantages are summarized in Table 1.

Table 1. Comparisons between Western Blot and Multi-Peptide ELISA

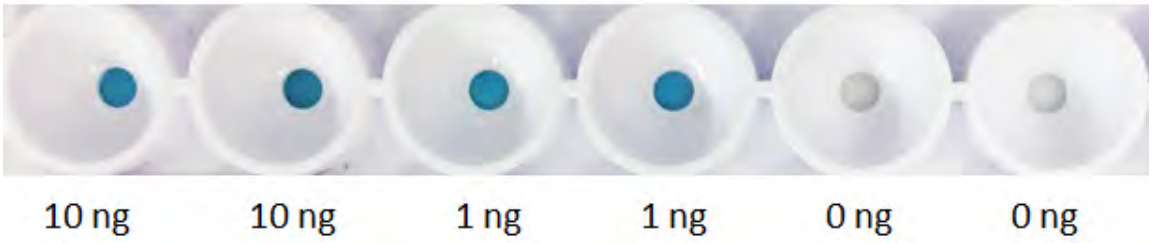
	Western Blot	Multi-Peptide ELISA
Detects antibodies against borrelial antigens prepared in culture	✔	✔
Measures antibody to antigens of *Borrelia* expressed during human infection		✔
Antigen used represents different life cycles of spirochete in infected tissue		✔
Detects antibodies against OspA and LFA to identify treatment-resistant arthritis		✔
Detects antibodies against DbpA from *Borrelia* subspecies *B. b. sensu stricto, B. afzelii,* and *B. garinii*, which indicates Lyme arthritis and neuroborreliosis		✔
Detects antibodies against *Borrelia miyamotoi* to identify hard tick-borne relapsing fever (HTBRF)		✔
Can detect antibodies against *Babesia, Ehrlichia* and *Bartonella*		✔
May provide predictive model for development of autoimmune inflammatory disease induced by *Borrelia*		✔
Method is sensitive		✔
Method is quantitative		✔
Method is subjective	✔	
Results can be used for follow-up treatment	✔	✔

Based on sera samples from the Centers for Disease Control and Prevention (CDC), 200 specimens from patients with Lyme disease, and more than 200 samples from healthy controls, **the sensitivity of the Multi-Peptide ELISA assay was found to be more than 80%, with a specificity greater than 95%.**

3D. Dot blot assays

Dot blot is a qualitative variation of ELISA that uses nitrocellulose or nylon membranes with which to bind the antigens, and is a good method for getting a simple yes or no answer.

The immunoblot assay or dot blot is a qualitative variation of the ELISA test that uses impure or a mixture of antigens. Instead of binding antigen to wells, antigen is covalently bound directly to a nitrocellulose or nylon membrane and is detected with labeled primary antibody, or indirectly with labeled secondary antibody. The result can only be a positive or negative (yes or no) reaction. This means this method cannot distinguish between positive, negative, and equivocal, borderline, or weakly positive results. This need to quantify the qualitative results of the dot blot test led to the development of the microarray method.



Different amounts of antigen give different results. While the dot blot assay can easily distinguish between negative (0 ng) and positive (10 ng), it is more difficult to distinguish between the color reactions of antibody to 1 ng and to 10 ng of antigens that were bound to the matrix.

3E. Microarray assays

Although many research articles have questioned the clinical utility of the microarray method for quantitative measurements of antigens or antibodies in the blood, it is still an excellent method for qualitative detection of DNA or tumor antigens in clinical specimens.

The need to quantify the qualitative results of the dot blot test led to the development of the microarray method, which converts the dot blot signal to a number. A microarray is a high-throughput miniaturized ELISA-based platform for efficient detection of nucleic acids and protein expressions in various types of clinical specimens. It is a 2D array on a solid substrate (usually a glass slide or silicon thin-film cell) that assays large numbers of biological materials. Using this technology, a tiny amount of biochemical, (protein, antibody, *etc*.) is spotted and fixed on a solid surface such as a microchip (silicon thin-film cell), glass, or plastic and the interaction between the biochemical, and its target protein (antigen), is detected via a light reader.

The basic principle of microarray is the dot blot assay. However, in the microarray method, instead of binding the antigens to a membrane or paper strip, hundreds of antigens can be bound to microscope-size slides, which requires very special chemistry. This chemistry may not be suited for every single protein or peptide. Many harsh chemicals are used to bind the antigens to the glass. The harsh process may affect the tertiary structure of proteins, and may result in either lack of binding (false negative), or excessive binding of the antibodies in the blood to the antigen on the chip. Furthermore, the dot blot is miniaturized in order to save

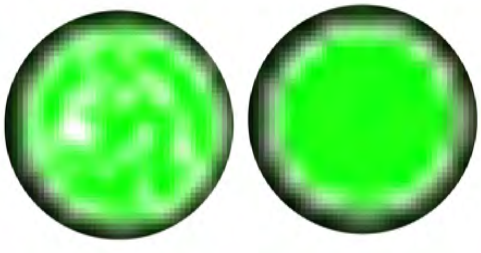
reagents. For example, if in a normal ELISA test 5 micrograms of antigen worth $10 is used, in a microarray the amount of antigen used would be about 100-fold less, say about 50 nanograms of antigen worth 10 cents. At the end of the assay procedure, the qualitative yes or no results are converted to quantitative results using additional equipment, mathematical formulation and calculation. For these reasons, microarray technology is considered experimental and is recommended for the detection of very small quantities of DNA or tumor markers in the blood, not for the determination of the level of antibodies that are present in the blood in milligram amounts. **Additionally, the miniaturization of the sample means that there is such a small amount of antigen on the testing chip that it may not react with the antibody in the blood sample.**

The concept and methodology of microarrays was first introduced and illustrated in antibody microarrays (also referred to as antibody matrix) by Tse Wen Chang in 1983 in a scientific publication and a series of patents. The "gene chip" industry started to grow significantly after the 1995 Science Paper by the Ron Davis and Pat Brown labs at Stanford University.

However, in the December 2018 issue of CAP Today, the monthly news magazine of the College of American Pathologists (CAP), one of the main regulatory and certification bodies for laboratories, an article said that **"In allergy testing, microarray technology offers speed and the benefit of smaller sample volumes, but it has a lower sensitivity and is unable to detect IgE antibodies of all specificities in a given extract unless all allergens are on the chip. For routine use, singleplex assays are here to stay."** The same article said that **"Microarray technology will remain a wonderful research tool but will probably not emerge into the clinical world in a serious way for a variety of reasons," among them a lack of FDA approval, said Robert G. Hamilton, PhD, D.ABMLI, director of the Dermatology, Allergy, and Clinical Immunology Reference Laboratory at Johns Hopkins University School of Medicine."**

Microarray technology offers speed and the benefit of smaller sample volumes, but it has a lower sensitivity. Microarray technology will remain a wonderful research tool but will probably not emerge into the clinical world in a serious way for a variety of reasons.

It should be noted that after more than three decades the microarray system is still regarded only as promising technology. For now, the process involved in microarray testing shows a comparatively greater chance of irreproducible results, for the reasons shown below, and in Figure 7.



Note that heterogeneity of color in microarray spots will give different results from spot to spot.

On the other hand, homogeneity of color in ELISA wells will give very similar results from well to well.

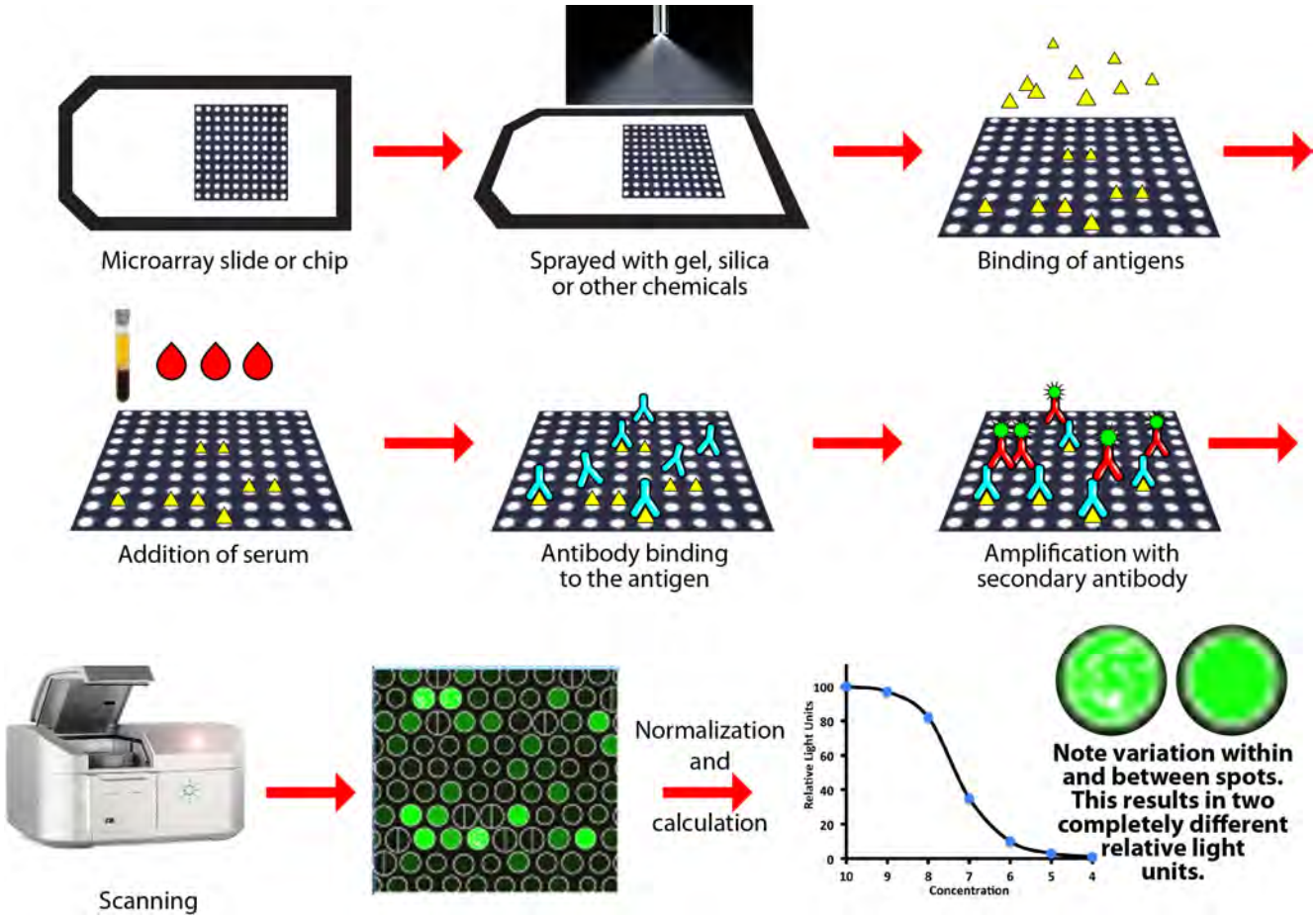


Figure 7. Steps involved in a microarray assay that generates irreproducible results.

