

Research Article

Improved Sensitivity of Lyme disease Western Blots Prepared with a Mixture of *Borrelia burgdorferi* Strains 297 and B31

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Abstract

Testing for *Borrelia burgdorferi* (BB), the spirochetal agent of Lyme disease, is problematic due to poor sensitivity of commercially available serological tests. We evaluated an in-house Western Blot (WB) prepared with a mixture of two strains of BB, B31 and 297. Sensitivity and specificity of IgG and IgM testing using the two-strain WB was compared to testing with a commercial single-strain WB, and interpretation of the in-house WB using in-house criteria was compared to interpretation using criteria recommended by the Centers for Disease Control and Prevention (CDC). A total of 364 control and patient sera (including 88 from treated patients with confirmed Lyme disease) were tested. The sensitivity of the combined IgG and IgM commercial WB using CDC criteria was 77.1%. When the in-house IgG and IgM WB and CDC criteria were used, the combined sensitivity improved to 88.6%, while use of the in-house IgG and IgM WB and in-house interpretation criteria resulted in a combined sensitivity that increased to 97.1%. Using CDC criteria, the specificity of the in-house IgG and IgM WB was 100% and 97.1%, respectively; using in-house criteria; the specificity was 95.3% and 93.1% respectively. By removal of patients who reacted to band 31kDa but tested negative for antibodies to recombinant OspA antigen, in-house IgG and IgM WB specificity increased to >97%. Based on these results, we conclude that the use of the two-strain IgG and IgM WB and in-house interpretation criteria significantly increased the combined sensitivity of the test without significant loss of specificity. In patients with indeterminate WB results, a confirmation test with recombinant OspA is recommended.

Keywords: Lyme disease; *Borrelia burgdorferi*; Western blot; Tick-borne disease

Introduction

Borrelia burgdorferi (BB) is the causative agent of Lyme disease – the most common tick-borne disease in North America and Europe. In Europe at least six species of BB (*B. burgdorferi sensu stricto*, *B. garinii*, *B. afzelii*, *B. valaisiana*, *B. lusitaniae* and *B. spielmanii*) are known to be pathogenic to humans [1,2], whereas *B. burgdorferi sensu stricto* is the only species known to cause human infections in the US [3]. The presence of a characteristic ‘bull’s-eye’ Erythema Migrans (EM) rash is generally considered the earliest and best indicator of acute infection. However, the rash may be absent or may go unrecognized in 20 – 40% of the patients [4]. If the initial infection goes untreated, patients can develop disseminated Lyme disease characterized by cardiac, musculoskeletal, and neurological manifestations months to years after the initial tick bite [5-11]. Diagnosis at this stage can be even more difficult, since the history of the rash and tick bite may be lacking and the symptoms are shared with a number of other diseases [6,8,10,12].

Direct detection of the agent of Lyme disease using microscopy, culture, nucleic acid amplification and antigen detection have limited sensitivity and/or specificity, except early in the disease when an EM rash is present [4,13]. Therefore, the clinical diagnosis of Lyme disease in the US [13] and Europe [14] is usually supported by antibody

detection using a two-tiered testing system. In this system, an Enzyme-linked Immunosorbent Assay (EIA) or Immunofluorescent Antibody (IFA) test is performed as a screen, followed by Western Blot (WB) confirmatory testing if the result obtained by EIA or IFA is indeterminate or positive [13]. The Centers for Disease Control and Prevention (CDC) guidelines for interpretation of the Western blot are based on the publication of Engstrom et al. for IgM [15] and Dressler et al. for IgG [16], and have been the standard for WB interpretation since the Dearborn conference in 1995 [13]. Two-tiered serologic testing has a reported sensitivity of 30 to 40% during the first week after presentation of the EM rash and 29 to 78% in convalescent stages after treatment [4,17]. Antibody response increases over time and the reported sensitivity in patients with neurological involvement or Lyme disease arthritis is 87% and 97% respectively [4,15,16].

Pathogens that cause diseases such as anaplasmosis, babesiosis and ehrlichiosis are transmitted by the same tick that transmits BB. Thus, Lyme disease patients may harbor these other tick-borne diseases. Therefore, it is important to determine which antibodies are specific for Lyme disease [8,12,18-21]. False positive IgM and IgG results have been reported in patients with illnesses such as rheumatoid arthritis, infectious mononucleosis, autoimmune diseases, bacterial endocarditis, syphilis, other spirochetal infections and *Helicobacter pylori* [16,22].

Table 1: Source and characterization of sera used in the study.

