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Recombinant protein immunoblots for differential diagnosis of tick-borne relapsing fever and Lyme disease

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ABSTRACT

Lyme disease (LD) is caused by a group of tick-borne bacteria of the genus *Borrelia* termed Lyme disease *Borreliae* (LDB). The detection of serum antibodies to specific LDB antigens is widely used to support diagnosis of LD. Recent findings highlight a need for serological tests that can differentiate LD from tick-borne relapsing fever (TBRF) caused by a separate group of *Borrelia* species termed relapsing fever *Borreliae*. This is because LD and TBRF share some clinical symptoms and can occur in overlapping locations. The development of serological tests for TBRF is at an early stage compared with LD. This article reviews the application of line immunoblots (IBs), where recombinant proteins applied as lines on nitrocellulose membrane strips are used to detect antibodies in patient sera, for the diagnosis and differentiation of LD and TBRF.

Key words *Borrelia burgdorferi*; borreliosis; Lyme disease; relapsing fever Borreliae; tick-borne diseases

INTRODUCTION

The detection of pathogen-specific antigens as well as the antibodies produced against them, are widely used for diagnosing many infectious diseases[1]. Tests that detect pathogen-specific nucleic acid sequences, e.g., PCR and fluorescence *in situ* hybridization, are alternative recent approaches[2]. Detection of antibodies in patient sera remains important for diagnosing particular infectious diseases, e.g., syphilis[3] and viral hepatitis[4]. Blood-feeding tick vectors transmit several viral, bacterial and protozoan pathogens that cause infections with relatively non-pathognomonic signs and symptoms in people, domestic animals and livestock[5–10]. Immunoassays that detect serum antibodies have a key role in diagnosing many tick-borne diseases[5–23].

The aetiological agent of Lyme disease (LD) was first identified in ticks in 1982[24] and in human LD patients in 1983[25] in USA as a spirochete bacterium that was subsequently named *Borrelia burgdorferi*. Many species of the genus *Borrelia*, classified as belonging to the *Borrelia burgdorferi sensu lato* complex, are now known to cause LD; however, the term Lyme disease *Borreliae* (LDB) is increasingly used because it includes the *Borrelia burgdorferi sensu lato* complex plus the other genetically-related *Borrelia* species identified in ticks and animals that may have the potential to cause LD in humans. LD is mainly prevalent in the temperate climate zone[26–29]. Some clinical manifestations of LD are shared with tick-borne relapsing fever (TBRF) caused by a different group of *Borrelia* species termed relapsing fever *Borreliae* (RFB)[6, 26, 30–34]. Recent observations show that a subset of patients who present with symptoms of LD may in fact be infected with RFB and not LDB[31–33, 35]. These findings expand the possible manifestations of TBRF and underscore the need for reliable laboratory testing that can identify and differentiate LD from TBRF. LDB and RFB, however, also have some distinct microbiological features[36]. The detection of serum antibodies to specific antigens of LDB is widely used to aid the diagnosis of LD in endemic countries[11, 23, 26, 37, 38]. LD and TBRF have an overlapping geographical distribution[5, 6, 26, 30–35, 39]. Not testing for TBRF in LD-endemic areas can therefore result in misdiagnosis or no diagnosis for patients. Hence, the use of serological tests specific for LD and TBRF can support a differential, and more complete clinical diagnosis. This article reviews recent applications of recombinant proteins from LDB and RFB in multiplex line immunoblot (IB) tests that may help diagnose and distinguish LD and TBRF.

Lyme disease

Background

LD is endemic in North America where it is mainly caused by the LDB species *Borrelia burgdorferi sensu stricto* (*Bbss*)[26], and sometimes *B. mayonii*[40–41]. Other LDB species detected in ticks and animals, including *B. americana*, *B. andersonii*, *B. californiensis*, and *B. carolinensis*, may potentially cause LD in the US[42–43]. The major vectors that transmit LDB in the US are the blacklegged or deer ticks, *Ixodes scapularis* in northeastern, mid-Atlantic and midwestern US states, and *I. pacificus* in the West coast[26]. LDB species established to cause LD in Europe

are *B. afzelii*, *B. garinii*, *B. spielmanii, B. baveriensis* and *Bbss*, while other LDB species may have the potential to do so[27, 38, 44]. Infections with *B. afzelii* and *B. garinii* have been reported in Mexico[45] and there is evidence to suggest that such infections may extend to contiguous US states[33, 35]. *Borrelia garinii*, *B. afzelii*, *B. baveriensis*, and potentially other LDB species, are responsible for LD in Asia[38, 46–47]. The well-established tick vectors of LD are *I. ricinus* and *I. persulcatus* in Europe, and *I. persulcatus* in Asia[38]. The clinical manifestations of LD in the US and Europe show variations that have been related to differences in infecting LDB species[37, 48]. Some of the symptoms of LD are shared with common illnesses as well as other tick-borne diseases, including TBRF[6, 26, 30–35, 39, 49–53]. Co-infections with other tick-borne pathogens e.g., *Anaplasma*, *Babesi*a, *Ehrlichia* and RFB, that can be transmitted by the same tick species that also transmit LDB, have been observed in LD[32–33, 35, 52–57]. These findings highlight the importance of accurate and complete diagnosis in tick-borne diseases.

