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# Promising alternatives for one-tier testing of Lyme borreliosis

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ABSTRACT

A main focus of human health studies is the early detection of infectious diseases to enable more rapid treatment and prevent disease transmission. Diagnosis of Lyme borreliosis has been always challenging because of the lack of specific, but simple assay formats. Two-tiered testing has been recommended by US Centers for Disease Control and Prevention to provide more specific results for diagnosis of Lyme disease. However, such a technique is time consuming and is not well suited for early stage detection. Therefore, many tests were proposed as alternatives to overcome these drawbacks. Simple assays, which are mainly performed in one-tier manner, could be conducted with better performance than the two-tiered testing. Proposed assays utilize both newly identified antigens and new platforms to improve detection performance. These assays can be classified into those based on employing a single antigen and assays based on using multiple antigens. In addition to assays to this type of assays, immunoassays on borreliosis-related biomarkers are available. We report here the most recent assays developed over the last 10 years, for detection of Lyme borreliosis in body fluids.

## 1. Introduction

Serologic tests on Lyme borreliosis are mainly immunoassays for detection of serum antibodies directed against *Borrelia burgdorferi* (*B. burgdorferi*). These antibodies were produced by the immune system as a relative fast response on *Borrelia* infection. Serologic diagnosis, as officially recommended, should be performed using a two-tiered testing in order to avoid false positive results caused by the cross-reactions with other spirochete infections [1]. A first test should employ a sensitive enzyme-linked immunoassay (ELISA) for screening followed by a western blot for confirmation. Two-tiered testing is highly specific (95%–100%) and highly sensitive (> 90%) in late stages of Lyme disease [2]. As a drawback, it has low sensitivity in the early acute phase of the disease (due to the lack phase in immunoresponse of the body). Also, the entire assay is time consuming and labor intensive. Numerous attempts were undertaken to develop alternatives to the two-tiered testing, which should be rapid as well as more sensitive in the early stage of the Lyme disease. Alternatives could be established in one-tier manner, which are principally based on highly specific recombinant antigens or synthetic peptides for antibodies detection. Such 'tailor made' antigens allow a highly specific detection of the disease even in early stages in combination with low cross-reactions [3]. Moreover, alternative assay platforms could be developed.

## 2. Lyme borreliosis

Lyme borreliosis (LB) is one of the most common infectious diseases in North America and Europe; it is transmitted to human by specific *Ixodes* spp. ticks. The transmission takes place during the tick feeding through injection of the tick saliva. 36 h as feeding period is usually needed for infection [4]. In 1982, Burgdorfer and colleagues successfully isolated and detected spirochete bacterial species from adult *Ixodes dammini*. These bacteria showed a specific binding to immunoglobulins of patients who were convalescing from Lyme disease. These bacteria were found in the *Ixodes* midgut, which were collected in an endemic Lyme disease breakout (Shelter Island, New York). The isolated bacteria were named later as *B. burgdorferi* and considered the cause of the LB [5]. Total of 12 species belong to the *B. burgdorferi*-group; at least five genospecies are pathogenic in human. *Borrelia afzelii* (*B. afzelii*) and *Borrelia garinii* (*B. garinii*) are most abundant in Europe; *B. burgdorferi* sensu stricto is the main reason for LB in North America. In addition to *Borrelia spielmanii*, *and Borrelia bavariensis* [6], *B. burgdorferi* is phylum spirochetes bacteria with helically shaped thin long cells, outer cell membrane, and flagella [7]. The outer cell membrane surrounds the protoplasmic cylinder, the inner cell membrane and the peptidoglycan [8]. The flagella are located in the space between the outer cell and the protoplasmic cylinder parallel to the long axis of the organism with average diameter of 18 nm. The flagella give the *Borrelia* its motility even in viscous medium [9].

Three clinical stages could appear and may overlap in patients with LB [9]: i) erythema migrans (EM), a rash increasing centrifugally, which appears after days up to few weeks after the tick bite [10], ii) spreading of *Borrelia* into bloodstream, causing clinical signs of an early

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dissemination. Many organs may be affected at this stage including nervous system, joints, and the heart; this stage appears after few weeks up to months after tick bite [11]. And iii), if LB is not treated, the third stage is characterized by chronic organ infections, persistent joint inflammation and joint swelling. In addition, direct involvement of the eye could take place at this stage [12]. Further on, other pathognomonic symptoms could express the presence of *Borrelia* infection: lymphadenosis cutis benigna, meningopolyneuritis Bannwarth, and acrodermatitis chronica atrophicans. A test on LB is recommended in any case.