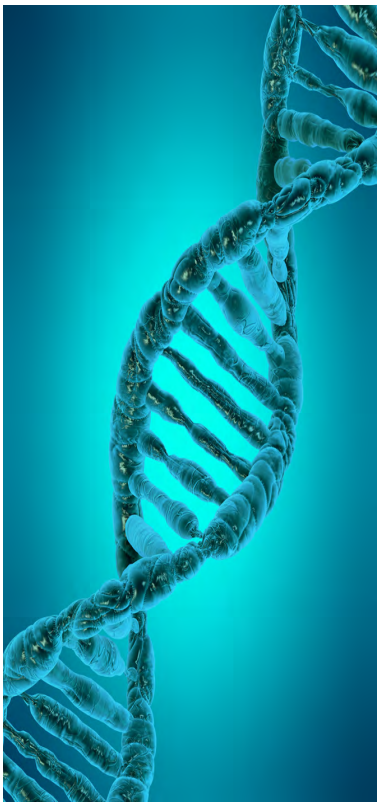
For these exact reasons, at Immunosciences Lab, regardless of the cost of materials, we use the gold standard ELISA method, not microarray, for the detection of Lyme disease and other infectious agents.

3F. Radioimmunoassay

This is an in vitro process whereby a radioisotope-labeled antigen (usually radiolabeled with gamma-radioactive iodine isotopes) is introduced and the radioactivity of the antibody is measured. Similar to ELISA, the radioimmunoassay is highly sensitive and specific. This method is also not restricted to merely the serum level of antigens; any biological molecule can theoretically be used in a radioimmunoassay. **Due to the use of radiolabeled reagents, there are potential radiation hazards with this technique**. This often means only specially trained individuals can handle this assay. Laboratories usually need licensure to handle this radioactive material as well as special protocols for storing and disposing of this material.

3G. Non-Antibody Testing Methods

3G1. Polymerase Chain Reaction (PCR)



The Polymerase Chain Reaction or PCR test is a powerful tool that can detect a single genome of an infectious agent in any body fluid with improved accuracy and sensitivity. Developed by Kary Mullis in 1983, it is an *in vitro* method that exponentially amplifies a specific region of a DNA strand to generate from thousands up to millions of more copies of that particular DNA segment. It is used in medical and clinical laboratory research for a broad variety of applications. PCR allows for rapid and highly specific diagnosis of infectious diseases, finding even the smallest trace of a pathogen and amplifying it for detection and identification. This allows for the detection of organisms for which conventional diagnostic techniques lack either sensitivity or specificity. In practice, PCR can fail for various reasons, in part due to its sensitivity to contamination, so that tainted samples would result in the amplification of spurious DNA products. The process requires rigorous purification and optimization protocols and very clean testing environments. An additional difficulty lies in that the *Borrelia burgdorferi* spirochete eventually migrates to the tissue, and unless the test is conducted soon after infection, a blood PCR would simply be looking in the wrong place. Therefore, PCR is not to be recommended as a methodology for detecting the presence of *B. burgdorferi* in the body.

3G2. ELISpot Assay

ELISpot is another variation of the ELISA method in which memory lymphocytes in blood are cultured in microwells coated with monoclonal antibodies against specific analytes such as interferon-gamma, tumor necrosis factor alpha, interleukin-2, or other cytokines. When a specific antigen, for example, *B. burgdorferi,* is added to the lymphocyte in culture, the memory lymphocyte that recognizes the antigen becomes stimulated and secretes a cytokine, such as interferon-gamma. The secreted cytokine binds to the specific antibody-coated wells and forms detectable colorful dots (see Figure 8). The color is measured by a special reader which converts the intensity of the color to the amount of cytokine released by the memory lymphocyte in response to the challenge by the *B. burgdorferi* antigen. However, because *B. burgdorferi* cross-reacts with many antigens from other spirochetes, with viruses such as Epstein-Barr virus, with bacteria such as *Yersinia enterocolitica*, with leukocyte function-associated antigen, cytokeratin-10 and more, the cross-reactive memory lymphocytes may react non-specifically to the *Borrelia* antigens and induce production of a cytokine (e.g., interferon), giving the false impression that the patient may be suffering from Lyme disease. Because of this type of possible false positivity, ELISpot is not recommended for the clinical diagnosis of Lyme disease.

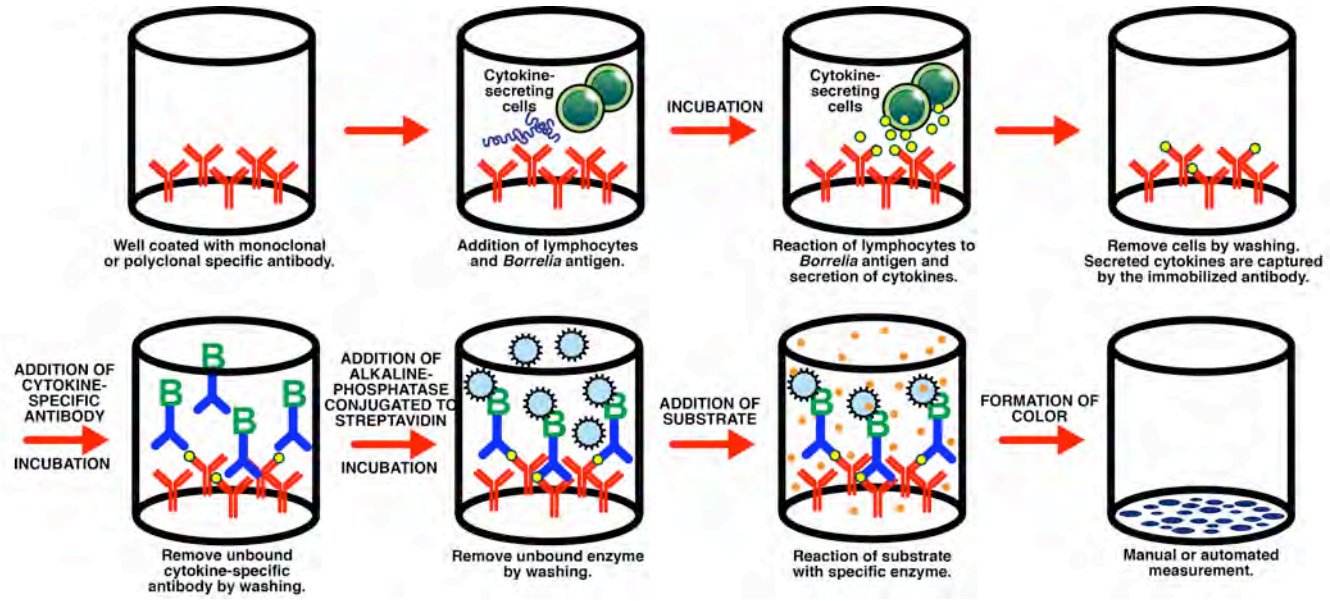


Figure 8. The ELISpot process. The memory lymphocytes used in this process may react non-specifically with cross-reactive antigens, resulting in possible false positivity.

4. It's Not Just The Test; It's How You Do It and What You Use

The reliable reporting of laboratory test results will still depend not just on the nature of the assay but also on what you put into the test or how meticulously the test is performed. This is why Immunosciences Lab spent years developing and perfecting the **4 Core Principles** for the reliable reporting of laboratory test results (Figure 9).

1. *Purity of the antigen* – The ELISA test or any other method for testing antigen-antibody reaction depends on the purity of the antigen. The quality and reliability of a test's results are only as good as the purity of the antigen used in the assay.
2. *Optimized antigen concentration* – Some labs blindly use the same volumes but not the same concentrations for the different antigens used for antibody measurements, assuming that apple and peanut both contain the exact same amount of protein. The problem with this is that indiscriminately using the same volume for all antigens runs the risks of false positivity and false negativity, even if two different antigens did contain the same amount of protein, unless conditions were optimized for each antigen during assay development. If not, this may increase the risk of obtaining erroneous results.
3. *Individual antigen-antibody validation* – According to the Federal Clinical Laboratory Improvement Amendments (CLIA) regulations, each method must have validated performance specifications for "accuracy, precision, analytical sensitivity and specificity; the reportable range of patient test results; the reference ranges; and any other applicable performance characteristic." However, not all labs perform all the above requirements for validation of their tests.
4. *Duplicate or parallel testing* – Each test should be run side-by-side or run twice. Many steps are involved in testing, and a potential for error exists with each step. One way to guard against these errors is to run duplicates from the same patient's sample in side-by-side wells on the same plate.

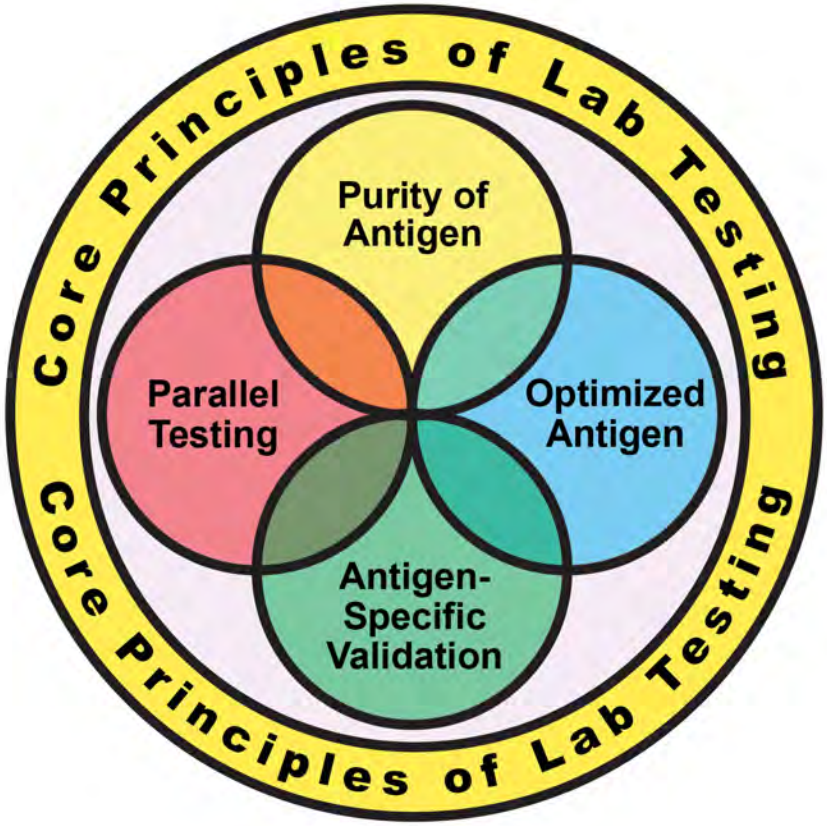


Figure 9. The 4 Core Principles for reliable reporting of laboratory test results.
The CLIA regulations established in 1988 apply to all facilities that perform tests on human specimens. The 4 Core Principles not only adhere strictly to these regulations but also ensure reliable and accurate reporting of laboratory test results.