The US Centres for Disease Control and Prevention (CDC) estimated, based on commercial insurance claims data, that approximately 476,000 persons were treated every year for LD in the US during the period 2010–2018[58]. This is likely to over-estimate the actual number of persons with LD. Cases of LD have been reported from many US states and Canada[59–61]. It is not mandatory, unlike in the US, to report all cases of LD to health authorities in European countries[38]. The number LD cases in western Europe is estimated to be >200,000 and increasing every year[37].

Diagnosis of Lyme disease

An Erythema Migrans (EM) rash, with a bull's eye-like appearance, resulting from an infected tick blood feed is a recognized indicator of an acute or early localized infection with LDB (sometimes termed stage 1 LD) although EM can be absent or not detected in some infections[11, 49, 62–64]. If LD is not treated at an early stage with antibiotics, patients can progress to disseminated LD that may be characterized by cardiac, musculoskeletal, and neurological manifestations. Symptomatic clinical diagnosis in the late stages of LD can be difficult without a history of EM rash and tick bite, because late-stage symptoms are not pathognomonic. Diagnosis of early disseminated LD with cardiac and neurological symptoms (sometimes termed stage 2 LD), as well as late disseminated LD with arthritis (sometimes termed stage 3 LD), therefore depends on ascertaining potential exposure to areas infested with the tick vectors (grassy, brushy and wooded locations)[26] as well as pertinent clinical manifestations and laboratory confirmation of infection[11, 26, 49].

Methods for detecting LDB in blood and other tissues by microscopy and culture isolation, or identifying LDB antigens, generally have limited diagnostic sensitivity and specificity LD[11, 23, 65]. This is because the concentration of bacteria and their antigens in tissues is often low, and culturing of LDB is a difficult time-consuming procedure[11, 23, 65]. Nucleic acid amplification by PCR is useful for demonstrating LDB in EM lesions in the skin and synovial fluid of patients with LD arthritis, but poorly sensitive for detecting LDB in blood or cerebrospinal fluid[11, 23, 65]. Efforts are being made to develop more sensitive PCR-based methods for detecting LDB[65]. One approach that first concentrated LDB DNA with a set of specific capture DNA probes followed by PCR amplification, detected 21.5% of LD patients who were negative by the CDC-recommended standard serological test for LD described in section 2.3.1 below[66]. Advanced multiplex PCR-based molecular diagnostic tests for simultaneously detecting LDB and other tick-borne pathogens are also under development[67].

Although the detection of antibodies to LDB in patients does not readily distinguish between active and resolved infections, its importance in supporting a clinical diagnosis of LD is well established[11, 26, 44]. The variety of LDB species that can cause LD in different countries, and infections that can be acquired during overseas travel, however, imply that immunoassays that incorporate antigens from diverse LDB species may generally be more advantageous for diagnosing LD in all endemic countries.

Serological tests for LD in USA

Standard two-tier test (STTT) in USA

Consideration of relevant immunoassay findings, notably western blot (WB) results[68–69], led the Second US National Conference on Serologic Diagnosis of Lyme Disease meeting, held at Dearborn, MI in 1994, to recommend a standard two-tier test (STTT) for the serological diagnosis of LD[70]. STTTs have since been widely used in the US for supporting a diagnosis of LD[11, 70–71]. The first tier of the STTT is an enzyme immunoassay (EIA) or immunofluorescence assay (IFA) on whole *Bbss* cell antigens, followed by a second-tier confirmatory WB on whole *Bbss* cell lysates for sera that give positive or equivocal results in the first-tier test[70–71]. The rationale for the STTT was that the first tier EIA or IFA was highly sensitive but inadequately specific, while the second tier WB was highly specific for detecting serum antibodies to LDB. Only *Bbss* cell antigens were used in STTTs because *Bbss* is the predominant cause of LD in the US. The EIA is more commonly used than IFA as a first-tier test. The CDC recommended a panel of *Bbss* protein antigens to be used for identification in the second-tier WB of a STTT[70–71].

The CDC recommended and US Food and Drug Administration (FDA) approved criteria for a positive reaction in the second-tier WB with whole *Bbss* cell lysate in the STTT[70–71] are (i) reaction with at least any two of the three antigens designated by relative molecular mass as P23 (OspC), P39 (BmpA) and P41 (FlaB) for IgM WBs, and (ii) reaction with at least any five of the ten antigens P18, P23 (OspC), P28, P30, P39 (BmpA), P41 (FlaB), P45, P58, P66 and P93 for IgG WBs. A positive IgM WB result was considered indicative of recent infection in patients presenting within 30d of the onset of symptoms, and was not recommended by the CDC for assessing sera from patients with symptoms lasting longer than 30d[70–71].

