TICK BORNE DISEASE-RELATED TESTING

Tick-borne infections are increasing globally - Lyme disease is among the most prevalent vector borne infection in the U.S. and Europe and is reaching epidemic levels (Kugeler et al. 2015; Sykes et al. 2014).

While most ticks have the capacity to transmit a number of pathogens that cause human disease, Lyme disease is the most widely known tick-borne disease and is caused by bacteria of the genus *Borrelia*; typically *Borrelia burgdorferi* which are gram-negative spirochete bacteria. Spirochetes are a group of phylogenetically distinct bacteria that have a unique mode of motility by means of axial filaments (endoflagella).

Borrelia are divided into "genospecies"; among the most common include *B. burgdorferi sensu stricto, B. garinii, B. afzelii, B. spielmanii, B. bavariensis* and the newly identified *B. miyamotoi*.

B. burgdorferi invades the blood and tissues of various infected mammals and birds via the bite of ticks of the genus *Ixodes*. The natural reservoir for *B. burgdorferi* is thought to be the white-footed mouse. Upon acquiring the spirochetes from an infected animal during a blood meal, the tick can transfer the spirochetes to deer, humans, and other warm-blooded animals. In most mammals, including humans, infection by *B. burgdorferi* can result in Lyme disease, but healthy carriers also exist (i.e. not everybody bitten by a tick get sick). However, it is noteworthy that all of *Borrelia* species are known to cause disease, 21 of which are associated with Lyme disease.

It is important to bear in mind that Lyme disease can be acute (i.e. occurring shortly after a tick bite) or late stage / persistent / chronic (i.e. occurring / persisting after the initial tick bite; Donta 2002). There has been an attempt to separate “late” Lyme disease from “chronic” Lyme disease; the former being manifested by objective signs of arthritis or neurological disease (Wormser et al. 2000). Some have denied the existence of chronic disease, inferring that these patients suffer from psychiatric disorders; some have used the term “chronic” to mean post-treatment disease (“post-Lyme”), assuming that the infection has been treated, and the remaining symptoms are in the same realm as those patients who have “fibromyalgia” or “chronic fatigue” (Seltzer et al. 2000; Steere AC 2001). That chronic Lyme disease actually exists, and is likely the most common form of the disease, is supported by epidemiologic studies demonstrating that 30-50% of treated and untreated patients go on to develop a multisymptom disorder typical of, and indistinguishable from, fibromyalgia and chronic fatigue (Asch et al. 1994; Shadick et al. 1994).

The distinction between acute and late / chronic / persistent disease is important given the diagnostic and treatment approaches might be different in these two situations.

Given the number of possible coinfections (i.e. tick-borne infections –TBI) transmitted by ticks (such as Bartonella, Babesia, Anaplasma, Ehrlichia, Rickettsia, etc), it is more correct to talk about Tick-borne disease instead of just Lyme disease which only refers to Borrelia infection. Frequently, we see the term “Lyme disease” broadly used for tick-borne diseases; however, Dr. Horowitz proposed that persistent/chronic infection with Borrelia and other coinfections be referred to as Lyme-MSIDS (Multiple Systemic Infectious Disease Syndrome).

Lyme borreliosis is a multisystemic disease with diverse manifestations which make clinical diagnosis difficult. Lyme disease exhibits a variety of symptoms that may be confused with immune and inflammatory disorders (see the book from Dr. Horowitz *Why Can't I Get Better*). Different checklists (from Dr. Horowitz or at the website www.lymedisease.org) are available for patients and doctors in order to take into account those multiple symptoms. A new, revised checklist, called HMQ (Horowitz MSIDS Questionnaire*, validated on over 1600 patients) is published in the new book of Dr. Horowitz (*How Can I Get Better).*

Inflammation around the tick bite causes skin lesions. Erythema (chronicum) migrans (ECM), a unique expanding skin lesion with central clearing that has a ring-like or bullseye appearance, is typically the first stage of the disease. Arthritis, neurological disease, and cardiac disease may manifest at later stages. While early treatment of a primary acute infection is straightforward, patients identified later can suffer from chronic Lyme and associated conditions, which are more difficult to diagnose and treat.

It is also important to bear in mind that the physician needs to assess the complete clinical picture. Diagnosing Lyme and TBI-related diseases is extremely challenging. TBI diagnosis complications are a result of inadequate testing. Lyme cases are commonly misdiagnosed with other illnesses and even when a proper diagnosis is made, it's often difficult to verify because accurate testing isn't always available. For that reason, some doctors base their diagnoses only on the presence of the classic "bullseye" rash, but don't pursue additional testing. Other doctors require laboratory confirmation before treatment. Very few tests for TBDs are approved for clinical diagnosis, thus most of available testing options are "investigational" or "research" tests, aiming to help the assessment of patients with Lyme-like complaints.

Laboratory testing not only helps to diagnose a disease, but also to manage the illness and follow treatment. A good test can help a doctor to assess the severity of disease, monitor the course of disease progression, stability or resolution, relapse, and select drugs or adjust therapy. Unfortunately, a test with this capability does not exist for Lyme disease. A significant amount of energy and money have been spent in order to improve testing efficacy, however, it still remains poor. Why? Several reasons exist, but the most significant is a consequent of the nature of these infectious agents which have develop multiple strategies in order to avoid the immune system. The most common tests are based on serology, but many patients do not produce antibodies, and thus produce a negative test result. There are many reasons why antibodies aren't observed when a person has an active Lyme infection, among which are: (i) test performed too soon (for acute infection), (ii) the tick's saliva contains specific immune suppressing components preventing the activation of an immune response, (iii) when Borrelias are in cyst form, there is no synthesis of surface components in order to make antibodies, etc. Furthermore, given the number of identified strains (and most likely not all have been identified), the available tests are not all inclusive, thus if a patient is infected by the strain not covered by a given test, the result will be negative. All this underscores the importance of a specialized physician who knows how to utilize the best of his knowledge in combination with the proper laboratory test and test results. An overall understanding of the limitations and significance of a given diagnostic is of the highest importance.