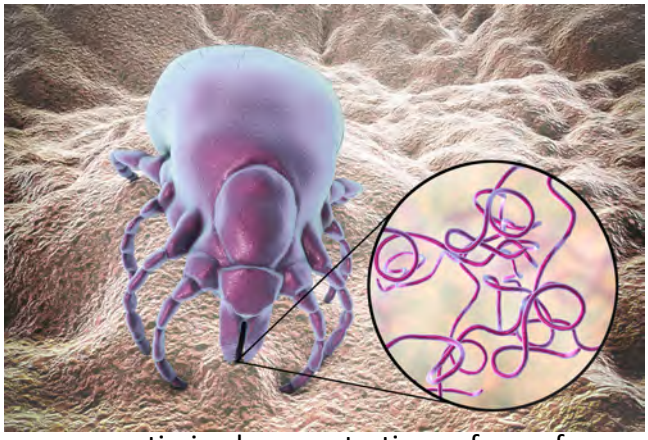
5. Comparative analysis of methods

In sum, then, the ELISA method still stands as the industry's gold standard because of its reliability, reproducibility, accuracy and ability to produce quantitative results. The Western Blot method offers the advantages of specific antibody reaction to antigens based on molecular size, but can only offer qualitative results, and its inability to distinguish between weak and strong reactions may generate false positives and negatives by an individual who reads the test results incorrectly. The microarray system has the advantage of speed and multiple antigens, but has been notably cited for low sensitivity and reproducibility because of the minute quantities of antigens contained on the sample chips. **In contrast the Multi-Peptide ELISA, as performed by Immunosciences Lab., Inc. (ISL), offers the advantages of the ELISA method's reliability, reproducibility and accuracy, and the antigen size specificity of the Western Blot method. The MPE also produces quantitative results for antibodies that are produced against different highly pure antigens and peptides prepared from *Borrelia* grown in culture, as well as antigens and peptides that are expressed in the *in vivo* system.**

6. Antigens included in ISL's Multi-Peptide ELISA and interpretation of the results

6A. Lyme-Specific Antibodies

6A1. Borrelia burgdorferi antigens prepared from B. burgdorferi grown in culture.



This double-membraned spirochete is the predominant causative agent of Lyme disease in the United States. It is transmitted by a deer tick's bite through its saliva into the human blood stream. The *B. burgdorferi* antigens used in this test are grown in culture, and a mixture of all these antigens are applied to the plates for testing.

Although good for screening purposes, the use of a mixture in testing naturally favors the detection of higher concentrations of antibodies to antigens rather than the lower concentrations. For this reason, we use optimized concentrations of pure forms of the rest of the antigen components used in our MPE.

6A2. Outer Surface Protein-A + Outer Surface Protein-C Peptides

Outer Surface Protein-A (OspA)

Outer surface protein-A (OspA) is a 30/31 kDa lipoprotein produced as *Borreliae* enter the tick vector and remains as a major surface antigen during colonization. This antigen was the first surface protein identified in Lyme disease spirochetes. OspA functions as a plasminogen binding protein, a function that is needed for efficient dissemination in the gut of the tick. OspA also functions to shield borrelial surface antigens. It would appear that *Borrelia* expresses OspA, first, to enable it to colonize and survive in the vector, and, second, to avoid immune attack through OspA's immunity-shielding function. Interestingly, in the inflammatory environment, *B. burgdorferi* shows an increase in OspA expression. Antibody against OspA appears in the blood 2-4 weeks after infection, and may be present in the blood during prolonged arthritic episodes which are observed in patients with Lyme disease. Antibody responses to OspA have been reported late in the course of Lyme disease. Furthermore, a study on blood samples from Lyme disease who had not been treated with antibiotics showed spiking OspA antibody titers that coincided with arthritic episodes. The strength of these anti-OspA titers also correlated with the severity and duration of arthritis in these patients. Since *B. burgdorferi* does not express OspA when it first infects the host, these antibody titers indicate that the spirochete can upregulate OspA expression within the host. This upregulation of OspA is crucial for the colonization of the tick by the spirochete, while the downregulation of this lipoprotein during infection of the host is important for the continuation of the spirochete's growth and differentiation. However, the immune system in turn is normally triggered into producing effective anti-IgM and anti-IgG responses to block the spirochete's growth.

Molecular mimicry between OspA and streptococcal M5 protein as well as human myosin has been shown to be involved in the induction of autoimmunity by this *B. burgdorferi* protein. OspA also cross-reacts with cytokeratin-10, which is present in the synovial microvascular endothelium and is a putative autoantigen in chronic, antibiotic treatment-resistant Lyme arthritis. This autoimmune reactivity to the self protein in the context of pathogen-induced inflammation and tissue injury could set in motion a feed-forward loop, which amplifies the inflammatory process, contributing to the chronic nature of the disease, even in the absence of the inciting pathogen. Additionally, OspA has been shown to cause polyclonal activation in B cells, which can result in the production of polyreactive IgM and IgG antibodies. These polyreactive antibodies can cross-react with self-tissue components, which may lead to the impairment of tissue functions and autoimmune activities.

Interpretation of Positive IgG or IgM against OspA

Because antibody against the 30/31 kDa lipoprotein OspA appears in the blood 2-4 weeks after infection, elevated OspA antibody may indicate recent exposure to the spirochete. Also, since OspA cross-reacts with LFA, the presence of OspA antibody may be responsible for the induction of prolonged arthritic episodes observed in some patients with Lyme disease. Additionally, OspA has been shown to cause polyclonal activation in B cells, which can result in the production of polyreactive IgM and IgG antibodies. These polyreactive antibodies can cross-react with self-tissue components, which may lead to the impairment of tissue functions and autoimmune activities.

Outer Surface Protein-C (OspC)

The majority of spirochetes in a feeding nymphal tick clear OspA from their surface and instead express another lipoprotein 23 kDa in size called outer surface protein-C (OspC). Prior to the tick bite and the blood meal, OspC is not present on the surface of the spirochete. However, after the bite, the *Borrelia* enters the host, and so do tick salivary proteins, which exert an immunosuppressive effect. In particular, the tick salivary protein-15 (Salp-15) binds to the OspC of *B. burgdorferi*, protecting it from immune attack. Salp-15 also binds to CD4 on helper cells and inhibits T-cell receptor ligation-induced T-cell signaling and immunosuppression. This allows the spirochete to move freely in the circulation, possibly reaching different tissues. Eventually, immune response to *Borrelia* and Salp-15 occurs, resulting in the production of antibodies as well as proinflammatory cytokines. Both IgG and IgM are produced against OspC during the early stages of Lyme disease.

Overall, antibodies against OspC are detected during early Lyme disease, and thus their diagnostic potential should be used during the early phase of this condition.

Interpretation of Positive IgG or IgM against OspC

Elevation in antibody levels, first of IgM, then of IgG, against OspC may indicate the early stages of Lyme disease.

6A3. Outer Surface Protein-E (OspE) Peptide

Outer surface protein-E (OspE) is a 26 kDa antigen that is found both in ticks and in mammalian tissue. It is highly antigenic and elicits a very strong immune response. This antigen has a hypervariable region which changes during infection to enable the spirochete to hide from immune attack. Factor H (FH) and factor H-like protein-1 (FHL-1) are soluble host serum proteins that are known to negatively regulate the complement system. OspE was the first FH-binding protein identified. It is thought that binding of FH/FHL-1 on the *B. burgdorferi* surface promotes evasion of the alternative pathway of complement, thus promoting the survival of the organism in the mammalian host. Normally, a strong antibody response against the OspE variable region has been observed in patients with ongoing Lyme disease. Therefore, the measurement of IgG and IgM antibodies against the variable region of OspE is important during an infection with *B. burgdorferi*.

Interpretation of Positive IgG or IgM against OspE

OspE is one of the major proteins that change during infection in an *in vivo* environment in order to provide the spirochete with the capacity to hide from defensive attacks by the immune system. High levels of IgG or IgM antibody against OspE in combination with *B. burgdorferi* antibodies may indicate ongoing Lyme infection.

6A4. Leukocyte Function Associated Antigen (LFA) + Cytokeratin 10 (CK10)

One of the manifestations of *B. burgdorferi* infection is Lyme arthritis. The development of antibody response to OspA occurs towards the beginning of an arthritic episode. However, the progression to an autoimmune disease begins with a cross-reactive response between OspA and a self-antigen such as leukocyte function associated antigen (LFA). OspA from *B. burgdorferi sensu stricto* contains HLA-DR4 restricted epitope that shares significant amino acid similarity with human LFA. In patients with HLA-DR4 this molecular mimicry may explain the rare occurrence of antibiotic-resistant chronic arthritis due to infection with *B. burgdorferi*. In fact, chronic arthritis can be induced after the injection of OspA or LFA peptide as a result of this cross-reactivity. Among patients whose T cells react to OspA or produce antibodies against OspA, almost all of them also react to LFA-1 peptide.

Cytokeratin 10 (CK10) is another tissue antigen that shares significant homology with OspA; it also cross-reacts with LFA-1 peptide. Cytokeratins are members of the intermediate filament family or cytoskeletal proteins that maintain the intracellular, structural, scaffolding of many cells. Antibodies to cytoskeletal proteins in general and to CK10 in particular are detected in several autoimmune diseases, including rheumatoid arthritis (RA), psoriatic arthritis, Lyme arthritis, and systemic lupus erythematosus (SLE). CK10 appears to be a relatively specific autoantigens in Lyme arthritis due to its cross-reactivity with OspA, and thus CK10 autoantibody appears to play a strong role in chronic Lyme arthritis. CK10 is present in the endothelial layer of synovial blood capillaries. The neurovascularity observed in chronic Lyme arthritis seems to contribute significantly to the presence of CK10 within the inflamed joints. This could be due to the initial acute arthritis that is triggered by *B. burgdorferi* and the accompanying pro-inflammatory cytokines that contribute to the endothelial cell damage, releasing CK10 in the joints. The reaction of B cells to CK10 and its cross-reactive antigens can initiate the polyclonal stimulation and activation of B cells that can react to the array of self-proteins that are

released in the context of inflammation-induced cell death. This results in the generation of high-affinity, self-reactive IgG or IgM antibodies with greater pathogenic potential in multiple autoimmune reactivities. Therefore, in addition to measuring antibodies against OspA and LFA, the determination of antibodies against CK10 is equally important.

Interpretation of Positive IgG or IgM against LFA + CK10

Elevation in IgG or IgM antibody against LFA + cytokeratin and its cross-reactive OspA may indicate chronic Lyme arthritis, RA, psoriatic arthritis, SLE or other autoimmune disorders.