All ten *Bbss* antigens recommended for identification in WBs by the CDC have recently been produced as recombinant proteins and applied as lines on nitrocellulose membrane strips for detecting IgG and IgM antibodies in IBs[72–74]. The line IBs are designed to replace the cumbersome second tier WB procedure of STTTs that requires growing *Bbss* in culture and preparing cell lysates. Line IBs detect antibodies reacting with purified antigens and do not have the disadvantage of WBs where reaction with a specific antigen has to be identified amongst many other antigens present in whole cell lysates. Several other advantages of line IBs over WBs for use in the second tier of STTTs have been extensively discussed elsewhere[11, 23, 72]. The FDA recently approved the use of line IBs with recombinant *Bbss* proteins for replacing WBs in STTTs for LD[73–74]. The use of recombinant antigens in line IBs to detect antibodies in US LD patients is described later.

The sensitivity of STTTs has been reported to be variable and as low at 46% in patients with LD in excess of six weeks duration[75]. Sensitivities of 60-90% in early disseminated stage 2 LD with neuroborreliosis and carditis prior to antimicrobial therapy have been observed[11, 76]. Sensitivity was reported to be lower in early stage 1 LD with EM, approaching 40% before and 61% after antimicrobial treatment[11, 76]. More detailed serological investigations in patients with culture or PCR confirmed LD are justified to better assess the sensitivity of STTTs in different stages of LD. The specificity of STTTs was ≥99% and ≥98% when tests were done with control sera from healthy persons living respectively in LD non-endemic and endemic locations[11, 76].

Modified two-tier tests (MTTTs) in USA

The Food and Drug Administration (FDA) in 2019 additionally approved the use of several modified two-tier tests (MTTTs) for diagnosing LD, where a positive or equivocal first-tier EIA test result necessitated a second tier EIA against a different antigen for confirmation[11, 71, 77]. One approved MTTT utilizes a first tier EIA based on the variable major protein-like sequence expressed (VlsE) present in LDB[78] and a 10 amino acid C-terminal peptide from the LDB P23 (OspC) protein (termed the C10 peptide)[79], accompanied by a second tier EIA test with whole *Bbss* cell sonicate[71]. Another MTTT, utilising a chemiluminescence immunoassay for VlsE as a first-tier test, followed by an EIA against a 25 amino acid long invariant region peptide C6 of the VlsE protein[80] as a second-tier test, reported comparable sensitivity and specificity to the conventional STTT in early LD[81]. The advantages offered by many MTTTs include the automated reading of EIA results and avoidance of the labour-intensive WB procedure that utilizes whole *Bbss* cell lysates.

Serological tests for LD in Europe

Infections with the LDB species *B. afzelii*, *B. garinii*, *B. spielmanii, B. baveriensis* and *Bbss*, and potentially other LDB species, cause LD in European countries[23, 27, 38, 44]. This differs from the situation in the US where *Bbss* is predominantly responsible for LD[26]. Serological diagnosis of LD is more complex in Europe than in the US for the following reasons (i) homologous proteins from different infecting LDB species can elicit antibodies in patients that do not readily cross-react with each other, (ii) the clinical manifestations of LD are more variable in Europe, and (iii) characteristics of antibody responses to LDB infections in European and US patients are reported to differ[21, 23, 37, 38, 44, 82–84]. Analysis of antibodies in European patients reacting in WBs with proteins of relative molecular masses between 40 to 100 kDa from *B. afzelli*, *B. garinii* and *Bbss* showed that combinations of particular antigen reactivities for each species gave specificities >96% and varying sensitivities for the three species[82–84]. Based on such results, two-tier serological tests with WBs in the second tier, analogous to the STTTs used in the US but requiring the recognition of fewer antigens, and varying combinations of antigens from different LDB species, have been utilized in Europe[44, 84].

The use of recombinant proteins from different European LDB species in a line IB format with the inclusion of additional antigens such as VlsE[78] and the decorin binding protein A (DbpA)[85] has since been reported to be diagnostically superior to WBs with whole cell lysates of LDB[23, 86]. Purified recombinant proteins rather than whole cell lysates are now preferred for the second tier of European LD tests, partly because LD is caused by multiple LDB species in Europe. The German Society for Hygiene and Medical Microbiology guidelines for LD recommended the recognition of two different antigens for seropositivity in both IgM and IgG LD IBs[87]. Reactivity with homolo-