** The new HMQ questionnaire can be obtained by mailing to tmijatovic@redlabs.be*

Most of the currently available diagnostic methods are presented below, they are used both for acute and chronic diseases evaluation. A separate part is dedicated to persistent / chronic disease seen it needs a wider approach based on multiple symptoms displayed by chronic patients.

MICROSCOPY

This is the examination of a blood smear or tissue sample using a high definition microscope to directly detect the presence of spirochetes. This is a lengthy and insensitive technique when used on blood because of the low numbers of Borrelia present, especially in the very early stage of infection. Additionally, very few clinical trials using this technique have been reported, making it difficult to ascertain the usefulness of this method in the analysis of those with very early infection or in a relapsing period of disease. Also, other spirochetal diseases would need to be excluded when relying on this method.

Focus floating microscopy has been developed for detecting spirochetes in tissue samples but this has not been used outside the research laboratory and has the practical difficulty that potentially infected tissues must be identified and biopsied.

CULTURE DETECTION

Because Borrelia have a fastidious growth requirements, these organisms are difficult to culture, and even under optimal conditions, their growth is very slow. Accordingly, culturing the bacteria, as in the diagnostic tool is not practical for Lyme disease, specifically for two reasons: first, Borrelia have evolved to propagate in a living host, therefore, culturing them is fraught with difficulties and these methods are often not reproducible with clinical rigor; and second, because they grow so slowly, results cannot be reported in a reasonable period of time.

BIOPSY

Approximately 60-80% of specimens isolated from the leading edge of a suspected erythema migrans lesion by means of saline-lavage needle aspiration or 2-mm punch biopsy reveal *B. burgdorferi*. However, because the presence of a lesion in combination with a confirmatory history and clinical presentation are sufficient to initiate treatment, these skin biopsy procedures are seldom performed.

SEROLOGY

The human immune system produces specific antibodies in response to foreign substance present in the body. Therefore, antibody-based tests identify these specific antibodies that are produced in response to a bacterial infection. Blood tests that identify antibodies specific to the bacteria *B. burgdorferi* are among the most commonly used diagnostics for Lyme disease. This method of diagnosis is referred to as serology. If the antibody-mediated immune response has not developed sufficiently, it is possible for these tests to yield negative results, despite an active

infection. Conversely, antibodies can persist for years after treatment, therefore, in the absence of an active infection, asymptomatic patients may produce a positive serology result.

Lab tests detect two different classes of antibody, IgM and IgG.

- Borrelia IgM (immunoglobulin M) antibodies are usually detectable in the blood about two to three weeks after exposure. IgM levels increase to maximum concentrations at about six weeks and then begin to decline.

- Borrelia IgG (immunoglobulin G) antibodies are not detectable until several weeks after exposure, increase to maximum levels at about four to six months, and may remain at high levels for several years.

Of note, *B. burgdorferi*, through gene recombination, can modify its surface antigens, creating different outer surface antigens, helping to avoid immune recognition leading to false negative test results.

The two most commonly used antibody-based tests are the enzyme-linked immunosorbent assay (ELISA) and the Western blot (immunoblot).

During the first four-to-six weeks of Lyme infection, these tests are often unreliable because most patients have not yet developed a sufficient antibody response for the test to detect. Even later in the illness, the two-tiered testing is highly insensitive missing roughly half of those who have Lyme disease.

In two-tiered Lyme disease testing the first tier is a screening test (i.e. ELISA), which would optimally identify anyone who might have the disease. Screening tests are typically designed to largely identify all infected individuals and are regarded as having high sensitivity. However, these tests often produce false positives. For this reason, this test is followed by a second confirmatory test (i.e. immunoblot) that is intended to make sure that only people with the disease are diagnosed. Tests that do this well have high specificity.

In Lyme disease, the second test (i.e. Western blot) is highly specific, assuming that antibodies have been produced as a result of the infection. So there are very few false positives. Unfortunately, the screening test for Lyme is highly insensitive and fails to accurately identify all patients who have Lyme disease. For this reason, the standard two-tiered Lyme test misses roughly 54% of infected patients (Stricker & Johnson 2010).

Serology is currently the most commonly used diagnostic method for Lyme disease. A positive serology test only suggests the patient has been exposed to the pathogen and is not diagnostic of an active infection. Utilizing an ELISA as a screening tool, followed, if positive, by a confirmatory Western blot, is not an adequate approach. The ELISA is not sensitive enough to serve as an adequate screen, and there are many patients with Lyme who test negative by ELISA yet have fully diagnostic Western blots. ELISA is the simplest, least expensive, easiest to perform, and most common Lyme test ordered. The typical Lyme test is based upon the detection of serum antibodies made in response to *Borrelia burgdorferi* exposure. It is a preferred test by laboratories, not because it is more accurate than other Lyme tests, but because can be easily automated. Therefore, many different patient samples can be performed by a single machine simultaneously. This allows for a faster turnover, less costs, and theoretically, standardized test results that are consistent from lab to lab. The ELISA test sounds simple and straightforward, but it has some major flaws. *Borrelia* species are some of the most polymorphic