6A5. Immunodominant Protein (C6 Peptide)

C6 is a 26-mer synthetic peptide that is associated with flagellin. This highly antigenic immunodominant 43 kDa protein is conserved across *B. burgdorferi* subspecies, including *B. b. sensu lato, B. b. sensu stricto,* and *B. b. garinii.* Humans react to this highly antigenic epitope during the early stages and persistently through the course of infection. Thus, patients with Lyme disease react strongly to these antigens during the early and late stages of the disease. Because this antigen is conserved across different *Borrelia* subspecies, it cannot be differentiated whether the reaction is against *B. b. sensu lato, sensu stricto, garinii* or others.

In 1999, a 26-AA peptide from the invariable region of the variable surface antigen VlsE of *B. burgdorferi* was discovered, and a very sensitive and specific ELISA was developed for serodiagnosis of Lyme disease. The sensitivity and specificity of this antibody assay was demonstrated first by the use of infected rhesus monkeys. By 5 weeks post-infection, 9 out of 10 or 90% of the test animals reacted to this peptide and produced both IgG and IgM antibodies; these determinants were consistent in the acute-phase serum obtained from humans infected with *B. burgdorferi*. This peptide ELISA was then applied to a panel of 210 samples collected from patients with clinically defined Lyme disease (some in the early or disseminated phase, some in the acute phase, some in the late phase, and some in convalescence). The sensitivities of this C6 peptide ELISA was as follows:

- Acute phase 74%
- Convalescent phase 85-90%
- Late phase 100%

This C6 peptide ELISA was also applied to 20 specimens obtained from patients with confirmed neuroborreliosis. 19 out of 20 or 95% of the specimens showed strong antibody reaction with this peptide. With post-treatment Lyme disease patients the sensitivity of this assay was 62%.

To assess the specificity of this peptide ELISA, 176 serum samples from patients with chronic infection, other spirochetal infections, autoimmune or neurologic diseases, and serum from patients residing in areas where Lyme disease was not endemic were tested. Only 2 out of 176 samples were found to be positive with this assay. Thus, the overall specificity of the C6 peptide ELISA was 99%.

It was concluded that this simple, sensitive, specific and precise ELISA may help to alleviate some of the problems associated with using the classic tandem of *Borrelia* lysate ELISA and Western Blot in the diagnosis of Lyme disease.

Interpretation of Positive IgG or IgM against Immunodominant Protein (C6 Peptide)

Elevated IgG or IgM antibodies against C6 or 26-mer synthetic peptide originated from the flagellin of *B. b. sensu lato*, *B. b. sensu stricto*, and *B. b. garinii* may indicate the presence of early or late stages of Lyme disease with one of these *Borrelia* subspecies. These C6 peptide antibodies are also detected in patients with neuroborreliosis.

6A6. Variable Major Protein E (VmpE)

Variable major protein (Vmp) is a spirochete lipoprotein with a predicted molecular mass of 34 kDa that is expressed on the surface of the *Borrelia* microorganism. The entry of the spirochete through the human skin initiates changes in the amino acid sequence that affects the antigenic properties of VMP. This helps the spirochete to bypass the immune system and invade the host's tissue. A gene cluster of Vmps is known as a Vmp-like sequence expression site (VlsE). To avoid confusion we shall henceforth simply refer to both as VmpE.

Normally, a strong IgG response to VmpE is observed by the fourth week of infection, and VmpE is also found circulating in the blood during the late stages of Lyme disease (see Figure 10). The figure shows the difference between the level of IgG antibody to VlsE epitope in healthy controls and the levels of IgG anti-VlsE antibody in subjects with single erythema migrans, multiple erythema migrans, early neuroborreliosis, late neuroborreliosis, respiratpry arthritis and refractory arthritis. The antibody to this antigen reacts with almost all subspecies of *Borrelia*, since its epitope is conserved across the different variants. This indicates that there are conserved epitopes among VmpE variants that are antigenic during infections. Based on these experiments, researchers employed recombinant VmpE in an ELISA test and demonstrated diagnostic sensitivities of 63% for culture-confirmed Lyme disease *erythema migrans* (EM) cases and 92% for late stage Lyme disease infections.

Due to the strong antigenicity of VmpE, immune response against it results in a significant amount of IgG and IgM antibody production. The antibodies directed against variable domains are normally able to effectively kill the organism that expresses the corresponding antigen. However, *Borrelia* escapes this killing capability of antibodies by successively expressing different variable domains to avoid identification by the immune system and thus invade the host. Therefore, the measurement of antibodies against invariable as well as variable antigens is very important in enhancing the serodiagnosis of Lyme disease.

Interpretation of Positive IgG or IgM against Variable Major Protein E (VmpE)

Detection of IgG or IgM antibodies against variable major protein E (VmpE) indicates that the *Borrelia* used stealth strategy in order to escape the immune surveillance mechanism and invade the host. These antibodies are detected in the blood in both the early and late stages of Lyme disease.

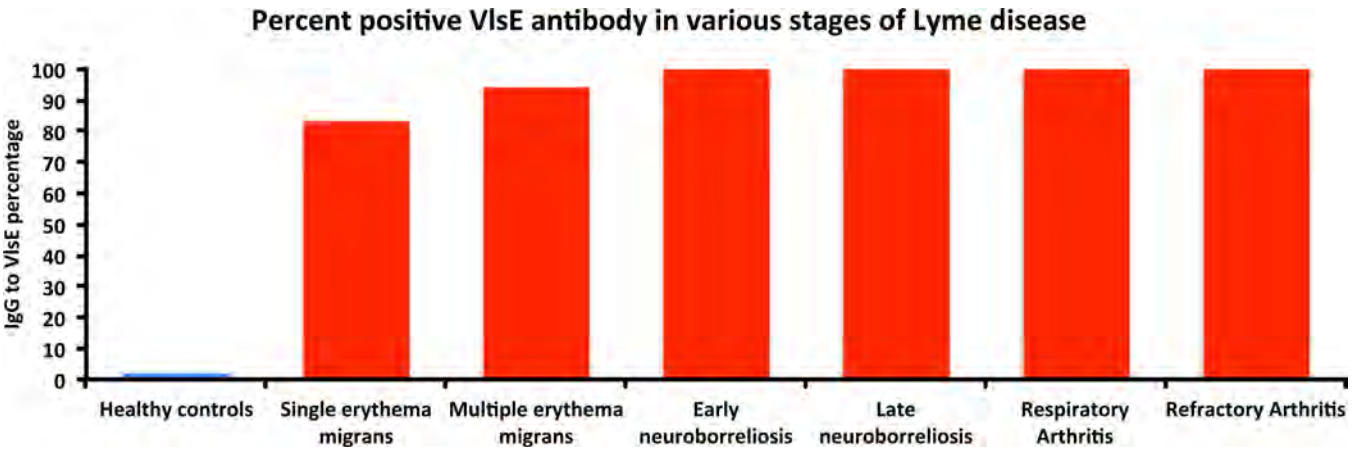


Figure 10. IgG antibody response to VlsE epitope in healthy controls ■ and patients ■ in various stages of Lyme disease.

6B. *Borrelia* Subspecies Antibodies

6B1. Borrelia burgdorferi sensu stricto
6B2. Borrelia garinii
6B3. Borrelia afzelii

Among individual borrelial proteins from different species, sequence heterogeneity varies up to 40%. The borrelial outer protein, Decorin binding protein A (DbpA), is one of the key proteins. This antigen elicits a strong antibody response during experimental murine borreliosis and has been suggested as a potential vaccine protein. DbpA from *B. b. sensu stricto, B. b. garinii* and *B. b. afzelii* were produced and tested as antigens by ELISA. One hundred percent of patients with neuroborreliosis (NB) and 93% of patients with Lyme arthritis (LA) reacted positively. Sera from the majority of patients reacted with only one DbpA and had no or low cross-reactivity with other variant proteins. In patients with culture-positive EM, the sensitivity of DbpA IgG or IgM ELISA was low. The DbpA seems to be a sensitive and specific antigen for the serodiagnosis of LA or NB, but not of EM. Therefore, the detection of high levels of IgG or IgM antibodies against one or all three of these peptides is more likely an indication of Lyme arthritis and neuroborreliosis. The process by which *B. burgdorferi* and its subspecies contribute to neuroborreliosis through breaks in the blood-brain barrier is shown in Figure 11.

Interpretation of Positive IgG or IgM against *Borrelia* Subspecies Antibodies

Elevated IgG or IgM antibodies against the borrelial outer protein named decorin binding protein from *B. b. sensu stricto, B. b. garinii* and *B. b. afzelii* may indicate the presence of chronic Lyme disease which may have resulted in Lyme arthritis, neuroborreliosis or both.

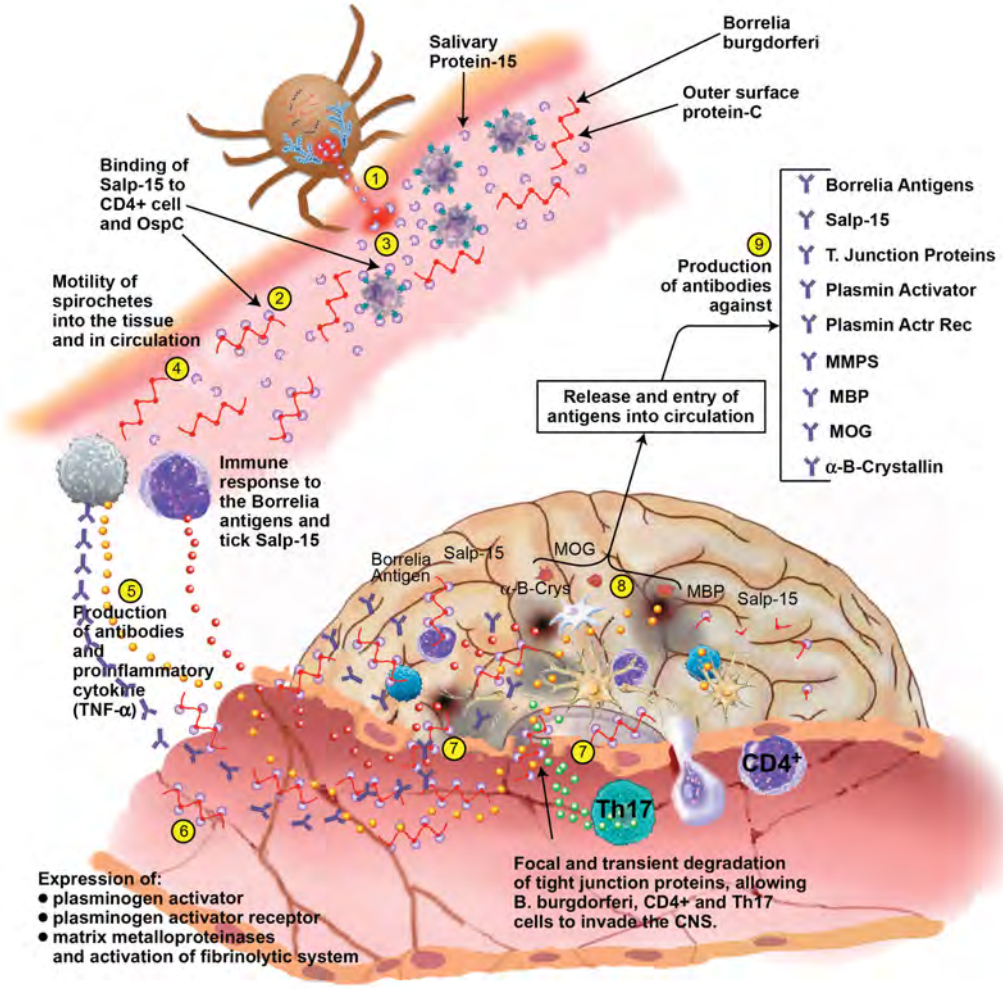


Figure 11. Breaking the blood brain barrier by *Borrelia* activation of the fibrinolytic system, which allows invasion of the CNS, resulting in neuroborreliosis.