gous proteins from different LDB species were judged to be equivalent to reaction with a single antigen band[87]. As an example, a line IB study in Germany used the following seven antigens (i) P100, a single protein from *B. afzelii*, (ii) P58, a single protein from *B. garinii*, (iii) BmpA, where proteins from *Bbss*, *B. afzelii* and *B. garinii* were applied separately in three different lines, (iv) VlsE, where proteins from *Bbss*, *B. afzelii* and *B. garinii* were applied separately in three different lines, (v) P23 (OspC), where four proteins from *Bbss*, *B. afzelii* and two different isolates of *B. garinii* were applied separately in four different lines, (vi) a P41 (FlaB) fragment, where two proteins from *B. afzelii* and *B. garinii* were applied separately as different lines, and (vii) DbpA, where four proteins from *Bbss*, *B. afzelii* and two different isolates of *B. garinii* were applied separately in four different lines[86]. Specificities of 99.1% and 98.2% and sensitivities of 84.7% and 73.8% for IgG and IgM antibodies were respectively achieved by applying the German criteria, but with the following modifications (i) a strong IgM reaction with P23 (OspC) alone in early LD with EM and early disseminated LD with neuroborreliosis was considered sufficient for positivity, and (ii) P100 reactivity was discounted in IgM IBs[86].

Except in patients with EM, a two-tier serological approach for the laboratory diagnosis of LD has generally been recommended in Europe, and the UK[87–88]. Serological studies on European patients with LD that compared US STTTs with European two-tier tests found comparable specificities (99–100%) but lower overall sensitivity with US (52%) than European tests (81%)[89]. This difference was primarily attributed to lower detection sensitivities in the disseminated stages of LD with US STTTs[89], probably due to the use of only *Bbss* antigens in US assays whereas European patients could have been infected with other LDB species. The use of US MTTTs with the same European sera, however, improved detection sensitivity to a level comparable to that achieved with European two-tier tests[89]. A European MTTT using a first tier EIA with *B. afzelii* whole cell antigens followed by a C6 EIA in the second tier were reported to yield approximately 70% sensitivity even in early-stage LD with EM, and comparable specificity to STTTs[90]. A 2016 meta-analysis of the serodiagnostic accuracy of all case-controlled studies on European LD reported that (i) sensitivity was lower in early stage LD with EM than in early and late disseminated stages of LD, (ii) results with single-tier and two-tier tests were comparable, (iii) detection of IgM and IgG antibodies was superior to detecting IgM or IgG alone, particularly in early stage LD with EM, (iv) recombinant proteins were better than whole cell lysates as antigens, and (v) better conformity with standards for reporting diagnostic accuracy studies (STARD) guidelines[91] was needed[92].

Recombinant proteins derived from European LDB species are now available from several commercial sources for diagnosing LD with IBs[93–95]. In one example of their use, a screening EIA followed by line IBs based on European LDB species proved helpful in diagnosing LD in Polish patients, although the IBs were unable to distinguish between co-infections with different LDB species or the presence of antibodies that cross-react with homologous antigens from different LDB species[96]. Also in Poland, infections with viruses such as cytomegalovirus and Epstein-Barr virus that cause polyclonal B cell activation, were reported to generate antibodies that cross-reacted in IBs with P41(FlaB) and P23 (OspC), as well as whole cell LDB antigens in the first-tier screening EIAs[97].

Serological tests for LD in Asia

LDB infections in vector ticks have been reported not only in temperate zone Asian countries *e.g.,* China, Mongolia and Japan, but also in wholly tropical Asian countries, *e.g.*, Malaysia and Thailand where there is little information on patients with LD[98]. Criteria for WB positivity for LD have been suggested only in China, based on WBs with whole cell lysates of *B. garinii*, which was reported as the predominant species causing LD in China[99]. A one band criterion was considered sufficient for detecting IgG (73.2% sensitivity and 99.4% specificity), and IgM (50.6% sensitivity and 93.1% specificity) antibodies in Chinese patients[99]. Sera from patients with *Treponema* and *Leptospira* spirochete infections on the other hand were found to cross-react with *B. garinii* P41(FlaB), and was a factor that had to be taken into consideration in serodiagnosis of LD in China[99].

Tick-borne relapsing fever

Background

TBRF is a relatively neglected disease prevalent in many areas of the world[39, 100–114]. *Borrelia hermsii, B. miyamotoi, B. parkeri*, and *B. turicatae* are principal RFB species that cause TBRF in USA[30, 39, 60, 100, 102]. Other RFB species may also be responsible for TBRF in USA, *e.g.,* a *B. johnsonii*-like species previously identified in bat ticks[103]. TBRF is prevalent in Central and South America[39, 103]. *Borrelia hispanica*, *B. persica*, and *B. miyamotoi* in Europe and Asia[39, 102, 105–106], and *B. hispanica*, *B. crocidurae*, and *B. duttonii* in Africa[39, 107], are also responsible for TBRF. Most RFB are transmitted by soft ticks of the genus *Ornithodoros*, typically found in mountain huts, caves and burrows in USA[6], but *B. miyamotoi* can be transmitted by *Ixodes* hard ticks that also transmit LDB[39, 60, 100]. Another hard tick, *Amblyomma americanum*, is a

vector of the RFB *B. lonestari* in southern USA[102, 109]. The distribution of LD and TBRF overlap in localities where their respective tick vectors are both present[6, 26, 30, 32–35, 60]. TBRF is not always considered when a patient presents with symptoms suggestive of LD, and serological testing only for LD may give negative results, risking misdiagnosis or a missed diagnosis of TBRF.