bacteria known to exist. In other words, most *Borrelia* can significantly change their surface proteins enough during cell division as to evade our immune system, and may differ from laboratory strains enough to result in negative tests, even if antibodies are present! Tom Grier (microbiologist and also former Lyme patient) wrote many reviews on Lyme-related tests. He presents a very compelling analysis as to why ELISA tests are imprecise: "The ELISA test depends on the active, free antibodies to attach to the free antigens that have been embedded on the walls of the test tube. If the antibodies in the serum being tested are already attached to antigens, then the enzyme reaction cannot take place. If we think of antibodies as sort of keys that fit into locks, and that on the surface of the bacteria are specific locks we now call antigens, you can see that once a key is inserted into a lock, the key is no longer available to open any other locks. What makes this test so misleading is that many doctors accept high readings as an indication that the patient must really be sick. This logic is exactly backwards. If a patient is really infected with lots of bacteria, that means they have a lot of bacterial antigens floating around in the blood that are complexing free antibodies. So, as free antigen increases, free antibody decreases. Since the ELISA test detects only free antibody, a negative test might actually indicate a more serious infection. Many times, I have seen totally asymptotic patients with ELISA titers over 1000 be treated as though they were on death's doorstep simply because they had a high titer, while patients with borderline titers who are practically disabled are ignored, because a low titer is perceived as meaning less infected! These conclusions are erroneous and actually opposite to the truth, which is that a high titer means greater natural immunity."

The Western Blot is specific because it provides a detailed map of the different antibodies the immune system produces to the bacteria. The map separates the antibodies by the weight of their respective antigens (bacterial proteins) and is reported in units called kilodaltons or kDa. For example, a Western blot may report bands at 22-, 23-, 25-, 31-, 34-, 39-, and 41- kDa. Each of these bands represents an antibody response to a specific protein found on the spirochete. The 41-kDa band indicates an antibody to the 41-kDa flagella protein and is nonspecific with respect to the bacterial species. The 31-kDa band represents the OSPA protein and is specific for just a few species of Borrelia, as is the 34-kDa OSPB band, and 23-kDa OSPC band.

Western blots are reported by showing which bands are reactive. 41-kDa band appear the earliest in the course of the disease but can cross react with other spirochetes. The 18-kDa, 23 to 25-kDa (Osp C), 31-kDa (Osp A), 34-kDa (Osp B), 37-kDa, 39-kDa, 83-kDa and the 93-kDa bands are the species-specific ones, but appear later or may not appear at all. You should see at least the 41-kDa and one of the specific bands. The 55-kDa, 60-kDa, 66-kDa, and 73-kDa are nonspecific and nondiagnostic. Different brands of Borrelia immunoblot are readily available. The kit from Mikrogen detects antibodies against four immunopathogenic genospecies (*B. burgdorferi sensu stricto, B. garinii, B. afzelii, B. spielmanii and B. bavariensis*) on one single test strip:

- VlsE from different genospecies
- OspC from all genospecies
- p18 (Decorin binding protein A = DbpA) from all genospecies

The advantages of using this method, assuming the patient develops an antibody response, are high sensitivity and specificity, easy and clear interpretation due to easy to read bands, optimum

presentation without cross-reacting Borrelia proteins, immunodominant antigens of the four genospecies: *B. burgdorferi sensu stricto, B. garinii, B. afzelii, B. spielmanii and B. bavariensis*, separate detection of IgG and IgM antibodies, and safe evaluation due to strip specific controls (cut-off and conjugate control). Thus, in the case where the patient was able to develop antibodies, this test will most optimally reveal their presence. However, in the case where the patient did not produce antibodies, this test will remain negative despite the infection. In order to ensure the most specific bands are included in the immunoblot, ask the Laboratory to inform on the antigens included in the test and to provide the full report, not only the final result (positive/borderline/negative).

Special attention is granted to *B. miyamotoi,* which usually does not cross react with *B. burgdorferi* tests (Branda & Rosenberg 2013; Lee et al. 2014). *B. miyamotoi* might be detected by PCR or by EliSpot.

Lastly, a new serology test for Lyme disease, called SeraSpot, has been heavily marketed. It is very similar to the Western blot but lends itself well to automatization and, therefore, high-throughput thus enabling quicker assessment in the clinical laboratory. Seen that both approaches enable quantification, there is no real improvement in serology-based testing with SeraSpot when comparing to it Western blot, providing that the Western blot contains very specific antigens, such as the Mikrogen kit, used in several laboratories or the diagnostic from IgeneX.

A major problem with Lyme diagnostics is that no single blood test provides definitive results. Two of the most commonly used tests, ELISA and Western blot, both rely on the presence of serum antibodies produced against *B. burgdorferi*. Unfortunately, the results of these tests can be inaccurate, and analysis methods are not always consistent from laboratory to laboratory. The ELISA test can produce a false negative if performed too soon, and a Western blot should always be performed to confirm positive ELISA result. Both tests indirectly detect infection by reacting with antibodies in the blood serum; however, the presence of antibodies doesn't always mean an active infection is present, but it does indicate exposure to the infectious agent. Conversely, the absence of antibodies cannot definitively confirm the absence of infection.

As a final note, Lyme bacteria can attach themselves to proteins, thereby masking the proteins previously recognized by antibodies. They also have the capacity to enter cells, including cells of the nervous system and immune system. Once inside of a cell, they are no longer accessible for antibody binding. It is important to know that Borreliosis is an immunosuppressive disease, which may prevent antibody production. So while a Western blot may be very specific in detecting Lyme-specific antibodies, neither it nor an ELISA tests is useful once the body has ceased creating antibodies.

C6 ELISA TEST

C6 is a synthetic peptide (C6 Peptide) derived from the VIsE protein, which appears in early as well as late stage Lyme disease. The assay identifies the presence of antibodies against this synthetic peptide. However, its sensitivity is reported to be only 70-74%, thus lower than that of a complete Western blot inclusive of highly specific antigens (23, 31, 34, 39, 83-93 -kDa bands); therefore, it is prudent to perform a confirmatory Western blot. In light of this, it is more efficient and less costly to only perform a Western blot. Furthermore, Embers et al. (PLoS

One 2012) demonstrated that the C6 peptide antibody test is unable to detect persistent infection, even when other methods confirm bacterial presence in the tissues of infected monkeys. Thus, the C6 peptide antibody test was shown to be an unreliable diagnostic tool in treated and untreated monkeys, given that a positive result can become negative.