Group #	Source	Antibodies to:
Group 1 (88)		Borrelia burgdorferi
1a	CDC (35)	Collected between 3 wks to 13 y after initial infection from treated patients. Four were collected within the first month of infection; 25 were collected between 2 nd to 12 month after infection and the remaining 6 were collected between one year and 13 years after initial infection.
1b	Columbia University – Dr. Fallon (30)	Collected between 9 m and 25 yrs after initial infection from patients diagnosed and treated for disseminated Lyme disease. Two were collected between 9m and one year. The remaining 28 were collected between 1 year and 25 years after initial infection.
1c	NYSDOH and CAP proficiency (6)	Expected to be positive for antibodies, but did not get 80% agreement in testing laboratories.
1d	Borreliosis Centrum, Augsburg, Germany – Dr. Schwarzbach (17)	Collected between 3 weeks after initial infection from untreated patients. One was collected within 6 months of infection, one within 6-12 months after infections and the remaining 15, more than one year after infection.
Group 2 (45)	CAP (10) and IGeneX (35)	Other Tick-borne Pathogens Babesia (10); Ehrlichia or Anaplasma (33); and Bartonella (2)
Group 3 (49)	NYSDOH and CAP proficiency	Autoimmune Diseases: Systemic lupus erythematosus (2); Nuclear antigen (2); Nuclear DNA (1); Rheumatoid factor (11); Allergy specific IgE (4); and Allergy specific IgG (29)
Group 4 (29)	New York Biologics	Rapid Plasma Reagin test positive
Group 5 (65)	New York Biologics	Viruses Epstein-Barr virus (24); Herpes simplex virus (10); Cytomegalovirus (13); Hepatitis C virus (12); HIV (2); Hepatitis B virus (2); and West Nile virus (2)
Group 6 (88)	New York Biologics (2) NYSDOH (71) CAP proficiency CDC (5) and Columbia University (10)	Toxoplasma gondii (2) Negative for antibodies to <i>B. burgdorferi</i> (71) Normal Controls (15)

The expression of different antigens depends on culture conditions and strains of BB [6,15,16]. Thus, WB assay sensitivity improves by using more than one strain of BB for preparing the WB [23,24]. Shah et al. [24] demonstrated that the IgG and IgM WB sensitivity improves without loss of specificity (1) if a mixture of two strains of BB, B31 and 297, are used for the preparation of WB strips, and (2) if WB results are considered positive when two of the following six bands are present: 23, 31, 34, 39, 41 and 93kDa.

One of the drawbacks of the two-strain WB study was that the analysis of specificity was limited to patients who were seropositive for other tick-borne diseases and healthy controls. Therefore, in the present study we evaluated 364 well-characterized specimens from healthy controls and patients with tick-borne diseases, other spirochetal infections, viral infections and autoimmune diseases. We also examined the results of WB band reactivity to: (1) determine if the in-house criteria for positive results could increase the sensitivity of the assay, and (2) the use of recombinant outer surface protein A (OspA) antigen WB could increase the WB specificity. Our goal was to provide an improved WB assay with increased sensitivity without loss of specificity.

Materials and Methods

Patient samples

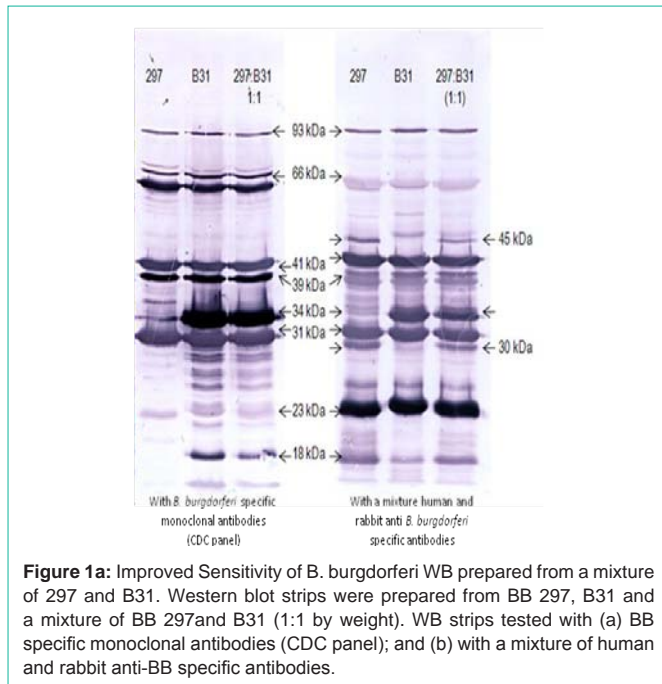
A total of 364 sera including 88 sera from patients with Lyme disease were tested. The source of these sera is described in Table 1. Once received, all specimens were stored at 4°C up to one week and then at -20 °C for longer storage. Testing was performed by laboratory personnel in the same manner as clinical samples without knowledge of the expected results.