Louse-borne relapsing fever (LBRF) is a similar disease to TBRF caused by the RFB species *B. recurrentis*, which is transmitted by the human body louse *Pediculus humanus humanus*. LBRF is not presently endemic in USA and Western Europe[39, 110-111], but typically prevalent in the Northeast Africa[39, 110-111], from where it is sometimes introduced to European countries by travellers and migrants[110].

Diagnosis of tick-borne relapsing fever and importance of its distinction from Lyme disease

Classic descriptions of the clinical manifestations of TBRF include repeating weekly cycles of acute febrile crises followed by a rapid defervescence, often accompanied by hypotension. However, recent publications[31, 33, 35] have demonstrated TBRF may also present as a LD-like illness. Because of this, and because EM is not always observed in LD[11, 49, 62–64] making the differential clinical diagnosis can be difficult in certain cases[6, 26–35, 39, 48, 63]. In addition, TBRF and LD can occur in the same locations[6, 26, 30, 39, 32–35, 60].

Antigenic variation in outer membrane proteins that subverts a protective antibody response is considered responsible for the recurring bacteremia and fever in TBRF[115–116]. The differential diagnosis of TBRF and LD is important because the pathology of late stage TBRF and LD can vary[26, 30 ,49, 108], and severe Jarisch-Herxheimer reactions are more common in TBRF than LD following antibiotic treatment[116].

Microscopic examination of Giemsa-stained blood smears is the most common laboratory diagnostic technique for TBRF with a reported sensitivity of 10^4-10^5 spirochetes per ml of blood[39]. It is particularly useful during acute febrile episodes when there is a high concentration of spirochetes in peripheral blood. Its sensitivity is reported to be poor, however, in *B. miyamotoi* disease[118]. At present standardised methods for PCR-based detection of RFB are unavailable[39]. The use of PCR in diagnosis of TBRF, although highly sensitive, is therefore mainly restricted to well-equipped laboratories in research settings at the present time[39]. Culturing bacteria for diagnosing TBRF, even during high blood bacteraemia, is difficult because *Borreliae* do not readily adapt to culture[11, 23, 39, 108]. RFB can sometimes localise in the brain causing neuroborreliosis and making direct detection more difficult[119]. Serological methods of detection at present are poorly developed for TBRF[39] compared with LD. Serological tests on whole cell lysates show considerable antigenic cross-reactivity between RFB and LDB due to their close genetic relationship[36, 120–121]. Cross-reactions between recombinant FlaB proteins from LDB and RFB species has been demonstrated in IBs[32, 72]. However, other RFB antigens have been reported to be useful for serodiagnosis in TBRF, and these include (i) glycerophosphodiester diesterase (GlpQ), a periplasmic enzyme[122], that is also present in *B. recurrentis*[123] but not in LDB[32], (ii) a 70-75kDa outer membrane lipoprotein termed the *Borrelia* immunogenic protein A or BipA[124–125], and (iii) a 20-23kDa outer membrane factor H binding lipoprotein or fHbp) that inhibits the alternative pathway of complement activation[126–128]. A serodiagnostic test for TBRF has not yet been approved by a national health authority in any country[21, 30]. The application of line IBs for the detection of serum antibodies in TBRF is discussed in more detail later.

Line immunoblots utilizing antigens from multiple LDB species for the diagnosis of LD in USA

A method for detecting IgM and IgG antibodies in US LD patients with line IBs utilizing a combination of purified recombinant proteins derived from US and European LDB species, to replace WBs with whole *Bbss* cell lysates in the second tier of the STTT, was recently reported[72]. The antigens used in this work included all ten *Bbss* target antigens recommended for assessment by the CDC for WBs in tier-2 of the STTT (section 2.3.1) with the following modifications (i) not all *Bbss* target antigens were applied as individual lines in the IB strips, (ii) a mix of P39 (BmpA) proteins derived from US LDB species, including *Bbss*, was applied in one line, and a mix of P39 (BmpA) from different European LDB species in a separate line, (iii) a mix of P41 (FlaB) from European and US LDB species including *Bbss* was applied as a single line, (iv) P23 (OspC) from different European and US LDB species including *Bbss*, were applied separately in different lines, (v) P31 (OspA), which is not on the list of WB antigens recommended by the CDC for tier-2 STTTs, from different European and US LDB species including *Bbss*, were applied separately in different lines, and (vi) the additional application of *Bbss* VlsE and its C6 peptide as well P34 (OspB), that are also not in the CDC list of WB antigens for STTTs, in separate lines[72].