CD57 CELLS COUNT

In chronic Lyme Borreliosis, the CD57 count is both useful and important. CD57+/CD3- cells are a subset of natural killer (NK) cells. NK cells play a crucial function in innate immunity, recognizing and killing virus-infected cells and tumor cells. Dr. Stricker and collaborators (Stricker & Winger 2001; Stricker et al. 2002) reported that the number of CD57+/CD3- cells was decreased in chronic (not acute) Lyme disease. Although acute infections can be treated with antibiotics, failure to treat may result in a chronic, debilitating illness characterized by musculoskeletal and neurologic symptoms. Chronic Lyme disease may be difficult to treat, but also to diagnose (Aguero-Rosenfeld et al. 2005).

The number of CD57+/CD3- cells is decreased in chronic Lyme disease patients, particularly those with pronounced neurologic symptoms. Patients with low CD57 have significantly more co-infections and persistent immunologic defects than patients with higher counts. In patients that respond to antibiotic therapy, the number will come back to normal following treatment, but in patients with persistent Lyme disease, CD57 levels remain low. The assay is a three-color flow-cytometry-based assay. Whole blood is stained with antibodies directed against the CD3 and the CD57 antigens; the absolute number of CD57-positive/ CD3-negative lymphocytes (cells per μl of whole blood) is determined by flow-cytometry. Result indicates the absolute number of CD57+/CD3- cells (cells/μl). The normal range is 60-360 cells/μl. Untreated, chronic Lyme disease patients have values below 60.

Of note, low CD57 count was also evidenced in autistic children (Siniscalco et al. 2016). This might also point to the importance of multiple infections in autism-spectrum disorders.

LYMPHOCYTE TRANSFORMATION TESTS

The Lymphocyte Transformation Test (LTT) was originally developed in the 1960s for evaluating histocompatible class II HLA antigens. The method was then modified for class II antigen typing and also applied extensively to detecting type IV allergies to drugs, metabolites, infectious organisms and metals. LTT became a common test for detection of allergy to beryllium, nickel, gold, cobalt, chromium and palladium.

These tests are specific for actual antigens, not antibodies, and are considered more accurate in Lyme diagnosis, particularly with regards to an active ongoing infection

1. MELISA LTT

In 1994, Stejskal and colleagues published a modification of the LTT for detecting metal sensitivity – the MELISA test. The MELISA technology is now applied to diagnose active Lyme disease (Valentine-Thon et al. 2006).

A positive reaction in the MELISA test demonstrates current active infection with *Borrelia burgdorferi sensu lato*. In addition to the standard four recombinant antigens derived from *B. afzelii and B. garinii*, the test usually includes three additional antigens derived from *B. burgdorferi sensu stricto* (a recombinant outer surface protein OspC, a recombinant p41-internal fragment, and a full antigen lysate). MELISA is a lymphocyte transformation test, which detects not antibodies but cellular immunoreactivity characteristic of active infections of *Borrelia burgdorferi*. The test improves laboratory diagnosis by confirming active disease in patients with clinical symptoms of Lyme. This test is based on use of peripheral blood mononuclear cells, incubated together with controls, in a multi-well culture plate coated with recombinant Borrelia antigens at three dilutions for five days at 37 °C with 5% carbon dioxide atmosphere. If the lymphocytes have previously encountered the antigen, the cells will divide and this division is measured through the uptake of radioactive methyl-3H-thymidine. The LTT-MELISA can measure disease activity in those infected patients who have not mounted an adequate antibody response, however, it is advisable to perform Western blot in parallel given that some Western blot-positive patients were tested negative by LTT-MELISA (Puri et al. 2014).

2. ELISPOT AND LYMESPOT REVISED

The EliSpot (Enzyme-linked Immunosorbent Spot Assay) measures the number of activated T-lymphocytes in cell cultures based on their release of cytokines upon being challenged by a reactive antigen. Presently, only a few publications are available to date and their conclusions are divergent (Forsberg et al. 1995; Nordberg et al. 2012; Jin et al. 2013).

The EliSpot (Interferon γ-test) is a test to detect an infection with Borrelia and various co-pathogens on a cellular level.

While the existing EliSpot test is exclusively based on the production of interferon-γ, the new LymeSpot test also evaluates the production of the cytokine interleukin (IL)-2. If the ratio of interferon-γ IL-2 is reversed, a latent disease can be assumed.

More and more laboratories perform LTT tests for Borrelia and some co-infectants. But, as for Western blot, it is very important to know which antigens are used in the test. Ask for a list of the antigens used and check for their specificity and the list of species covered. Not all laboratories use multiple antigens.

PCR TESTS

Targeting DNA using polymerase chain reaction (PCR) may be useful since it is a direct measurement of the pathogen and not based on indirect serology.

PCR is a blood test that amplifies a key portion of DNA from the Lyme bacteria so that it can be detected. While PCR is highly accurate when the Lyme DNA is detected, it produces many false negatives. This is because the Lyme bacteria are sparse and may not be in the sample tested. Instead of identifying antibodies to the Borrelia bacterium, the PCR is a direct measurement of the organism itself. Unfortunately, PCR testing commonly produces false negatives because Lyme bacteria tend to only reside in the blood for short periods of time, preferring to dwell in tissues with reduced vascular circulation.