Western blot membrane strips

In-house WB strips

Preparation: WB strips were prepared from a mixture of two strains of BB, B31 and 297, as described by Shah et al [24]. An aliquot equivalent to 50 µg of pooled B31 and 297 BB sonicated cell lysate (~100 µg/ml) was fractionated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on 11.3% acrylamide gel and transferred to Protran BA nitrocellulose membrane (Schleicher & Schuell, Keene, NH) in Tris-glycine-methanol transfer buffer pH 8.3 [25 mM Tris, 192 mM glycine, 20% (vol/vol) methanol]. Following transfer, the membranes were washed in deionized water and blocked in 5% non-fat dry milk for one hour. Membranes were dried and sliced into 5 mm strips.

Immunoblotting: An aliquot of control or patient serum was tested with the in-house WB strip. Prior to use, each strip was labeled and then soaked in 1 ml of diluent (100 mM Tris, 0.9% NaCl, 0.1% Tween-20 and 1% non-fat dry milk) for 5 minutes in a trough. An aliquot of 10 µl of the test serum or control was added to the corresponding WB strip in the trough. The strips were incubated at room temperature for one hour, followed by three washes with lx wash buffer (KPL, Gaithersburg, MD) at room temperature. After aspirating the final wash solution, the IgG and IgM strips were incubated with alkaline phosphate-conjugated goat anti-human IgG and IgM, respectively (KPL, Gaithersburg, MD) at 1:5000 dilution for IgG and 1:1000 dilution for IgM, for one hour. After three washes, the bands were visualized with 5-bromo-4-chloro-3-indolylphosphatnitro-blue tetrazolium (BCIP/NBT, and Gaithersburg, MD). The reaction was terminated by washing with distilled water after 15 minutes or when the calibrator produced a faint visible band at 39 kDa. The strips



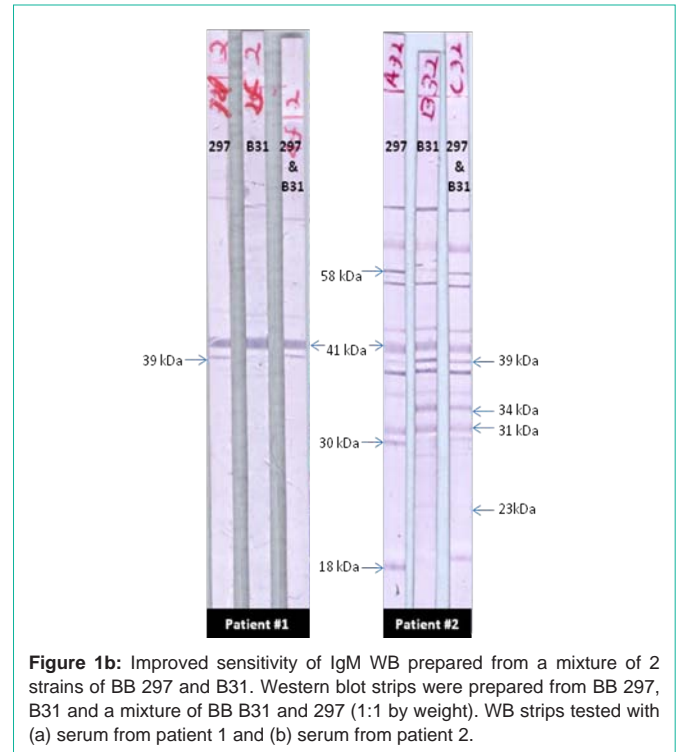
were allowed to dry completely before being scored.

The WB strips were also tested with a positive control, a mixture of human and goat anti-BB antibody (KPL, Gaithersburg MD) along with a series of monoclonal antibodies provided by the CDC to determine presence and location of bands. Diluted alkaline-phosphatase labeled rabbit anti-BB antibody to 39/93kDa that showed a faint but visible band at 39kDa (Strategic Biosciences, Stow, MA) was used as a calibration control. Bands with less intensity than those of the calibration control were reported as negative. The following bands were scored for each patient serum on the in-house WB strip: for IgG 18, 23 (OspC), 28, 30, 31 (OspA), 34 (OspB), 39 (BmpA), 41 (FlaB), 45, 58, 66 and 93kDa; for IgM 23 (OspC), 31 (Osp A), 34 (OspB), 39 (BmpA), and 41 (FlaB) and 93kDa. By CDC criteria, IgM WB reactivity with two of three BB antigens (23, 39 and 41kDa) or IgG WB reactivity with five of ten BB antigens (18, 23, 28, 30, 39, 41, 45, 58, 66 and 93kDa) was considered a positive result [13]. By in-house criteria, IgG or IgM WB reactivity with any two of six BB antigens (23, 31, 34, 39, 41 and 93 kDa) was considered a positive result [24].