## 3. Laboratory diagnosis of Lyme borreliosis

Laboratory diagnosis is required when the patient shows the typical borreliosis symptoms, EM with fever following a tick bite in an endemic Lyme disease area. The diagnosis is typically based on a direct or an indirect detection of the infecting agent. *Borrelia* detection can be performed by four different techniques: i) the direct detection of intact *Borrelia* spirochetes using microscopy [13], ii) cultivation of *Borrelia* followed by microscopic detection of spirochetes [14], iii) detection of *Borrelia* specific deoxyribonucleic acid (DNA) sequences by polymerase chain reaction (PCR) [15], and, as indirect method, iv) immunocaturing of *Borrelia* specific immunoglobulin G (IgG) or immunoglobulin M (IgM) antibodies using serological immunoassays like ELISA [16]. In case of LB suspicion, direct microscopic detection of *Borrelia* species in clinical samples should come as a first approach; however, this method has some disadvantages because of limited sensitivity arose from the sparseness of bacteria in clinical samples, in addition to the subjective interpretation of the microscopy results. As an alternative approach, cultivation of the causative agent followed by the microscopic detection of the spirochetes could be performed. *Borrelia* species can be cultivated in Barboure-Stoennere-Kelly (BSK) medium [17], a liquid medium of CMRL-1066 with bovine serum albumin fraction V, rabbit serum and *N*-acetylglucosamine supported by other ingredients; the cultivation takes place at 30° to 34 °C up to 12 weeks. The presence of *Borrelia* spirochetes is then detected out of the culture supernatant by dark-field microscopy or by fluorescence microscopy after specific staining. Cultivation of bacteria is a time-intensive process; patients have to wait up to 12 weeks before being declared positive or negative. Other limitations should be also considered; for example, cultivation of bacteria out of human samples other than skin and blood is limited due to the scarcity of those, and slow growth of *Borellia*. The low number of *Borrelia* in clinical specimen can be overcome by detection of PCR-amplified *Borrelia*-DNA in early Lyme disease in skin, cerebrospinal fluid (CSF), and synovial fluid. As disadvantages, diagnosis of blood and CSF samples using PCR has shown to be uncertain because of reduced sensitivity the test showed in this special case (usually, PCR is very sensitive). Also PCR based methods do not differ between dead and living organisms, as well as accidental contamination with target DNA. These scenarios would lead to false positive results even after clinical treatment [18].

Due to their high specificity, the three techniques mentioned above are still in use. Nevertheless, more simple and specific assays are required to overcome these draw backs. Immunoassays for serological diagnosis as an indirect detection method could be a promising alternative. Immunoassays as a ligand binding technique have a wide range of applications, ranging from small molecules [19] up to intact cells [20]. The performance of these assays depends on the specificity of the capturing molecule (antigen for indirect detection of borreliosis) and its binding strength (as tighter the binding as higher the sensitivity).

## 4. Serological diagnosis of borreliosis

Serological diagnosis on borreliosis is the only test, which has been approved by the US Food and Drug Administration (FDA) for borreliosis diagnosis. Serological diagnosis comes mainly as a confirmatory test of the disease when a clinical diagnosis suspects a LB from the symptoms. The test is based on detection of the immune response to the *B. burgdorferi* infection, particularly the detection of IgG and IgM antibodies. The human body needs 2 to 6 weeks after the vector bite to produce antibodies against *Borrellia*. During this stage of infection, serological testing is mainly positive with one tier test; however, it might yield false positive results because of other spirochete infections. Therefore, two-tiered serological testing [21] has been recommended by US Centers for Disease Control and Prevention (CDC) as well as European guidelines [1]. Two-tiered testing starts with a sensitive ELISA on IgG or IgM, followed by immunoblotting of the positive or intermediate samples acquired from the first test. The two-tiered testing is recommended also in particular when the patients are not presenting with EM or the infection took place in area, where LB is not an endemic disease.

Using whole-cell lysates for ELISA might give cross-reactivity with other pathogenic bacteria that led to false positive results. Because of this, it I recommended that the used ELISA should be at least a second-generation test, i.e. based on a recombinant antigen with low cross reactivity [22]. In addition, the used antigen should cover the wide antigenic variety of several relevant *Borrelia* subspecies. In addition, it should avoid cross reaction with the other non-pathogenic *Borrelia* strains and other spirochetes. Also, the antigen should show high binding to IgG and IgM and should distinguish between fresh, past or healed infection. Second generation ELISA with high-quality recombinant antigens are suitable for the early detection of the disease [23]. Safe one-tire detection seems to be possible with this strategy. As a further advantage, validation of ELISA's is possible by standard routines, which are much more difficult for immunoblots as essential part of a two-tire strategy [24].

Hence, in the presented work, we report about different approaches developed either as alternative to the two-tired testing or as alternative to the second immunoblotting test for detection of LB. Thus, we report here about the most recent developments for detection of LB in body fluids based on a one-tier approach.

## 5. One-antigen based assays for detection of Lyme borreliosis

For this assays-type, only one antigen (a protein that is ether produced by gene-modified organisms or a synthetic peptide) is employed to improve both sensitivity and specificity of the test. Favorably, the amino acid epitope of the antigen is used as recognition element of serum antibodies, since the whole protein antigen might possess cross-reactivity. Yet, both antigen approaches have shown the potential to propose a one tier-method that could be used as alternative to conventional two-tier testing. Bacon et al. developed a one-tier testing which showed at least equal performance as the two-tiered testing on infections caused by *B. burgdorferi* [25]. The assay exploits the high specificity of a recombinant variable lipoprotein surface-exposed protein (VlsE) antigen (named rVlsE1), a 10-mer conserved portion (named pepC10) of the outer surface protein C (OspC), or the C6 peptide region of VlsE antigen [26] in order to detect IgG antibodies and to avoid false positive results. The assay is based on a conventional ELISA platform, where the antigen is immobilized inside the wells of a microtiter plate and samples are incubated subsequently, enabling a specific recognition of infection. Finally, signal can be quantified after an enzyme-catalyzed (mainly horseradish peroxidase) color reaction. Results revealed that the assay is simpler and more readily standardized than two-tiered analysis. Besides this, the specificity for an assay based on recombinant VlsE antigen and synthetic peptide C6 was 99%. Similarly, via ELISA on microtiter plate, a synthetic conserved 20-aminoacid peptide epitope, OspC1 [27], decorin binding protein A (DbpA) and DbpB [28], and oligopeptide permease A2 (OppA2) [29] could be utilized for the detection of *Borrelia* antibodies in early stages, proposing a one-tier test.

It is also recommended that immunoblots should be us as second assay to confirm the presence of *Borrelia* antibodies in order to exclude potential false-positive findings. Yet, bringing together the immunoblot

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