PCR is a specific and sensitive method for rapid and direct detection of *B. burgdorferi*. It has shown utility for detection of Borrelia DNA from skin biopsies of ECM lesions, as well as DNA from synovial and cerebrospinal fluid in late-stage of the disease. Borrelia DNA can also be detected from blood but PCR results should be correlated with clinical presentation of the patient. Due to the clinical sensitivity limitations of the PCR assay, a negative result does not preclude the presence of the organism or active Lyme disease. Also, a negative result does not rule-out Lyme disease, since inhibitory substances may be present in the specimen. Through proper design of degenerate PCR primers, multiple species can be detected in a single test or, conversely, primers can be designed to identify a specific species. PCR test results should be used as an aid in diagnosis and not as a stand-alone diagnostic. These results should be correlated with clinical presentation of the patient. Concurrent infections with multiple tick-borne pathogens, including *Bartonella, Ehrlichia chaffeensis/Anaplasma phagocytophilum* and *Babesia microti* have been reported, and consideration should be given to testing for other pathogens if clinically indicated.

Although PCR is an inherently sensitive and specific method, results are frequently negative in spite of the patient having an infection. This is typically a consequence of a lack of bacteria in the assayed sample. Blood may be negative for Lyme DNA because when Borrelia is present in the tissue in cyst-form, it rarely releases any genetic material into circulation. Also, individuals with Lyme disease frequently have periods of time when they are symptomatic and then asymptomatic, which is reflective of the bacteria's activities. The PCR test can come back positive or negative depending on the activity level of the infection. In later stages of the disease, it is more likely that bacteria will move to tissues thus testing blood samples will give negative results.

ANTIGEN DETECTION TESTS

Antigen detection tests look for a unique Lyme protein in fluid (e.g. blood, urine, synovial fluid). Sometimes people whose indirect tests are negative are positive on this test.

Recently, the Nanotrap® LA Test was received national notoriety when a 1 million USD grant was awarded by Bill and Melinda Gates Foundation. This test uses the Nanotrap® technology to directly measure Lyme antigens in urine using a Western blot format. The Nanotrap® test is designed to provide high sensitivity and accuracy, delivering confident results at the earliest stages of infection. The Nanotrap® LA test uses a direct approach identifying a Lyme bacterial antigen, which is present in the body and can be detected within days of initial infection. Conversely, the Nanotrap® LA Test will provide a negative result indicating no presence of the antigen if the infection has been eliminated with effective treatment.

ENERGETIC TESTS

There is no one "gold standard" test for all Lyme disease infections that is 100% accurate. For this reason, many Lyme-literate doctors diagnose Borrelia and co-infections based upon symptoms, lab tests and unconventional, but sometimes more sophisticated types of alternative testing, such as energetic testing.

Electrodermal screening devices (such as the ZYTO or ASYRA), for instance, use a software program and the body's galvanic skin response to detect energetic imbalances in the body. They can also be used to detect the energetic frequencies of a wide variety of pathogenic microbes, and therefore, the presence of those microbes. A software program connected to a hand cradle or other device scans the body for infections and other imbalances and then displays a report of the different pathogenic organisms that are suspected to be in the body, and at what levels. Many Lyme-literate practitioners, such as Lee Cowden, consider the ZYTO to be over 90% accurate. Other devices may be more or less accurate.

Applied Kinesiology, or muscle strength testing methods, such as Autonomic Response Testing (ART), which was developed by Lyme-literate physician Dietrich Klinghardt, are another way to test the body for infections using the energy of the human body and the autonomic nervous system. Applied Kinesiology can be very useful for helping to establish a diagnosis.

For ART and some other common muscle testing methods, the practitioner applies strength to one of the patient's muscles (usually the arm), while holding a substance (in this case the energetic signature of, or physical substance of a pathogen) against the patient's body. He or she will then ask the patient to resist. The autonomic nervous system will respond to that substance or pathogen by creating either a strong or weak muscle response in the arm of the person being tested, thereby indicating to the practitioner whether the pathogen is in the body. However, accurate results depend largely upon the skill and experience of the practitioner.

TESTING FOR CO-INFECTIONS

In Lyme disease concurrent infections frequently occur. The clinical and pathological impact of co-infections were first recognized in the 1990s (Mitchell and al. 1996). Their pathological synergism can exacerbate Lyme disease or induce similar disease manifestations. Co-infecting agents can be transmitted together with *Borrelia burgdorferi* by tick bite resulting in multiple infections but a fraction of co-infections occur independently of tick bite. Infections caused by these pathogens in patients not infected by *Borrelia burgdorferi* can result in clinical symptoms similar to those occurring in Lyme disease. This applies particularly to infections caused by *Bartonella henselae*, *Yersinia enterocolitica*, and *Mycoplasma pneumoniae*. *Chlamydia trachomatis* primarily causes polyarthritis. *Chlamydophila pneumoniae* not only causes arthritis but also affects the nervous system and the heart, which renders the differential diagnosis difficult. The diagnosis is even more complex when co-infections occur in association with Lyme disease (Berghoff 2012).

Concerning their testing options, they are quite similar to the tests for Lyme borreliosis. PCR, ELISA and/or immunofluorescence diagnostics are available for most of them but they do not cover all species. Also, they all need to be combined with clinical presentation.

-Babesia – FISH (fluorescence in situ hybridization – most specific and most sensitive), Giemsa smear, Immunofluorescence (IFA), Serology, PCR.