Commercial WB strips

Preparation: A commercial test kit for detection of IgG and IgM antibodies against BB, the Marblot Test System (MarDx Diagnostics, Inc., Carlsbad, CA 92008) was used as a reference standard by CDC. The results provided by CDC were compared with the in-house WB. As described by the manufacturer, the commercial Marblot WB strips are prepared from BB strain B31. The antigens of BB are separated by SDS-PAGE, and the resolved proteins bands are transferred by electrophoresis to a nitrocellulose membrane. The membrane is dried, cut into strips and packaged for commercial sale.

Immunoblotting: An aliquot of patient serum or control was tested with the Marblot WB strip as per manufacturer’s instructions. With each run, 3 controls were included: a positive control, a weakly



reactive control and a negative control. Prior to use, each strip was labeled and then soaked in 2 ml of sample diluents/wash solution in a trough for 5 minutes. An aliquot of 20 µl of the test serum or weakly reactive control and negative control or 80 µl of positive control was added to the corresponding strip in the trough. The strips were incubated at room temperature for 30 minutes, followed by three washes with 1x wash solution at room temperature. After aspirating the final wash solution, the IgG and IgM strips were incubated with 2 ml alkaline phosphatase-conjugated anti-human IgG and IgM, respectively for 15 minutes. After three washes, the bands were visualized with color developing solution. The reaction was terminated by washing with 2 ml distilled water when the weakly reactive control produced a faint but visible band at 41kDa (4-15 minutes). The strips were allowed to dry completely before being scored. Bands with less intensity than those of the weak reactive control were reported as negative. The following bands were scored for each patient serum on the Marblot WB strip by CDC: 18, 23 (OspC), 28, 30, and 31(OspA), 34 (Osp B), 39 (BmpA), 41 (FlaB), 45, 58, 66 and 93kDa. By CDC criteria, IgM WB reactivity with two of three BB antigens (23, 39 and 41kDa) or IgG WB reactivity with five of 10 BB antigens (18, 23, 28, 30, 39, 41, 45, 58, 66 and 93kDa) was considered a positive result.

OspA antigen WB

Confirmation of antibodies to OspA antigen: Purified recombinant OspA antigen was purchased from Biodesign International (Saco, ME) and used to prepare WB strips. These strips were used to test sera with bands at the 31kDa location on WB. As a positive control, monoclonal antibody to OspA (provided by CDC) was used. If a band at the same position as the positive control was present on the blot, the sample was considered positive for antibodies to OspA antigen. If there was no band present at the same position as the positive control, the sample was considered negative for

antibodies to OspA antigen. All the samples that had a band at 31kDa on the WB were tested. None of the patients included in the study had received the OspA vaccine. Goat anti-*E. coli* antibodies (Biodesign International, Saco, ME) gave no signal on the OspA WB strip.

Statistical analysis

The two-tailed Fisher's exact test for small samples was employed to compare assay sensitivity and specificity of different blots.

Results

BB and WB observations

Expression of 18, 30, 45 and 58kDa antigens is better in BB strain 297 than in strain B31; and expression of Osp B (34kDa) is better in strain B31 than in strain 297 (Figures 1a & 1b). When the WB strip is prepared from the mixture of these 2 strains, all the antigens were well expressed (Figure 1a). As shown in Figure 1b, two representative patients were positive on the IgM blots prepared from a mixture of B31 and 297. However, patient #1 was negative by CDC criteria on the IgM WB prepared from BB strain B31 only, while patient #2 was negative by CDC criteria on the IgM WB prepared from BB strain 297 only. This data clearly demonstrates that the assay sensitivity of the IgM WB increases if a mixture of both BB strains is used compared to a WB prepared from either BB strain 297 or B31 alone.

Patient testing

A set of 88 samples (including 71 sera from treated patients with Lyme disease from the US and 17 from Europe), was tested by the in-house WB using 2 strains of BB, 297 and B31 (Table 3). A subset of 35 sera from treated patients with Lyme disease was provided by the CDC. CDC also provided Lyme IgG and IgM Marblot WB results on all the sera. CDC Marblot WB results were compared with the in-house WB (Table 2). The sensitivity of the combined IgG and IgM Marblot WB using CDC criteria was 77.1%. Using the in-house IgG and IgM WB, the combined sensitivity increased to 88.6% using CDC criteria, and 97.1% using the in-house interpretation criteria. The sensitivity of the in-house IgG WB ($p < 0.1$) improved with interpretation by in-house criteria as compared to CDC criteria. The sensitivity of the in-house IgM WB was significantly higher than Marblot IgM WB ($p < 0.05$). As shown in Table 3, for the 71 sera from the US, the IgG sensitivity was 78.9% using CDC criteria. Using the in-house WB criteria the assay sensitivity improved to 91.5% ($p < 0.1$). The sensitivity of IgM was between 71.8-73.2% by CDC criteria and in-house WB criteria. All 17 sera received from the European patients were positive by the in-house WB. For the IgG WB, 16 (94.1%) were positive by CDC criteria and all 17 (100%) were positive by in-house WB criteria. For the IgM WB, 12 (70.6%) were positive by CDC criteria and 14 (82.4%) were positive by in-house WB criteria. Overall 16 European patients