Illustrative published findings with LD patient and control human sera from the US using these investigational LD IgM and IgG IBs[72] are shown in Figure 1. Sera in lanes 2-5 were positive and in lanes 1, 6-10 were negative

for IgM and IgG antibodies when the CDC criteria for positivity in tier-2 of STTTs were applied. The positive sera variably recognized P23 (OspC) from different LDB species in the IBs. Three of the four positive sera recognized P39 (BmpA) from both US and European LDB species in the IgG IBs. The variable recognition of P23 (OspC) and P39 (BmpA) was consistent with varying antibody cross-reactivity after exposure to one or more LDB species. The four positive sera also had antibodies recognizing P31 (OspA), VlsE and C6, suggesting their potential value for diagnosis, and particularly because antibodies to VlsE and its C6 domain are reported to be produced early in infection[11].

The LD IBs as stand-alone, single tier test demonstrated a sensitivity of 88.5% and specificity of 99.3% utilizing the CDC-recommended STTT antigens, when (i) well-characterized external serum samples from patients with all stages of LD, patients with potentially confounding autoimmune conditions and other infections, as well as healthy controls were tested (ii) the presence of either IgM or IgG antibodies was considered to be a positive reaction, (iii) the 30d limit after the onset of LD symptoms was not taken into consideration for IgM antibodies, and (iv) recognition of any one of the different P23 (OspC) or P39 (BmpA) proteins was considered to be a positive reaction for that antigen[72]. Furthermore, rabbit antisera raised against cultured whole bacteria from the two RFB species, *B. hermsii* and *B. coriaceae,* were shown not to react with antigens used in the LD IBs other than with

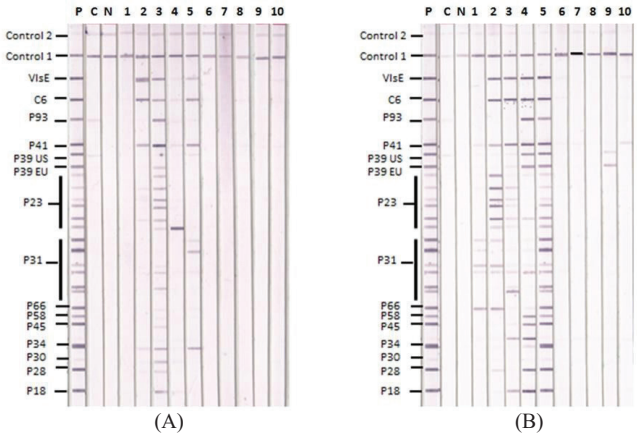

Fig. 1: Representative A. IgM and B. IgG LD IBs. P - positive control, C - threshold control, and N - negative control. Controls 1 and 2 indicated addition of conjugated second antibody and human serum. Positions of target antigens used in the IB strips are shown. P39 EU - A mix of P39 (BmpA) from European LDB species, P39 US - A mix of P39 (BmpA) from US LDB species including *BBss*. P23 (OspC) and P31 (OspA) from different LDB species were applied as separate lines. Fig. 1 is reproduced with permission under the creative commons licence from reference 72.

P41 (FlaB)[72], demonstrating the potential of LD IBs for differential diagnosis of LD and TBRF.

Detection of only IgM antibodies in STTTs >30d after possible infection is presently not recommended by the CDC because IgM antibodies are more prone to cross-reaction with irrelevant antigens and robust IgG antibody responses are normally thought to be present by 30d[11, 70–71]. Results with IBs using recombinant proteins that demonstrate specific IgM antibody responses to LDB[72], interference by LDB of host adaptive immune responses[129], and instances of delayed IgM antibody production in LD[130–132], suggest that it may be useful to re-evaluate the 30d limit and the target antigens for detecting IgM antibodies in LD.

Other reports have documented the use of recombinant antigens in a variety of test formats, including EIAs and IBs, to detect IgG and IgM antibodies to LDB for diagnostic purposes[23, 73–74, 86, 93–97]. These include commercially available tests in Europe[93–95] and USA[74]. A single step, multiplex, serological IB test for LD that is simpler to use will have many advantages over the present FDA-approved STTTs and MTTTs. However, a single tier test for LD has yet to be approved by the FDA for use in USA. The CDC has established a Lyme serum repository of well-characterized sera from LD patients, healthy controls and patients with pertinent other illnesses, to support the development of improved serological tests for LD in the US that may help in this context[76].

Line immunoblot tests utilizing antigens from multiple RFB species for diagnosing tick-borne relapsing fever

Multiplex IgM and IgG IB tests utilizing the recombinant proteins BipA, GlpQ, fHbp, and FlaB from several RFB species have also been explored in TBRF[32]. Recombinant proteins from five human-infecting US and European RFB species for BipA, four for GlpQ, two for fHbp, and three for FlaB were used in this investigation[32].