-Bartonella –For Bartonella there is only serology testing for *B. henselae* and *B. quintana* and there is no Western blot available. Bartonella is very difficult to uncover in that multiple species are known (over 30) and frequently organized in biofilms (containing cells and extracellular polymeric substance, thus creating a matrix that provides a physical barrier for testing and

treatment). Among available diagnostics, there are also IFA (quite unreliable), PCR (need for multiple sets and usually fail in the case of biofilms), blood smear, rarely: culture + PCR tests. As an indirect option, vascular endothelial growth factor (VEGF) testing is often useful in that Bartonellosis is accompanied by a stimulation of blood vessel formation, inducing granulomas in various regions of the skin (Kempf et al. 2001). VEGF levels will rise in presence of Bartonella infection but only if mold infections are absent. If present, characteristic Bartonella skin rash is of great diagnostic value.

-**Brucella** – BrucellaCAPT, agglutinating antibodies, PCR.

-**Ehrlichia / Anaplasma** – ELISA, EliSpot, PCR, Giemsa smear.

-**Rickettsia** – PCRs, serology/IFA testing.

-**Coxiella** – PCRs, IFA testing

-**Tularemia (Francisella tularensis)** – antigen detection assays, ELISA, PCR, culture, direct fluorescent antibody.

-**Leptospira -** serology, immunochromatography.

-**Leishmania** – serology.

-**Mycoplasma -** serology (ELISA), PCRs (on blood, sputum, swaps), bacterial culture

-**Chlamydias** –Western blot, ELISA, PCR, EliSpot.

-**Yersinia -** Western blot (*Y. enterocolitica* and *Y. pseudopneumoniae*), EliSpot, antigen testing in stool (*Y. enterocolitica* only).

Of note, an immune-mediated response to Yersinia antigens may play an important role in the pathogenesis of chronic undifferentiated arthritis and in chronic inflammation (van der Heijden et al., 1997; Saebo & Lassen 1994).

-**Epstein-Barr Virus (EBV) -** Western blot, PCR, EliSpot, serology, antigen test.

-**Cytomegalovirus (CMV)** – Western blot, PCR, EliSpot, serology, antigen test.

-**Coxackie virus** – PCRs, antigen test, serology.

-**Herpesviruses** – serology, PCRs.

The list of possible co-infectants is not exhaustive. New pathogens are frequently evidenced, as well as opportunistic infections. Also, new investigational testing is in development and hopefully will complete and improve the existing testing offered.

INTEGRATIVE APPROACH FOR LATE / PERSISTENT / CHRONIC TICK-BORNE INFECTIONS

In order to offer better management of patients with late/chronic and/or persistent infections that are very difficult to uncover, R.E.D. Laboratories (www.redlabs.com) focuses on an integrative approach, inclusive of both direct pathogen detection as well as indirect supportive tests. The overall high failure rate of TBI-related testing, especially in late / persistent / chronic

patients, underscores the necessity to focus on the patients' self-reported symptoms and consider and fully investigate any potential dysregulations and disabilities resulting from these TBI.

Chronic TBDs can mimic every disease process including chronic fatigue syndrome (myalgic encephalomyelitis), fibromyalgia, autoimmune conditions, including sero-negative rheumatoid arthritis and MS, psychiatric conditions, including depression and anxiety, and significant memory and concentration problems that mimic early dementia. It is for this reason it was called the "Great Imitator" by Dr. Richard Horowitz, in his book, "Why Can't I Get Better".

Persistent TBIs have been reported for many autistic patients (Bransfield et al. 2008; Kuhn et al. 2012; Kuhn & Bransfield 2014). Indeed, many specialized physicians focus on TBDs when assessing autism-spectrum disorders.

If an individual has any chronic health condition, ranging from arthritis to chronic fatigue syndrome to fibromyalgia, it is important to rule out or diagnose Lyme disease. It is apparent that many cases of fibromyalgia and chronic fatigue syndrome are actually Lyme disease in disguise (Nicolson & Nicolson 1998).

Chronic Lyme sufferers also frequently house "co-infections" such as Mycoplasma, Chlamydias, Ehrlichia, Bartonella and Babesia. These are different types of "bugs" that enjoy the company of *B. burgdorferi*.

Patients with Lyme and TBDs may present primarily with gastrointestinal (GI) manifestations. These patients may have complex or persistent GI symptoms involving the upper, mid, or lower GI tract. The number of patients presenting with such symptoms is probably reaching epidemic proportions (Dr. Rahbar, ILADS Conference Augsburg 2015). Testing for gastrointestinal problems need to be included.

Accordingly, the **initial integrative panel** focuses on:

- *Borrelia serology IgG & IgM (immunoblot)*

This is an immunoassay (immunoblot) for the detection of IgG and IgM antibodies against *Borrelia burgdorferi* in human serum, plasma or CSF. It detects antibodies against four immunopathogenic genospecies (*B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. spielmanii* and *B. bavariensis*). Precisely, it enables detection of p100, VisE, p58, p41, p39, OspA (without distinction of genospecies), OspC from all genospecies and p18 from all genospecies. The goal of the test is not to distinguish genospecies but to offer the largest coverage in terms of Lyme disease detection.

- *Chlamydia serology IgG & IgA/M (immunoblot)*

This is an immunoblot for the detection of IgG and IgA antibodies against *Chlamydia trachomatis*, *Chlamydophila pneumoniae* and *Chlamydophila psittaci*. Of note, ticks do not carry Chlamydia but Chlamydias are reactivated with tick-borne infections.

- *Yersinia serology IgG & IgA/M (immunoblot)*

An immunoblot for the detection of IgG and IgA antibodies against all pathogenic Yersinia by means of Yersinia outer proteins (YOPs). Serological differentiation of *Y. enterocolitica* and *Y.*

pseudotuberculosis infections is possible for the first time with the use of new species-specific Yersinia antigens (PsaA, MyfA).