(94.1%) were positive by CDC criteria and all 17 (100%) were positive by in-house WB criteria.

The overall sensitivity of the IgG WB was 81.8% using CDC criteria. Using the in-house WB criteria the assay sensitivity improved to 93.2% ($p < 0.05$). The significant increase in positivity rate (from 58.1% to 83.9%) was in patient sera collected within the first year of infection ($p < 0.05$). More than 93% of samples collected between one and 25 years post-infection were positive by IgG WB by both CDC and in-house WB criteria. The sensitivity of the IgM WB was 71.8% by CDC criteria and increased slightly to 73.2% by in-house criteria. The IgM WB sensitivity was 84.8% in samples collected within the first year of infection and decreased to about 64% (63.3-65.3) in samples collected between one and 25 years after infection.

As shown in Table 4, 29 sera from patients with no history of Lyme disease (16 positive on IgM WB, 10 on IgG and three on IgM and IgG) gave a positive result with the in-house WB criteria, whereas only eight of these 29 subjects gave a positive IgM result by CDC criteria. This included one patient with antibodies to *Ehrlichia*, three with antibodies related to autoimmune diseases, seven with antibodies to syphilis by Rapid Plasma Reagin (RPR) test, 15 with antibodies to viruses and three controls. Thus the specificity of the in-house IgG and IgM WB were 95.3% and 93.1% respectively. By CDC criteria, the specificity of IgG and IgM WB was 100% and 97.1% respectively.

OspA WB confirmation

Of the 29 patients with no Lyme-like symptoms, 20 had a band at 31kDa on WB; 19 however, did not react with the recombinant OspA antigen strip. For these 19 patients, the band present at the same position as OspA was not BB specific. This suggests that a non-specific antibody present in the sera is binding to a protein from the cell lysate that co-migrates at the same position as OspA. One serum (IgG and IgM positive) with antibodies to EBV that had bands at 23, 31, 41 and 58kDa, also had antibodies to OspA antigen. Thus, this sample was considered positive for BB-specific antibodies. After removal of the OspA-negative 31kDa-positive samples and "one true positive" there were still 13 samples (eight IgM and five IgG) that were considered to be false positive samples. This included serum from one patient with autoimmune disease, four sera from patients with antibodies to syphilis by RPR test, seven sera from patients with antibodies to viruses and one normal control serum. Thus the in-house WB specificity improved from 95.3% to 98.2% for IgG WB and from 93.1% to 97.1% for IgM WB if the OspA confirmation test was included for any sample that was positive with 31kDa band and one other band used in the in-house criteria of interpretation.

Table 2: Comparison of in-house prepared WB with commercially prepared WB (Marblot Test System). A set of 35 sera from patients with confirmed Lyme disease were provided by CDC. CDC also provided Marblot IgM and IgG WB test results. All 35 sera were tested by in-house prepared WB and read by CDC and in-house criteria.

WB Strip Source	Number (percent) positive interpreted with CDC criteria			Number (percent) positive interpreted with in-house criteria			2 tailed Fisher Test**
	IgG	IgM	IgG+IgM	IgG	IgM	IgM+IgG	
Marblot WB tested by the CDC	17 (48.6)**	18 (51.4)*	27 (77.1)	23 (65.7)**	18 (51.4)*	28 (80)	p=0.22
In-house WB tested in-house	22 (62.9)**	27 (77.1)*	31 (88.6)	30 (85.7)**	28 (80)*	34 (97.1)	p<0.1
2 tailed Fisher Test*	p<0.05			p<0.05			

Table 3: Performance of in-house prepared WB on sera from patients confirmed with Lyme disease. A set of 88 sera, 71 from US and 17 from Europe were tested by in-house prepared WB and read by CDC and in-house criteria.