1. After a tick bite, in addition to *Borrelia burgdorferi*, tick salivary proteins enter the host and exert an immunosuppressive effect.
2. On one hand, tick salivary protein-15 (Salp-15) binds to the outer surface protein-C (OspC) of *B. burgdorferi*, protecting it from immune attack.
3. On the other hand, Salp-15 binds to CD4 on helper cells and inhibits TCR ligation-induced-T-cell signaling and immunosuppression.
4. This way the spirochete can move freely in the circulation and possibly in different tissues.
5. Eventually, immune response to *Borrelia* and Salp-15 occurs, resulting in antibody as well as proinflammatory cytokine production.
6. Simultaneously, a few spirochetes may make contact with the endothelial cells of the blood brain barrier, stimulating expression of plasminogen activators, plasminogen activator receptors and matrix metalloproteinase, all of which contribute to activation of the fibrinolytic system.
7. This in turn results in focal and transient degradation of tight junction proteins, allowing B. burgdorferi, CD4+ and Th17 to invade the central nervous system.
8. This invasion of the CNS may result in the destruction of neuronal cells and the release of neural cell antigens.
9. This invasion may also lead to the production of antibodies against MBP, MOG, α-B-crystallin and other neural cell antigens.

6B4. Borrelia miyamotoi

Borrelia miyamotoi is a relapsing fever spirochete that is found in several *Ixodes* tick species. This is unusual in that *Ixodes* ticks generally carry and transmit *Borrelia* spirochetes that cause Lyme disease, while ticks of the soft *Ornithodoros* genus carry and transmit tick-borne relapsing fever (TBRF). While the condition caused by *B. miyamotoi* is similar in most ways to TBRF, it is different enough that it has been given a name, hard tick-borne relapsing fever (HTBRF), that reflects its characteristic carrier or vector, the hard-shelled *Ixodes* tick. *B. miyamotoi* shares this peculiarity of vector with very few other spirochetes, but it is the only one whose pathogenicity to humans has been clearly established. It was first discovered in 1995 in ticks in Japan by Masahito Fukunaga, who named the new *Borrelia* species after Kenji Miyamoto, who first isolated spirochetes from ixodid ticks in Hokkaido, Japan. Although at the time the *B. miyamotoi* in Japan was found only in wild rodents, human cases of infection with the species were found in Russia in 2011, and in North America and Europe by 2013.

B. miyamotoi is distantly related to the bacteria that causes Lyme disease. Because *B. miyamoto*i infection has only fairly recently been found in humans, establishing even a definitive animal model for the spirochete and how it causes HTBRF has been difficult. The *B. miyamotoi* strain can express various variable major proteins (Vmps) to evade humoral immunity, and these Vmps have been found to be antigenic to humans. TBRF spirochetes are able to switch serotypes by nonreciprocal gene transfer of these immunogenic Vmps, allowing them to evade the host's antibody response and enabling relapses to occur (Figure 12).

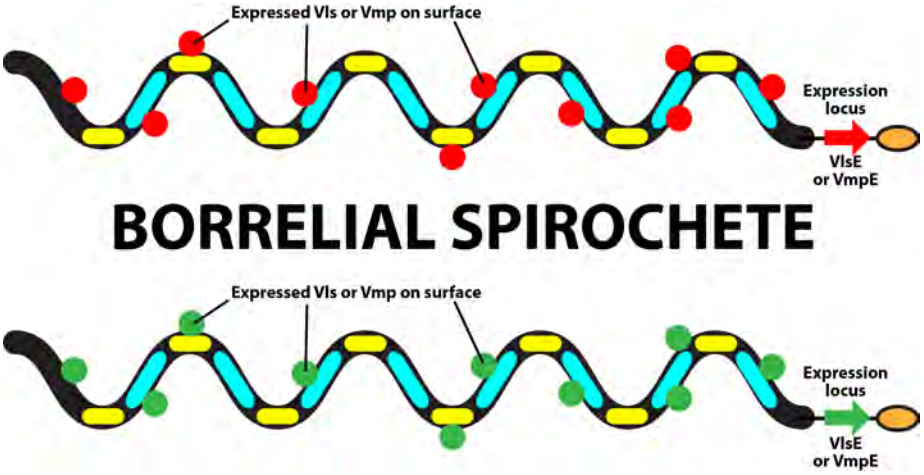


Figure 12. Antigenic variation of borrelial Lyme VlsE and TBRF VmpE systems.
Both Lyme and TBRF spirochetes are composed of cassettes (blue and yellow capsules) numbering approximately 16-60 depending on the strain. The red and green circles are the Vls or Vmp expressed on the surface of the spirochete. For the Lyme VlsE system, random recombination of the 16 or so cassettes results in changing the VlsE expression locus from red to green, so that the spirochete will now express green Vls on the surface. Thus, this random recombination of cassettes can result in thousands of unique VlsE variants. In contrast, for the TBRF VmpE system, antigenic variation is achieved when the entire VmpE expression locus (red) is deleted and replaced with a new VmpE expression locus (green), so that the spirochete will now express green Vmp on the surface. The immune system will typically target spirochetes with these immunodominant (green) Vmps, but a small number of spirochetes expressing different Vmps will remain, proliferating until they reach the point where the patient experiences the characteristic recurrence of TBRF. It is presumed that *B. miyamotoi* follows this same mechanism.

A 2016 study by Wagemakers et al. used a combination of PCR, ELISA, Western Blot and dark-field microscope methodologies to gather data. The study successfully identified Vsp1 as a dominant antigenic target and showed that although anti-Vsp1 antibodies killed most *B. miyamotoi* spirochetes, spirochetes that expressed a different Vmp were resistant to the anti-Vsp1 mediated killing. The study was also able to detect Vmp-specific antibodies in the sera of patients with HTBRF, showing that these Vmps are associated with HTBRF pathogenesis, and proving the value of *B. miyamotoi* VMPs as early serodiagnostic markers. The researchers concluded that by combining different Vmps, a serologic test could be developed to diagnose early infection caused by various *B. miyamotoi* serotypes in humans.

Interpretation of Positive IgG or IgM against *Borrelia miyamotoi*

Elevated IgG or IgM antibodies against the Vmp of the relapsing fever spirochete *Borrelia miyamotoi* indicate that this spirochete, by using immune invasion strategy, could evade the host antibody response, enabling relapses to occur. Anti-Vmp antibody is an early biomarker of relapsing fever.

6C. Lyme Co-Infections

6C1. Babesia

Babesia is a genus of intracellular protozoan parasite that infects and multiplies in red blood cells. It was named after the Romanian bacteriologist Victor Babes, who in 1888 identified the microorganisms as the cause of febrile hemogloinuria in cattle. The *Babesia* parasite causes severe hemolytic anemia, cerebral babesiosis, abortion and even death in susceptible animals. Babesiosis is a malaria-like disease quite common in animals including cats, dogs, deer, and horses, and can also affect humans. Out of 100 species of *Babesia, Babesia microti* is the most common cause of human babesiosis. *Babesia duncani, B. bovis* and *B. divergens* can also cause disease in humans. Agricultural workers, cattle breeders and other individuals in contact with *Ixodidae* ticks are more prone to develop babesiosis and other tick-borne diseases.

Babesiosis is commonly transmitted by tick bite, so that it often presents with other tick-borne diseases such as Lyme disease (Figure 13). After the bite, *Babesia* penetrates into the blood capillaries and red blood cells. Trophozoites of *Babesia* feed on the hemoglobin of the red blood cells, multiplying and going on to destroy even more and more red blood cells. The destruction of red blood cell membranes by the parasite results in blood byproducts within the bloodstream that can cause strong toxic symptoms. A massive release of red blood cells' free hemoglobin and other factors may result in accumulation of indirect bilirubin in the blood and jaundice, as the liver is unable to metabolize the excess bilirubin. The parasite and the blood products negatively affect the functioning of all organs and body systems.