- *Babesia FISH Test*: Immunofulorescence in situ detection of Babesia infection

- *PCR testing Mycoplasma infections*

Ticks have been found to carry Mycoplasmas. These infections are exacerbating, particularly in chronic patients, and especially those with autoimmune manifestations. *Mycoplasma spp.* cause B cells to be overstimulated, promoting autoimmune and Rheumatoid Disease. Mycoplasma increase production of IL-1beta & IL-6.

- *An activity test (LTT-MELISA or ELISPOT LTT)*

- *CD57 absolute cell count*

$CD57^+/CD3^-$ cells are a subset of NK cells. The absolute number of $CD57^+/CD3^-$ cells is low in patients suffering from chronic Lyme disease. Patients with very low CD57 have significantly more co-infections and persistent immunologic defects than patients with higher counts.

- *PGE2 levels*

PGE2 is a compound derived from membrane phospholipids, is also a key mediator of immunopathology in chronic infections and cancer. PGE2 enhances its own production but suppresses acute inflammatory mediators, resulting in its predominance at late/chronic stages of immunity. PGE2 selectively suppresses effector functions of macrophages and neutrophils and the Th1-, CTL-, and NK cell-mediated type 1 immunity, but it promotes Th2, Th17, and regulatory T cell responses. PGE2 is observed as significantly upregulated in chronic TBD patients (Professor De Meirleir, ILADS workshop Antwerp 23/4/2016).

-*IL-8*

When Borrelia migrates, a multisystemic inflammation is initiated. Chemotaxis is exerted by IL-1 and TNF alpha. This migration induces upregulation of cytokines, e.g. IL-8. IL-8 is observed as very significantly upregulated in chronic TBD patients (Professor De Meirleir, ILADS workshop Antwerp 23/4/2016).

- *sCD14*

CD14 is expressed in monocytes/macrophages and plays a critical role in the recognition of bacterial cell wall components (LPS). The extracellular part of CD14 can be cleaved and released in the plasma, where it will inactivate circulating LPS. Serum soluble CD14 levels are significantly elevated in patients with inflammatory bowel disease and Crohn's disease, but also in patients suffering from Brucellosis or Lyme disease. Patients with early or untreated late Lyme disease had significantly higher levels of sCD14 than did healthy controls (Lin et al. 2000).

- *VEGF*

VEGF plays a significant role in pathological conditions that are associated to autoimmune diseases such as in systemic lupus erythematosus (SLE), rheumatoid arthritis (RA), and

multiple sclerosis (MS). Abnormally high levels of VEGF in a mold-free environment would suggest Bartonella infection (Kempf et al. 2001).

- *CD38*

CD38, which has an important role in dendritic cells (DC) chemotaxis and migration to lymph nodes, was strongly up-regulated by LPS but practically not at all by *Borrelia garinii* (mostly inducing neuroborreliosis). This finding was confirmed with quantitative RT-PCR and with flow cytometry at the protein level. In addition, RT-PCR showed that CCR7 (shown to stimulate dendritic cell maturation) expression was 11-fold greater in LPS-stimulated than in *Borrelia garinii*-stimulated cells. These findings suggest that *Borrelia garinii* may affect crucial DC functions by blocking the up-regulation of important molecules in DC migration to lymph nodes, thus affecting further immune responses in Lyme borreliosis infection (Hartiala et al. 2007). Furthermore, to determine whether the inability of *B. garinii* to induce CD38 expression would be related to other *B. burgdorferi* genospecies as well, Hartiala and colleagues (J Immunol. 2010) stimulated DC with *B. burgdorferi sensu stricto* and *B. afzelii*. Neither of these Borrelia genospecies induced CD38 upregulation.

-Tests for gastrointestinal problems

1. MSA stool test

R.E.D. Labs scientists have developed and validated a new procedure to analyze bacterial populations in a stool sample: the MSA assay is a new molecular technique involving sequencing of specific regions of bacterial DNA (metagenomics). This test can be performed on dead organisms (exposure to oxygen and freezing are not a problem). Until recently, research into microbiota composition relied almost exclusively on culture, while (i) 40 to 80% of gut bacteria cannot be cultured, (ii) identification of colonies can be difficult, (iii) bacteria must be alive: studies of anaerobes very difficult, major loss during collection and processing of samples, (iv) culture approach may address only a small fraction of all bacterial species (10%?). In contrast, identification of each bacteria by comparing sequence with public databases is extremely precise, not subjective, and high-throughput technology allows identification of tens or even hundreds of organisms in a single sample.

2. Calprotectin in stool

Calprotectin is a neutrophil cytosolic protein with antimicrobial properties, which is present at increased concentration in stool during bowel inflammation.

3. D-lactate in serum

D-lactate is a product of bacterial metabolism, it is neither produced nor metabolized by mammalian cells. Typically, elevated D-lactate levels are due to bacterial infection or short bowel syndrome in humans. Due to slow metabolism and excretion, high D-lactate can cause acidosis and encephalopathy.

Given that persistent infections promote significant damage to the body, which need to be repaired, the following tests should also be considered for a **broader integrative approach** focusing not only on the identification of causal infection but also on global damages:

-Testing for Tularemia (search for antibodies against *Francisella tularensis*)

-Testing for Brucellosis (BrucellaCapt assay is very sensitive and specific)

-Testing for Coxsiella, Anaplasma, Rickettsia, Bartonella (PCRs and/or serology)

-Testing for EBV (preferably by immunoblot and PCR)

-Testing for Herpesvirus infections (HHV6)

-Testing for MOLD infections (especially Candida)

- Testing for inflammation (cytokine levels (see Grab et al. 2007) and oxidative stress) : Inflammation creates free radicals and oxidative stress which damages cell membranes, mitochondria, and nerve cells, creating the "sickness syndrome" (fatigue, pain, cognitive dysfunction, mood disorders).

-Testing for complement (C3a and C4a): C4a appears to be a valuable immunologic marker in patients with persistent symptoms of Lyme disease (Stricker et al. 2009).