Date and source of serum collection after initial diagnosis	US Samples				European Samples			All Samples			
	<1 year (n=31) 29 (CDC) 2 neuroborreliosis patients	1 to 25 years (n=34) 6 (CDC) 28 neuroborreliosis patients	Unknown (n=6)	Total (n=71)	<1 year (n=2)	≥1 year (n=15)	Total (n=17)	<1 year (n=33)	≥1 year (n=49)	Unknown (n=6)	Total (n=88)
CDC Criteria	# Positive Patients (%)				# Positive Patients (%)			# Positive Patients (%)			
IgG	18 (58.1)*	32 (94.1)	6 (100)	56 (78.9)*	2 (100)	14 (93.3)	16 (94.1)	20 (60.6)	46 (93.9)	6 (100)	72 (81.8)*
IgM	26 (83.9)	19 (55.9)	6 (100)	51 (71.8)	2 (100)	10 (66.7)	12 (70.6)	28 (84.8)	31 (63.3)	6 (100)	65 (73.9)
IgG or IgM	27 (87.1)	33 (97.1)	6 (100)	66 (92.9)	2 (100)	14 (93.3)	16 (94.1)	29 (87.9)	48 (97.9)	6 (100)	83 (94.3)
In-house Criteria	# Positive Patients (%)				# Positive Patients (%)			# Positive Patients (%)			
IgG	26 (83.9)*	33 (97.1)	6 (100)	65 (91.5)*	2 (100)	15 (100)	17 (100)	28 (84.8)	48 (98.0)	6 (100)	82 (93.2)*
IgM	26 (83.9)	20 (58.8)	6 (100)	52 (73.2)	2 (100)	12 (80)	14 (82.4)	28 (84.8)	32 (65.3)	6 (100)	66 (75)
IgG or IgM	30 (96.8)	33 (97.1)	6 (100)	69 (97.2)	2 (100)	15 (100)	17 (100)	32 (97.0)	48 (98.0)	6 (100)	86 (97.7)
2 tailed Fisher test*	p<0.05			p<0.1							p<0.05

Table 4: Summary of Sera from patients without Lyme-like symptoms. A total of 276 sera from patients without Lyme-like symptoms were tested by in-house prepared WB and read by CDC and in-house criteria. 20/29 samples positive by in-house WB had a band at 31kDa. These were tested with recombinant Osp A WB. 19 did not have Osp A antibodies. ** One sample (positive by IgM and IgG WB) positive for antibodies to EBV had bands at 23, 31, 41, and 58 kDa also had antibodies to Osp A. Therefore, it was considered a true positive. 13 samples remained positive after Osp A WB testing. These were considered false positives.

Antibodies (Number of Sera tested)	Number of Positive Sera by CDC		Number of Positive Sera by in-house Criteria				
	IgM WB	IgG WB	IgM WB	IgG WB	IgG and IgM WB	Total	After Osp A testing (IgM and/or IgG WB)
Tick Borne Diseases other than <i>B. burgdorferi</i> (45)	0	0	0	1 (Ehrlichia)	0	1	0
Autoimmune Diseases (49)	1 (RF)	0	2 (RF)	1 (IgG)	0	3	1
RPR (29)	3	0	4	2	1	7	4
Viruses (65)	3 (2 HSV, 1 EBV)	0	9 (4 EBV, 3 HCV, 2 HSV)	5 (3 EBV, 1 HCV, 1 CMV)	1 (EBV)**	15	7
Controls (88)	1	0	1	1	1	3	1
Total (276)	8	0	16	10	3	29	13 (8 IgM & 5 IgG)
Specificity	97.1%	100%	93.1%	95.3%			IgM – 97.1% IgG – 98.2%

Discussion

In this study of 364 serum samples from a diverse group of patients and controls, we found that the sensitivity of the combined IgG and IgM commercial WB using CDC interpretation criteria was 77.1%. With the use of in-house IgG and IgM WB and CDC criteria, the combined sensitivity improved to 88.6%; and by using the in-house WB and in-house interpretation criteria, the combined sensitivity increased to 97.1%. Using CDC criteria, the specificity of the in-house IgM and IgG WB was 97.1% and 100%, respectively; using in-house criteria; the specificity was 93.1% and 95.3% respectively. By removal of patients who were positive for band 31kDa but tested negative for antibodies to recombinant OspA antigen, in-house IgG and IgM WB specificity increased to >97%. Thus, the use of the two-strain WB and in-house interpretation criteria significantly improved IgG and IgM WB sensitivity without appreciable loss of specificity, and specificity could be further increased with confirmatory testing for OspA antigen reactivity.