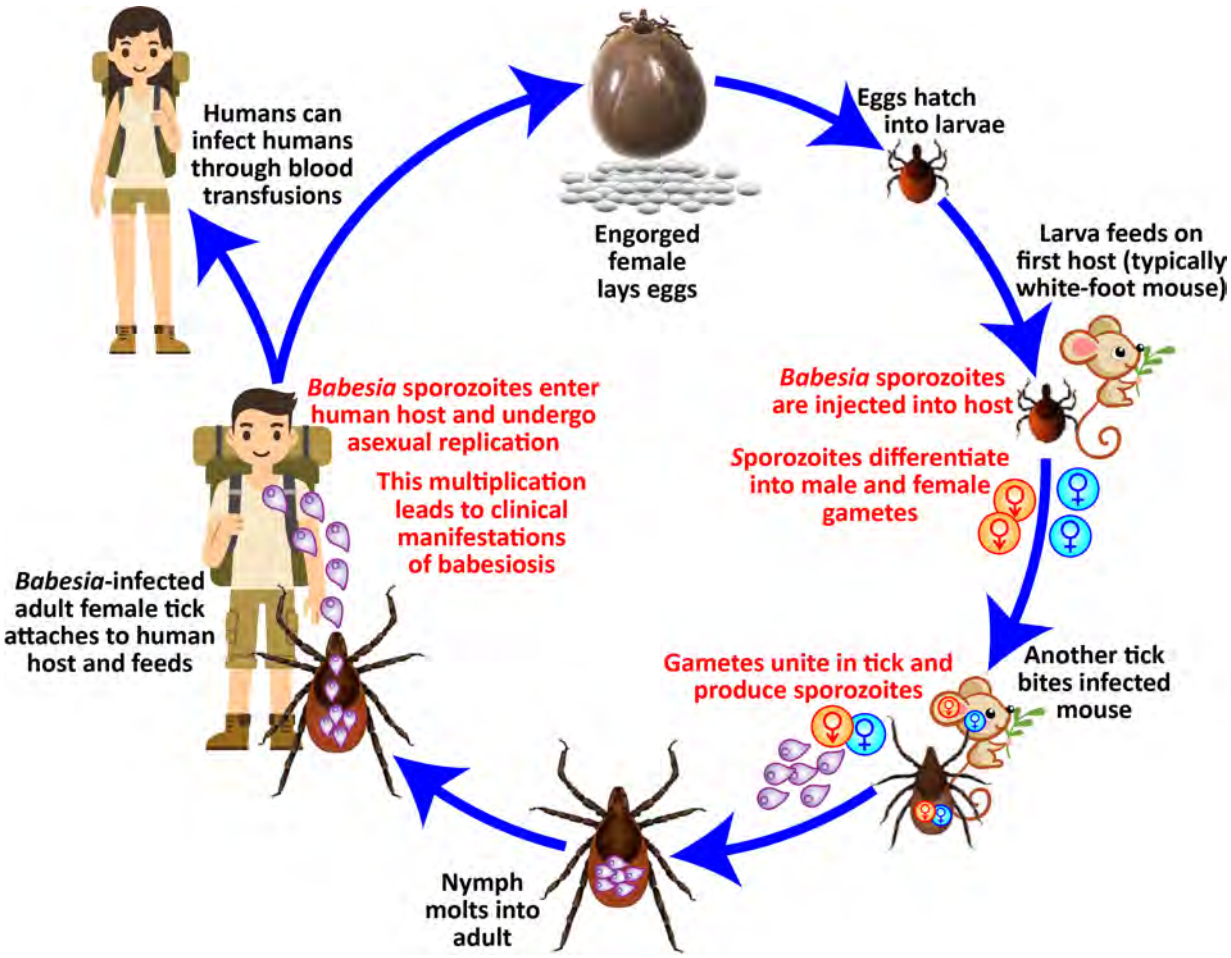
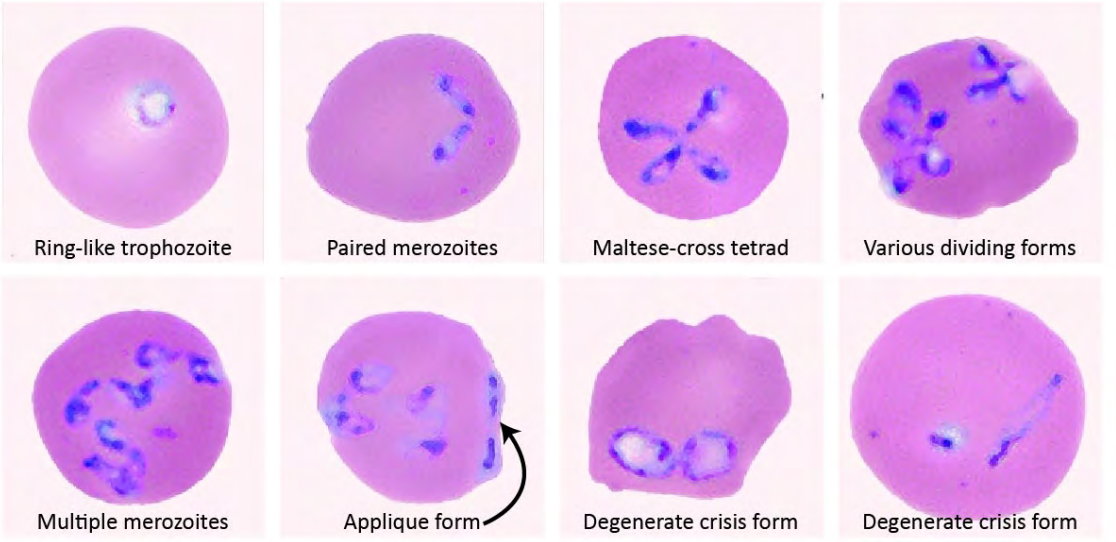


Figure 13. Life cycle of ticks and transmission of *Babesia.*

Like many diseases caused by parasites and opportunistic pathogens, babesiosis affects individuals with reduced immune function, such as people of advanced age, patients with cancer, and overall those suffering from immunodeficiency. Individuals with healthy immune systems and efficiently functioning B cells, T cells, and natural killer cells who are infected by babesiosis are generally asymptomatic, since the host's cell-mediated immunity will not allow the spread of the parasite, constituting an asymptomatic case of babesiosis.

A diagnosis of babesiosis is based on clinical symptomatology such as anemia with fever, hemoglobinuria, jaundice and kidney malfunction, and laboratory test results such as elevated lactate dehydrogenase, decreased serum haptoglobin, high bilirubin in the blood, detection of *Babesia* in a blood smear, identification of the organism by microscope using Wright stain, and other urine and blood tests. The host immune response to the pathogens results in the production of IgM and IgG antibody against a variety of antigens, including the merozoite surface antigen 1 protein (MSA_1 or MSP-1). This antigen is so important that it is used for the development of vaccines for animals against babesiosis.



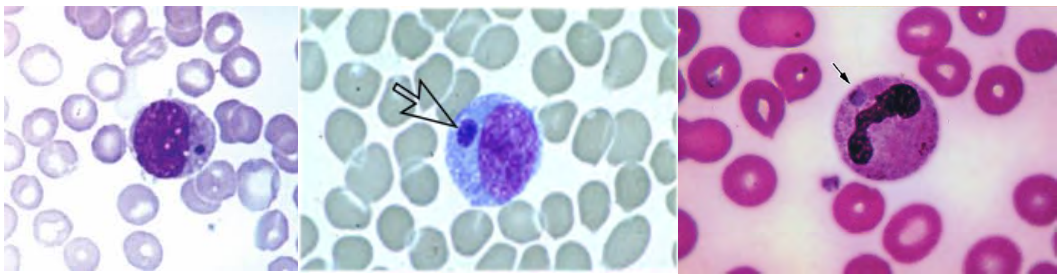
Babesia-infected erythrocytes.

Interpretation of Positive IgG or IgM against *Babesia*

Although babesiosis is a common malaria-like disease in cats, dogs, deer, and horses, it can also affect humans. Elevated IgG or IgM antibody against *Babesia microti, B. equi,* and *B. bovis* antigens may indicate host immune response against this intracellular protozoan. Routine blood tests such as blood stain, free hemoglobin, and indirect bilirubin level, and tests such as elevated lactate dehydrogenase, decreased serum haptoglobin, high bilirubin in the blood, detection of *Babesia* in a blood smear, identification of the organism by microscope using Wright stain, and other urine and blood tests should be performed in order to find the symptomatic cases of babesiosis.

6C2. Ehrlichia

Ehrlichia is a genus of *Rickettsiales* bacteria named after the German microbiologist Paul Ehrlich. In 1925 it was initially named *Rickettsia ruminantium*, but the name was later changed to *Ehrlichia ruminantium*. *Ehrlichia* causes the disease ehrlichiosis, an infectious disease transmitted by the bite of different ticks, especially lone star ticks, deer ticks and dog ticks. The bite of a tick transmits the *Ehrlichia* from the tick into the body of an individual, causing not only ehrlichiosis but other disorders such as typhus fever and Rocky Mountain spotted fever (Figure 14).



In the US there are two main types of ehrlichiosis:

- *Human monocytic ehrlichiosis (HME)* – HME is caused by the rickettsial bacteria species *Ehrlichia chafeensis*. This bacteria affects the cells that are involved in the immune system's first line of defense, such as macrophages and monocytes, which protect the body against invading microorganisms and debris. This disease is more common in the regions where white-tailed deer and lone star ticks flourish.

- *Human granulocytic ehrlichiosis (HRE) or human granulocytic anaplasmosis (HGA)* – HRE or HGA is a disorder that results from infection by the rickettsial bacteria known as *Anaplasma phagocytophilum*, which is transmitted by the bite of ticks belonging to the *Ixodes ricinus* species complex, which includes *Ixodes scapularis* and *Ixodes pacificus*.

Both monocytic and granulocytic ehrlichiosis produce similar symptoms, such as fever, headache, muscle ache, shaking and chills. In some cases, affected individuals suffer from nauseating, vomiting sensations and diarrhea.

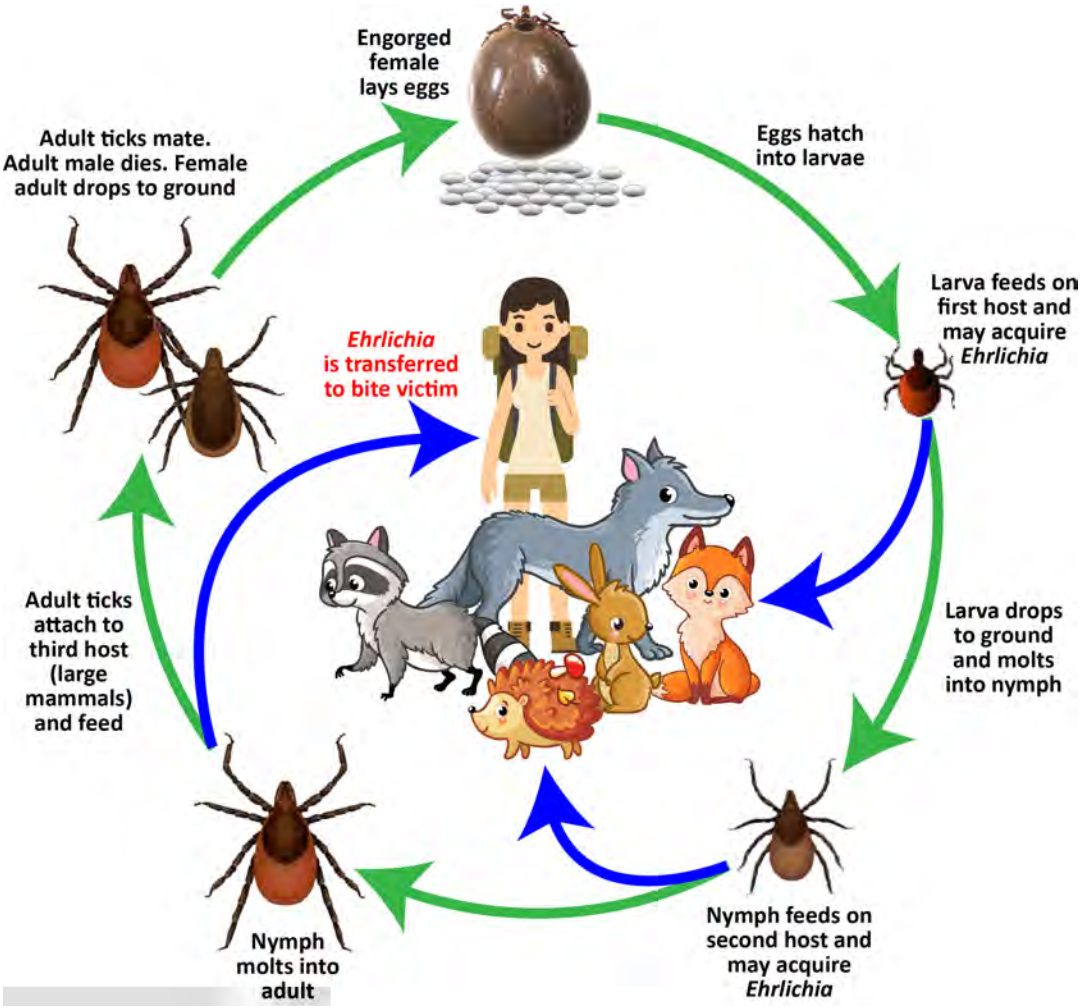


Figure 14. Life cycle of ticks and transmission of *Ehrlichia.*

Ehrlichiosis is diagnosed based on these symptoms and a physical examination of the patient. A complete blood cell count and granulocyte stain examination are also helpful. Additionally, measurement of the IgM and IgG antibodies against different components of rickettsial pathogens, especially the immunodominant major surface protein-2 that is expressed in the repeated cycles of rickettsemia, as well as proteins that localize with actin filaments during intracellular infection, would greatly aid in the successful diagnosis of the disease.