The specificity of TBRF IBs was assessed with sera from patients with likely confounding non-infectious (fibromyalgia, and autoimmune and allergic conditions) and infectious (LD, anaplasmosis, babesiosis, bartonellosis, ehrlichiosis and other bacterial and viral illnesses) diseases, as well as healthy controls living in TBRF endemic and non-endemic areas[32]. The sensitivity determined using sera from patients with PCR-confirmed RFB infections[32]. The optimal criterion reported for TBRF test positivity was the recognition of FlaB plus at least two of the three antigens BipA, GlpQ and fHbp with either IgG or IgM antibodies[32]. The use of this criterion yielded a specificity of 99.5% and sensitivity of 70.6% in the TBRF IB tests[32]. Importantly, six rabbit antisera raised separately against six different cultured LDB species (*Bbss, B. afzelli, B.*

californiensis, B. garinii, B. spielmanii, and *B. valaisiana*) reacted only with RFB-derived FlaB and not with BipA, GlpQ and fHbp, while three rabbit sera similarly raised against three cultured RFB species of widely different origins (human *B. hermsii,* bovine *B. coriaceae* and tortoise *B. turcica*) gave positive reactions in the TBRF IBs[32]. These findings demonstrated the potential of TBRF IBs to help diagnose TBRF and differentiate it from LD.

Potential of IBs for the differential diagnosis of LD and TBRF

The parallel use of LD and TBRF IBs for differential diagnosis has been investigated in patients with clinical symptoms LD from Australia, Ukraine and USA[32], and in another study from Mexico and the adjoining US state of California[35]. Both studies reported three types of IB test results, *viz*., positive reactions in (i) TBRF IBs only, (ii) LD IBs only, and (iii) both TBRF and LD IBs. Representative examples of the three types of reactions from one of the two studies[32] are reproduced in Figure 2. Sera of both patients in lanes 1 and 2 were only positive in TBRF IBs. Serum in lane 3 was positive in both TBRF and LD IBs. Serum in lane 4 was negative in both IBs. Serum in lane 5 was only positive in LD IBs.

Findings from two studies suggest that patients with LD-like symptoms in different parts of the world may have been solely exposed to, or else infected with, either LDB or RFB, and in other cases with both types of *Borreliae*[32, 35]. The parallel use of LD and TBRF IBs can therefore help identify which pathogen or pathogens are responsible for causing the LD-like clinical symptoms, some of which are shared with TBRF[6, 26–35, 39, 48, 63]. Further IB studies with PCR-characterized patient samples from multiple locations will help to corroborate these early findings.

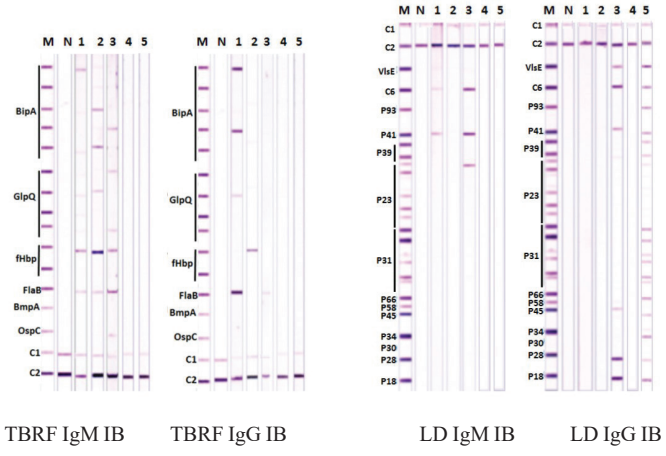

Fig. 2: Representative TBRF and LD IgM and IgG IBs with five sera from patients with LD-like symptoms. M: positive control (rabbit antiserum for TBRF IBs and human serum for LD IBs); N: negative control (human sera for TBRF and LD IBs); C1 and C2: controls for confirming addition of conjugated secondary antibody and human serum respectively. The positions of the different target antigens used in the IB strips are indicated. Fig. 2 is reproduced with permission under the creative commons license from reference 32.

Infection with *B. duttonii* and *B. crocidurae* in Africa causes TBRF with intermittent high fevers, hepatomegaly, splenomegaly, and anemia, and because such symptoms are also characteristic of malaria[133], antimalarial drugs are sometimes inappropriately administered[134]. TBRF IBs may therefore be useful in parts of the world where malaria and TBRF are co-endemic. In a related context, the applicability of TBRF IBs for the serodiagnosis of LBRF caused by *B. recurrentis* also merits investigation.