All the samples from Lyme disease patients were collected after treatment. This might have altered the immune response to some antigens more than others [25]. Nonetheless, lack of sensitivity early in the disease could be a problem, because immune response is not

fully developed. ELISA tests that are currently recommended for screening lack sensitivity, especially early in the disease [15]. Variable rates of antibody response (4% to 84%) by patients have been reported in WBs prepared from different strains of BB, especially to the chromosomally encoded 39kDa protein BmpA [4,15,16,25,27]. The 39kDa antigen was detected in 84% of patients with early Lyme disease using a WB prepared from BB strain 297 [15] but in only 4% of these patients using a WB prepared from BB strain G39/40 [16]. Marvin et al. [23] have reported that using a mixture of *B. afzelii* and a reference strain of BB sensu stricto improved WB sensitivity when compared to WB prepared from the reference strain only. For improved sensitivity especially early in the disease, use of a mixture of two strains of BB, 297 and B31, appears to be beneficial as demonstrated by improved sensitivity of the IgM WB (Figure 1b).

IgG WB sensitivity improved significantly ($p<0.05$), especially within the first year of infection (Table 3) using the in-house criteria of interpretation as compared to CDC criteria of interpretation (58.1 to 83.9%). For IgM WB, there was no significant increase in sensitivity by including bands 31 and 34kDa in the scoring criteria with the current set of samples. However, the specificity dropped from 100% to 95.3% for IgG WB and 97% to 93.1% for IgM WB. Addition of the

confirmation test for OspA for any sample that had a band at 31kDa improved assay specificity to >97% for both IgG and IgM.

Antibodies to the OspA antigen banding at 31kDa is present in vaccinated patients and in late stage Lyme disease [28,29]. A large number of serum samples from patients with symptoms of Lyme disease have only bands at 31kDa and 41kDa on either IgG or IgM WB. We reviewed results of Osp A confirmation test results performed on 500 patients with bands 31 and 41kDa on IgG and/or IgM WBs. About 50% of the patients were positive by the Osp A confirmation test. The negative patient samples probably had cross-reacting antibodies to a non-specific BB protein that co-migrates with Osp A at 31kDa. While the truly positive samples would be missed if band 31kDa is not included, the confirmation test was able to resolve a large number of false-positive results. We also observed that 30% of the patients with IgM antibodies to EBV had band 93kDa present with or without band 31kDa on the IgM WB. Removal of band 93kDa from IgM WB interpretive criteria did not affect sensitivity of IgM WB. Thus, the interpretive criteria for Lyme WB have been changed. The in-house WB is considered positive if two bands from the following six bands are present: 23, 31,34, 39, 41 and 93kDa, with the following exception: indeterminate if only bands 31 and 41kDa or 31 and 93kDa are present; and IgM WB is considered negative if only bands 41 and 93kDa are present.

In patients with late Lyme disease, antibodies to OspA are present [27]. In a prospective study performed in-house on over 400 PCR positive patients with late-stage Lyme disease, 71% had a positive WB (interestingly, 43% of these patients only had a positive IgM WB). Of these patients, 67% had bands at 31kDa (confirmed as OspA) and 41kDa only on the WB (unpublished data). This data clearly demonstrates the importance of antibodies to OspA in late-stage Lyme disease. Thus, any serum sample with an indeterminate WB result (except from vaccinated patients) should be tested with recombinant Osp A WB.

In conclusion, we have demonstrated that WB sensitivity improves significantly with (1) use of a mixture of 2 strains of BB and (2) interpretation by in-house criteria. The specificity of the WB improves to >97% when Osp A confirmation testing is included in patients who react to band 31kDa on the two-strain WB. Because positive reactivity to a two-strain WB by in-house criteria only suggests exposure to BB, patient results should be used in conjunction with patient history and symptoms to determine whether or not a patient has Lyme disease.