The IgM and IgG antibodies are produced against the major surface protein 2 (MSP2) to control rickettsemia. T-helper response to this conserved region of MSP2 also enhances the primary antibody response against newly emergent variants. The immunodominant peptide component of this protein induces the production of high levels of interferon gamma, a cytokine that is not only associated with protection against *Ehrlichia*, but is also needed for rapid generation of variant-specific IgG2.

Both sera from patients infected with *Ehrlichia phagocytophila* and sera from cattle infected with *Anaplasma marginale* react with MSP2, indicating that this is the dominant antigen that shares more than 85% amino acid sequence similarity with the bacteria.

The detection of antibodies against the rickettsial antigen called appendage-associated protein, which localizes with F-actin during intraerythrocytic infection may explain the mechanism by which a pathogen uses a host's self-protein to influence the cycle of infection, replication, and egression in the host cell. Furthermore, this could be an additional mechanism showing that localization of pathogen proteins with actin filaments may be responsible for immune response against this complex and the induction of autoimmune reactivity against human F-actin, which is involved in several autoimmune disorders.

Interpretation of Positive IgG or IgM against *Ehrlichia*

Monocytic and granulocytic ehrlichiosis is rickettsial disease that is transmitted by the Lone Star tick. Ehrlichiosis is diagnosed based on symptoms and physical examination. However, elevation of IgG or IgM antibody against major surface protein-2 of *Ehrlichia phagocytophila* and *Anaplasma marginale* may confirm the diagnosis. Furthermore, due to cross-reactivity between *Ehrlichia* F-actin and human F-actin, immune response against *Ehrlichia* antigen may ignite friendly fire that may result in autoimmunity.

6C3. Bartonella

Bartonella is a genus of bacteria that can cause several different infections and diseases in humans. There are at least 26 species and subspecies of *Bartonella* that are transmitted to humans by a vector. A significant cross-reactivity among *Bartonella* species occurred in 94% of patients with suspected cat-scratch disease. Thus it is sufficient to measure *B. henselae* as the main agent of bartonellosis.

***Bartonella henselae* is the primary cause of cat-scratch disease.**

Cats are the primary reservoir host for *Bartonella henselae*, while dogs are the primary host for *Bartonella vinsonii. B. henselae* was confirmed to be the primary cause of cat-scratch disease, which is characterized by regional lymphadenopathy and self-limiting fever. Epidemiologic evidence supports an association between rheumatic symptoms and cat-scratch disease. Indeed, among patients examined by rheumatologists the prevalence of antibodies against *Bartonella* was as high as 62%. These individuals suffer from arthralgia, chronic fatigue and fibromyalgia. Some of these patients can also suffer from blurred vision, headaches, subcortical neurologic deficits, numbness in the extremities, memory loss, balance problem, and muscle pain, which is why they are commonly referred to a neurologist. Overall, more than half of the patients examined by rheumatologists reported a prior diagnosis of Lyme disease, babesiosis or bartonellosis.

Individuals who have direct contact with cats and dogs are more prone to bartonellosis. In the presence of symptoms such as severe fatigue, neurologic and neurocognitive abnormalities, myalgia and arthralgia, occupationally at-risk patient populations should be referred to medical specialists such as rheumatologists and neurologists. The bacteria is commonly transferred from cat or dog to human when the animal breaks the human's skin, allowing the bacteria to enter the human's system. *Bartonella* can also be transferred directly to humans by fleas, ticks, lice and mosquitos (Figure 15).

The detection of high levels of IgG or IgM antibodies against *Bartonella* may indicate exposure to *Bartonella* or co-infections and confirm the presence of neurological and rheumatological symptoms in individuals occupationally exposed to this organism.

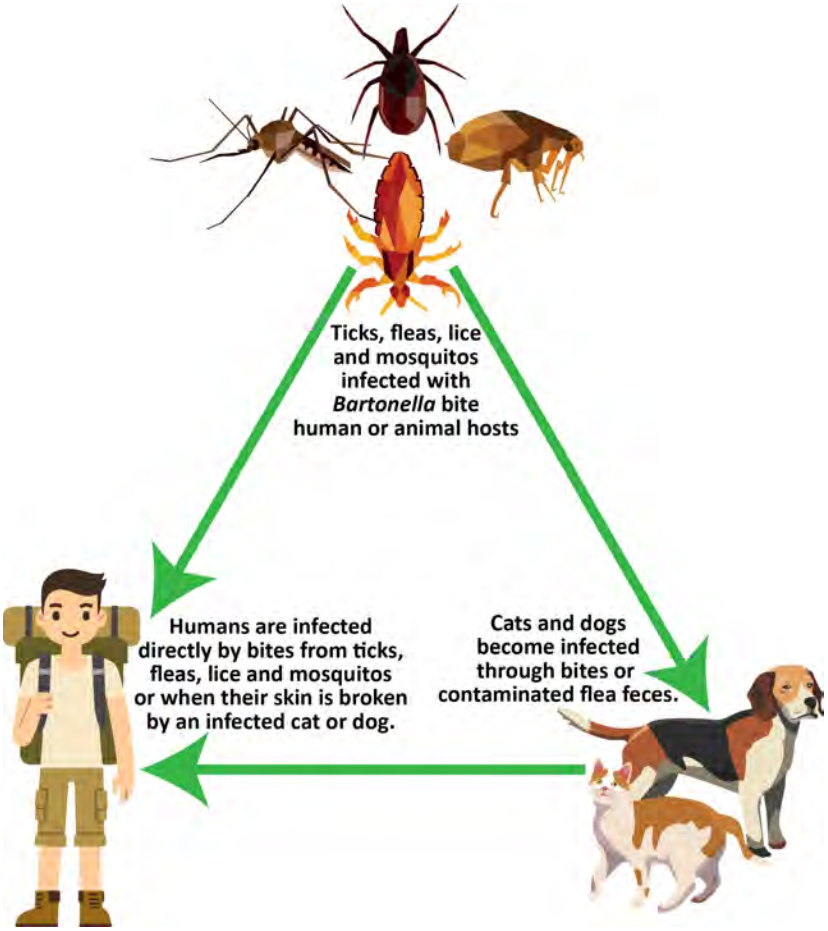


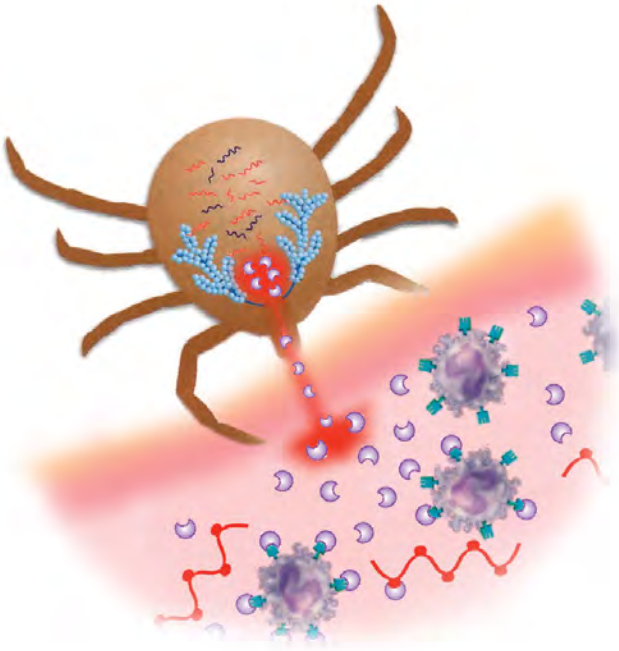
Figure 15. Mode of transmission of *Bartonella.*

Interpretation of Positive IgG or IgM against *Bartonella*

Individuals who have direct contact with cats and dogs are more prone to bartonellosis. The detection of high levels of IgG or IgM antibodies against *Bartonella henselae* may indicate exposure to *Bartonella* or co-infections and confirm the presence of neurological and rheumatological symptoms in individuals occupationally exposed to this organism.

7. Conclusions: The advantages of the Multi-Peptide ELISA

Each method for detecting the antibodies associated with Lyme disease and its co-infections has its advantages and disadvantages. However, in the Multi-Peptide ELISA, we have combined the major advantages of Western Blot, which is the use of pure proteins or antigens, with the quantitative nature of the ELISA assay. We also measure antibodies against a multiplicity of antigens known for their involvement with Lyme disease, including antibodies against the *Borrelia* subspecies *Sensu stricto, Afzelii, Garinii* and *Miyamotoi,* as well as the co-infections *Babesia*, *Ehrlichia* and *Bartonella*. And in this patented methodology, we use not only a mixture of *Borrelia* proteins but also measure separately antibody levels against proteins or peptides of different molecular sizes, such as OspA, OspB, OspC, OspE, LFA, VMP, DbpA and more. We use both *Borrelia* antigens grown in culture and the various component proteins expressed *in vivo*, enabling us to combine the advantages of both environments to improve the accuracy of our determinations. It is vital to correctly determine as early as possible whether someone is developing Lyme disease or something else. False positive results could lead to years of incorrect treatment and unnecessary medications and their side effects, while false negative results could lead to years and even a lifetime of suffering down the road. Our combination of the gold standard of the ELISA method, the variable antigens expressed *in vivo*, and our own 4 core principles of testing has resulted in a system for detecting Lyme disease in the most reliable way.