- Testing for heavy metals poisoning and nutritional deficiencies (minerals, vitamins, etc).

- Testing for toxic metabolites (Ammonia, Kynurenic and Quinolinic acids).

- Testing for autoantibodies for connective tissue diseases (ANA/ENA immunoblots).

- Testing for leaky gut (Zonulin in stool, antibodies against intestinal bacteria in serum)

IgA/IgM against intestinal bacteria: an antibody screening assay for antibodies (IgA and IgM) directed against antigens from intestinal pathogens. IgA are secreted from intestinal cells, IgM are produced by immune cells in the blood. In healthy individuals pathogenic bacteria are only found in low quantities in the gut, and antibody titers in the blood are very low. However, in the case of bacterial overgrowth (dysbiosis), large quantities of IgA are produced and some IgA will be found in the bloodstream. In case of leaky gut, bacterial proteins may make their way to the bloodstream, and specific IgM will be produced. Therefore, a high titer of IgM for intestinal bacteria is an indicator of increased intestinal permeability.

ZONULIN ELISA tests in stool: Zonulin modulates the permeability of tight junctions between cells of the wall of the digestive tract. As the Zonulin level rises, the seal between the intestinal cells diminishes, opening up spaces between cells that allow numerous macromolecules to pass right through. This is called "leaky gut".

- Testing for intestinal inflammation (sIgA, EDN/EPX, beta-defensin-2)

sIgA ELISA tests in stool samples: sIgA key function is to bind to invading microorganisms and toxins and entrap them in the mucus layer or within the epithelial cells, so inhibiting microbial motility, agglutinating the organisms and neutralizing their exotoxins and then assist in their harmless elimination from the body in the fecal flow. The concentration of sIgA gives us information about the intestinal immune defense: a lack of sIgA indicates a diminished activity of the intestinal immune system, an increased level of sIgA shows intestinal inflammation.

EPX/EDN ELISA tests in stool samples: The accumulation of EDN in the intestine is associated with inflammation and tissue damage. Measuring of EDN in stool can serve as an objective parameter for a current clinical or sub-clinical chronic inflammation located in the gastrointestinal area. Fecal EDN is considered the best of the cytotoxic granule proteins for

assessment of gut inflammation, as it most accurately reflects clinical, endoscopic, and histologic scores of disease activity and mucosal damage. Elevated levels of fecal EDN are linked to multiple inflammatory conditions, like food allergy/sensitivity, pathogenic infections (C. difficile and H. Pylori), IBS, and Eosinophilic Gastrointestinal Disorders.

ß-defensin-2 ELISA tests in stool samples: The ß-defensins are an integral part of the congenital immune system and contribute through their antimicrobial effect to the barrier function of intestinal epithelial cells. Defensins exert a variable degree of antimicrobial activity against bacteria, fungi, and some enveloped viruses. The expression of ß-defensins is induced by pro-inflammatory cytokines and also through microorganisms (e.g. E. coli, H. pylori or P. aeruginosa) and by probiotic microorganisms. A ß-defensin-2 deficiency can, for example, be observed in the intestinal mucous of patients with Crohn's disease. The defense system of the mucous membrane is therefore restricted and allows an increased invasion of bacteria, which could possibly lead to a typical infection in Crohn's disease patients. Recent results imply that ß-defensin-2 is overexpressed in active intestinal inflammation, especially in ulcerative colitis.

These are not the only tests that might be useful to consider for an integrative approach. Based on clinical examination, the physician will select other ones that might apply. A wide range of potentially useful considerations are listed in the books from Dr Horowitz. Specialized medical and scientific conferences are also a great source of valuable information.

FUTURE POSSIBILITIES FOR DIAGNOSTIC TESTS

Because treatment is more effective in the early stages of Lyme disease, there is a great need to develop simple, fast and accurate diagnostics to determine whether people have been infected. According to the National Institute of Allergy and Infectious Diseases (NIAID), "NIAID-supported scientists have identified genome sequences of multiple strains of *B. burgdorferi*. Greater advances in diagnostics are anticipated as genetic information is combined with advances in microarray technology, imaging, and proteomics. These growing fields of science are expected to lead to improved diagnostic tools as well as provide new insights on the pathogenesis of Lyme disease. Examples of future tools being developed with NIAID support include use of metabolomics to characterize new biomarkers of infection, next generation T-cell based measurements, and novel antigens for improved measurement of effective treatment."

TickPlex, a new nanotechnology-based method from a Finnish group, is currently being elaborated as a multiple infection diagnostic tool.

Some recent developments, that are almost ready to be included in the testing panels, are the HybriSpot technique (from Master Diagnostica) with their TICK-BORNE BACTERIA FLOW CHIP that allows the simultaneous detection (by PCR and blot) of 7 tick-borne bacteria genera: Rickettsia, Erhlichia, Anaplasma, Francisella, Bartonella, Borrelia and Coxiella. This technique is compatible with human, animal, and tick samples. While extremely promising, the technique still does not offer good reproducibility, showing lot-to-lot differences and flaws when multiple bacterial types are mixed together.

Finally, with increasing attention that is granted today to TBI, existing tests (like PCRs) are also constantly improved and are leading to better results.

Sharing the information and spreading the knowledge is the very first step towards improved testing.

FINAL REMARKS

Summarizing TBI-related testing is a huge enterprise. Many articles, books, websites, blogs, etc are available today (see below a short selection), and multiple conferences are held each year. The goal of the present contribution was to put some of this information together as a convenient resource for patients in order to promote an understanding of the usefulness but also the limitations of available tests and the underlying reasons of failure. The final aim was also to further emphasize the need for a global, integrative approach for a better management of TBIs.

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