IBs in the diagnosis of other tick-borne diseases

Serodiagnosis has an important role in the diagnosis of other tick- borne diseases, *e.g.,* babesiosis, ehrlichiosis, anaplasmosis, and rickettsiosis, as well as bartonellosis transmitted by fleas[5–10]. Several tick-borne diseases are transmitted by the same ticks that transmit LDB, and are not easily distinguished by clinical signs and symptoms from LD[5–10]. The causative pathogens, like in LD, are difficult to isolate and detect in direct tests. IFA tests for IgM and IgG antibodies are commonly applied in the serodiagnosis of babesiosis, bartonellosis, ehrlichiosis, anaplasmosis, and rickettsiosis[7]. IFA tests have been used to help differentiate human infections with *Babesia duncani* and *Babesia microti* in the US and other countries[135–136]. However, IB tests based on purified recombinant protein antigens, analogous to LD and TBRF IBs, have recently become available for the serodiagnosis of babesiosis and bartonellosis[7]. Detection of serum antibodies to specific pathogen antigens is also important for the diagnosing tick-and flea-borne diseases that afflict domestic and farm animals[137]. IB tests, analogous to those discussed here for LD and TBRF, can be helpful for this purpose.

CONCLUSION

Recombinant proteins have many advantages as target antigens in serological diagnosis of infectious diseases, and have been employed in multiple assay formats in different diseases, e.g., lateral flow immunochromatography in syphilis[138]; lateral flow immunochromatography, EIA and IB in lymphatic filariasis[139]; and EIA in Chagas' disease[140]. The utilization of recombinant proteins in IBs for the serodiagnosis of LD and TBRF therefore builds on well-established precedence.

The detection of antibodies is an indirect method for diagnosing infections. Finding serum antibodies in IB

tests at a single point in time does not readily distinguish between active infection, convalescence after antibiotic therapy or a past resolved infection because antibodies continue to be produced by bone marrow resident plasma cells long after the resolution of infection[141]. IB tests therefore need to be always used in conjunction with clinical signs and symptoms in diagnosing LD and TBRF. Repeating IB tests at a later time can provide pertinent information *e.g.*, in distinguishing active and past infections as well as ensuring early infections with nascent antibody responses are not missed. Testing for both IgG and IgM antibodies is reported to be necessary for optimal diagnostic sensitivity in both LD and TBRF[11, 23, 32, 70–72].

Protein antigens applied on nitrocellulose strips for the IB tests are stable at ambient temperature for several months. The IB strips are readily manufactured to yield reproducible results. The alkaline phosphate substrate 5-bromo-4-chloro-3-indolyl phosphate nitro-blue tetrazolium produces a visible IB band that is stable at ambient temperature for several months[32, 72] - a property which enables the IB results to be re-assessed if needed. The IB procedure as well as the reading of reactive antigen bands by densitometry for greater objectivity, can be readily automated. These properties make IB tests attractive for the serodiagnosis of LD and TBRF as well as potentially other tick-borne diseases for medical and veterinary medical purposes under the One Health concept[137].

The development and validation of single-step serodiagnostic tests for LD and TBRF that can also help differentiate the two diseases, similar to that described in here, will be an important diagnostic advance. Additional investigations, *e.g.,* the use of a weighting score for individual antigens[142–143], and further selection of target antigens, may help optimize LD and TBRF IB tests in this regard. Determining clinical diagnostic parameters of the LD and TBRF IB tests with serum samples from patients with PCR-confirmed infections at different stages of the two diseases and appropriate control sera in different clinical settings is also important.

It is relevant that LD has been reported in widely different parts of India[144–146]. TBRF has also been observed in India[147–148]. The development of sensitive and specific LD and TBRF IB tests can therefore contribute to differentiating the two diseases and establishing their epidemiology in the country.

ABBREVIATIONS

Bbss - *Borrelia burgdorferi sensu stricto*; BipA - *Borrelia* immunogenic protein A; BmpA - *Borrelia burgdorferi* basic membrane protein A; CDC - US Centres for Disease Control and Prevention; EM - erythema migrans; EIA - enzyme immunoassay; FDA - US Food and Drug Administration; fHbp - factor H binding protein; FlaB - flagellin B; GlpQ - glycerophosphodiester diesterase; IB - immunoblot; IFA - immunofluorescence assay; LBRF - louse-borne relapsing fever; LD - Lyme disease; LDB - Lyme disease *Borreliae*; MTTT - modified two-tier test; OSP - outer surface protein; PCR - polymerase chain reaction; RFB - relapsing fever *Borreliae*; STARD - standards for reporting diagnostic accuracy studies; STTT - standard two-tier test; TBRF - tick-borne relapsing fever; VlsE - variable major protein-like sequence expressed; WB - western blot;

Ethical statement: Not applicable

Conflict of interest: ID-FISH and IGeneX Inc., to which the authors are affiliated, develop and apply serological and nucleic acid tests for diagnosing tick-borne and many other infectious diseases

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