INTERPRETATION OF RESULTS FOR MULTI-PEPTIDE ELISA

Antibody against	Indication
***B. burgdorferi* antigens**	Elevated IgM or IgG antibody against *B. burgdorferi* antigens may indicate recent or past exposure to the spirochete or cross-reactive antigens.
Outer Surface Protein A (OspA), 30/31 KDa	Elevated IgM or IgG against OspA may indicate exposure to the spirochete or cross-reactive antigens. Arthritic episodes observed in some patients may confirm exposure to the organism.
Outer Surface Protein C (OspC), 23 KDa	Both IgM and IgG are produced against OspC during the early stages of Lyme disease. Elevation in antibody against OspC may indicate recent exposure to *B. burgdorferi* or cross-reactive antigens.
Outer Surface Protein E (OspE), 26 KDa	IgM and IgG antibodies against OspE are detected in patients with ongoing Lyme disease. Simultaneous detection of antibodies against OspE and *B. burgdorferi* antigens may indicate exposure to an agent of Lyme disease or its cross-reactive antigens.
Leukocyte Function Associated Antigen (LFA)	Detection of high levels of antibodies against LFA, its cross-reactive antigen (OspA) and Cytokeratin-10 may indicate chronic Lyme arthritis, rheumatoid arthritis, psoriatic arthritis, lupus or other autoimmune disorders due to exposure to the Lyme spirochete.
C6 Peptide originated from Immunodominant Protein, 43 KDa	Elevated levels of antibodies against flagellar antigen or C6 Peptide may indicate exposure to *B. b. sensu lato, B. b. sensu stricto, B. garinii* or their cross-reactive antigens during the early stages, and persistently through the course of infection. The C6 antibodies are also detected in patients with neuroborreliosis.
Variable Major Protein E (VmpE), 34 KDa	IgM and/or IgG antibodies against VmpE, which present in almost all subspecies of *Borrelia*, may indicate recent or late exposure to *B. burgdorferi,* its subspecies, or their cross-reactive anrtigens.
Borrelia* subspecies: *B. b. sensu stricto, B. garinii, B. afzelii, B. miyamotoi	Elevated antibodies against *Borrelial* outer protein or Decorin Binding Protein indicates not only exposure to one of these subspecies but the presence of chronic Lyme which may have resulted in neuroborreliosis or Lyme arthritis. Elevated antibodies against Variable Major Protein (Vmp) of *B. miyamotoi* may indicate exposure to hard tick-borne relapsing fever.
Babesia microti, B. equi, B. bovis	Antibody elevation against *Babesia* antigens, particularly in individuals with reduced immune function, anemia, hemoglobinuria, elevated liver enzymes, jaundice and kidney malfunction may indicate exposure to the organism and host immune response against this intracellular protozoan or its cross-reactive antigens.
Ehrlichia phagocytophila, Anaplasma marginale, Major Surface Protein 2	Elevation in IgM or IgG antibodies against Major Surface Protein 2 of *Ehrlichia* may indicate exposure to this microorganism or cross-reactive antigens such as human F-actin
Bartonella henselae	Elevation in IgM or IgG antibodies against *Bartonella henselae,* especially in individuals who have had direct contact with cats and dogs and exhibit symptoms of fatigue, myalgia, arthralgia, and/or neurologic abnormalities may indicate exposure to *Bartonella* or its cross-reactive antigens.

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PANEL A, ISL#2017

IMMUNOSEROLOGY OF LYME DISEASE BY MULTI-PEPTIDE ELISA

	Neg.	Pos.
B. burgdorferi lysate	0.15	2.70
OspA + OspC	0.16	0.75
OspE	0.18	0.29
Leukocyte function associated antigen	0.17	1.75
Immunodominant protein of invariable region	0.20	2.30
Variable major protein	0.17	0.64
Decorin binding protein of *B. b.* sensu stricto	0.14	2.38
Decorin binding protein of *B. garinii*	0.18	0.26
Decorin binding protein of *B. afzelii*	0.19	0.19
Major protein of *B. miyamotoi*	0.18	2.50
Babesia peptide	0.20	0.22
Ehrlichia peptide	0.15	0.46
Bartonella antigen	0.19	0.64

Lyme-Specific Antibodies (IgG, IgM)

- *B. burgdorferi* Antigens
- Outer Surface Protein A+C Peptides
- Outer Surface Protein E Peptide
- Leukocyte Function Associated Antigen + Cytokeratin 10
- Immunodominant Protein C6 Peptide
- Variable Major Protein E

Borrelia Subspecies Antibodies (IgG, IgM)

- *Borrelia burgdorferi sensu stricto*
- *Borrelia garinii*
- *Borrelia afzelii*
- *Borrelia miyamotoi*

Lyme Co-Infection (IgG, IgM)

- *Babesia*
- *Ehrlichia*
- *Bartonella*

Specimen requirement: 2 mL Serum

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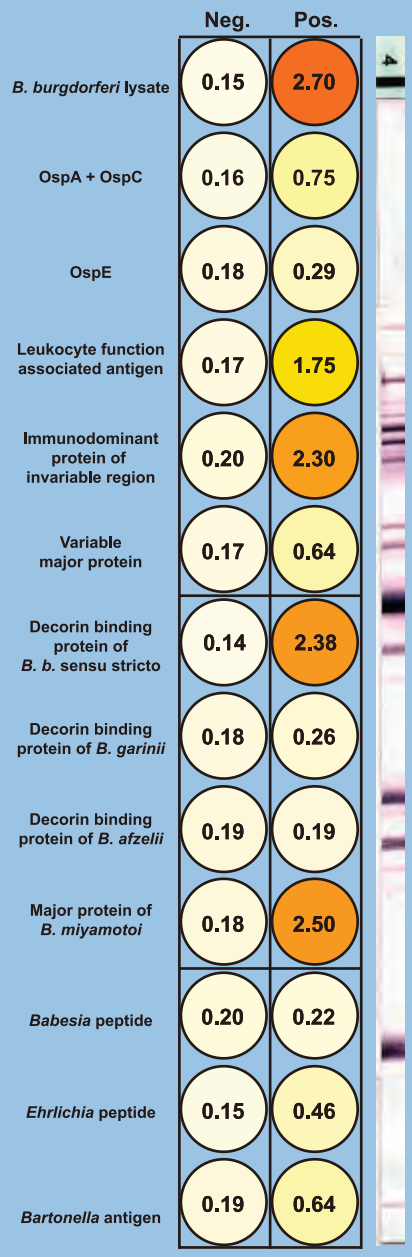
PANEL B, ISL#2018

IMMUNOSEROLOGY OF LYME DISEASE BY MPE & WESTERN BLOT



Lyme-Specific Antibodies (IgG, IgM)

- *B. burgdorferi* Antigens
- Outer Surface Protein A+C Peptides
- Outer Surface Protein E Peptide
- Leukocyte Function Associated Antigen + Cytokeratin 10
- Immunodominant Protein C6 Peptide
- Variable Major Protein E

Borrelia Subspecies Antibodies (IgG, IgM)

- *Borrelia burgdorferi sensu stricto*
- *Borrelia garinii*
- *Borrelia afzelii*
- *Borrelia miyamotoi*

Lyme Co-Infection (IgG, IgM)

- *Babesia*
- *Ehrlichia*
- *Bartonella*

WESTERN BLOT ASSAY (IgG, IgM)

- *B. burgdorferi*

Specimen requirement: 2 mL Serum

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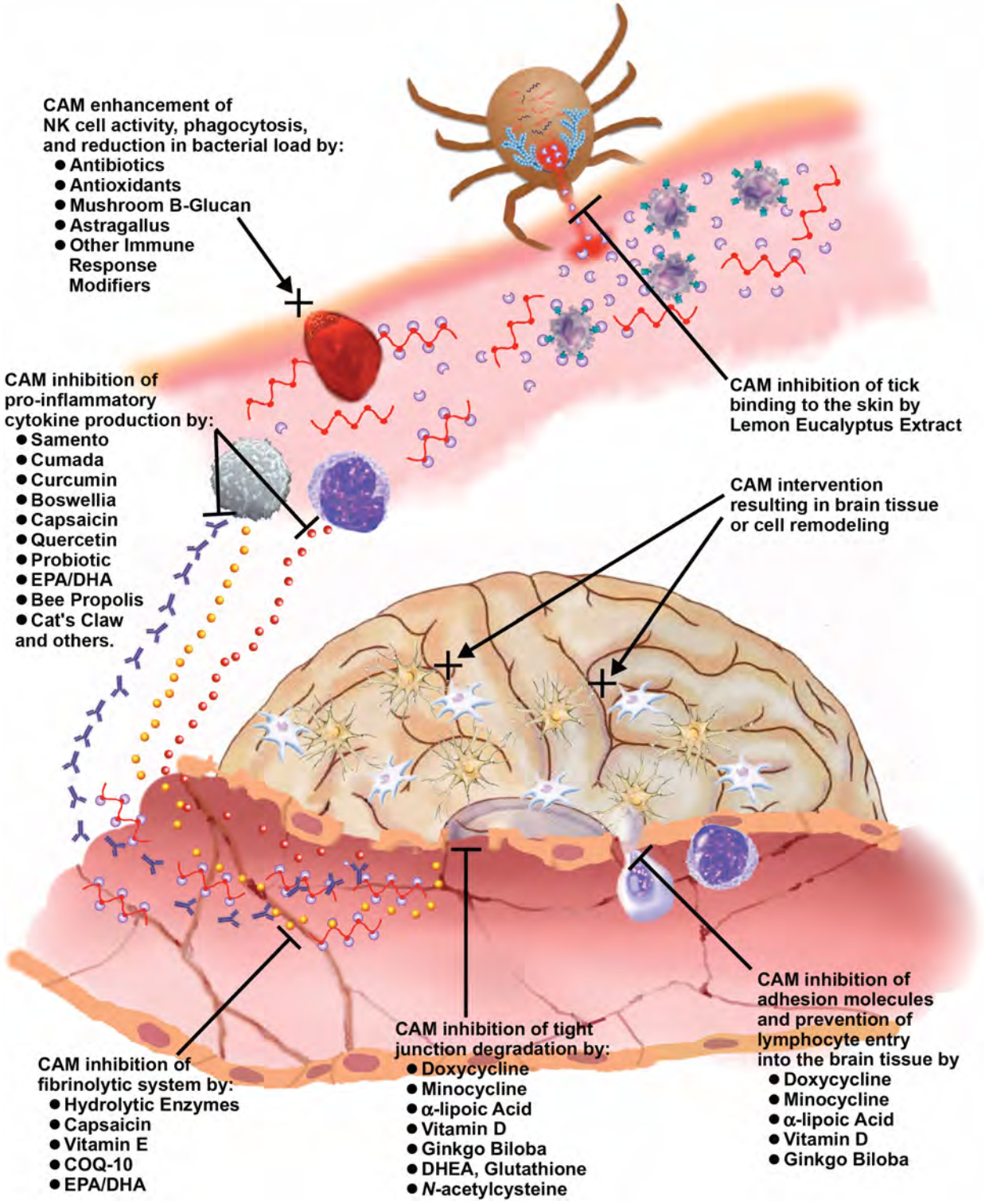

Using CAM treatment could prevent different processes ranging from the attachment of the tick to the skin, to inflammatory events, CNS invasion, and the induction of neuroborreliosis. CAM can act through the enhancement of natural killer cell activity, macrophage function, inhibition of pro-inflammatory cytokine production, inactivation of the fibrinolytic system and repair of blood brain barriers.

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