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References

1. Wilhelmsson P, Fryland L, Borjesson S, Nordgren J, Bergstrom S, Emerud J, et al. Prevalence and diversity of *Borrelia* species in ticks that have bitten humans in Sweden. *J Clin Microbiol*. 2010; 48: 4169-4176.
2. Fingerle V, Schulte-Spechtel UC, Ruzic-Sabljic E, Leonhard S, Hofmann H, Weber K, et al. Epidemiological aspects and molecular characterization of *Borrelia burgdorferi* s.l. from southern Germany with special respect to the new species *Borrelia spielmanii* sp nov. *Int J Med Microbiol*. 2008; 298: 279-90.
3. Mathiesen DA, Oliver JH Jr, Kolbert CP, Tullson ED, Johnson BJ, Campbell GL, et al. Genetic heterogeneity of *Borrelia burgdorferi* in the United States. *J Infect Dis*. 1997; 175: 98-107.
4. Aguero-Rosenfeld ME, Wang G, Schwartz I, Wormser GP. Diagnosis of Lyme borreliosis. *Clin Microbiol Rev*. 2005; 18: 484-509.
5. Fallon BA, Keilp JG, Corbera KM, Petkova E, Britton CB, Dwyer E, et al. A randomized, placebo-controlled trial of repeated IV antibiotic therapy for Lyme encephalopathy. *Neurology*. 2008; 70: 992-1003.
6. Hsieh YF, Liu HW, Hsu TC, Wei JC, Shih CM, Krause PJ, et al. Serum reactivity against *Borrelia burgdorferi* OspA in patients with rheumatoid arthritis. *Clin Vaccine Immunol*. 2007; 14: 1437-1441.
7. Logigian EL, Steere AC. Clinical and electrophysiologic findings in chronic neuropathy of Lyme disease. *Neurology*. 1992; 42: 303-311.
8. Marcus LC, Steere AC, Duray PH, Anderson AE, Mahoney EB. Fatal pancarditis in a patient with coexistent Lyme disease and babesiosis. Demonstration of spirochetes in the myocardium. *Ann Intern Med*. 1985; 103: 374-376.
9. Nadelman RB, Wormser GP. Lyme borreliosis. *Lancet*. 1998; 352: 557-565.
10. Stanek G, Strle F. Lyme borreliosis. *Lancet*. 2003; 362: 1639-1647.
11. Steere AC. Lyme disease. *N Engl J Med*. 2001; 345: 115-125.
12. Krause PJ, McKay K, Thompson CA, Sikand VK, Lentz R, Lepore T, et al. Disease-specific diagnosis of coinfecting tickborne zoonoses: babesiosis, human granulocytic ehrlichiosis, and Lyme disease. *Clin Infect Dis*. 2002; 34: 1184-1191.
13. Centers for Disease Control and Prevention (CDC). Recommendations for test performance and interpretation from the Second National Conference on Serologic Diagnosis of Lyme Disease. *MMWR Morb Mortal Wkly Rep*. 1995; 44: 590-591.
14. Wilske B, Fingerle V. [Diagnosis of Lyme borreliosis. How to corroborate suspected borreliosis]. *MMW Fortschr Med*. 2000; 142: 28-31.
15. Engstrom SM, Shoop E, Johnson RC. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. *J Clin Microbiol*. 1995; 33: 419-427.
16. Dressler F, Whalen JA, Reinhardt BN, Steere AC. Western blotting in the serodiagnosis of Lyme disease. *J Infect Dis*. 1993; 167: 392-400.
17. Steere AC, McHugh G, Damle N, Sikand VK. Prospective study of serologic tests for Lyme disease. *Clin Infect Dis*. 2008; 47: 188-195.
18. Benach JL, Coleman JL, Habicht GS, MacDonald A, Grunwaldt E, Giron JA. Serological evidence for simultaneous occurrences of Lyme disease and babesiosis. *J Infect Dis*. 1985; 152: 473-477.
19. De Martino SJ, Carlyon JA, Fikrig E. Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis. *N Engl J Med*. 2001; 345: 150-151.
20. Nadelman RB, Horowitz HW, Hsieh TC, Wu JM, Aguero-Rosenfeld ME, Schwartz I, et al. Simultaneous human granulocytic ehrlichiosis and Lyme borreliosis. *N Engl J Med*. 1997; 337: 27-30.
21. Stricker RB, Harris NS, Yong DC, Winger EE. Clinical and seroepidemiologic characteristics of *Babesia WA-1* coinfection in patients with Lyme disease in California. *J Invest Med*. 2003; 51: S145.
22. Reed KD. Laboratory testing for Lyme disease: possibilities and practicalities. *J Clin Microbiol*. 2002; 40: 319-324.
23. Mavin S, Milner RM, Evans R, Chatterton JM, Joss AW, Ho-Yen DO. The use of local isolates in Western blots improves serological diagnosis of Lyme disease in Scotland. *J Med Microbiol*. 2007; 56: 47-51.
24. Shah JS, Du Cruz I, Wronska D, Harris S, Harris NS. Comparison of specificity and sensitivity of Igenex Lyme western blots using Igenex criteria and CDC criteria for a positive western blot. *Townsend Letter*. 2007; 275: 129-135.

25. Aguero-Rosenfeld ME, Nowakowski J, Bittker S, Cooper D, Nadelman RB, Wormser GP. Evolution of the serologic response to *Borrelia burgdorferi* in treated patients with culture-confirmed erythema migrans. *J Clin Microbiol.* 1996; 34: 1-9.
26. Marangoni A, Sparacino M, Cavrini F, Storni E, Mondardini V, Sambri V, et al. Comparative evaluation of three different ELISA methods for the diagnosis of early culture-confirmed Lyme disease in Italy. *J Med Microbiol.* 2005; 54: 361-367.
27. Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis by western immunoblot: reactivity of various significant antibodies against *Borrelia burgdorferi*. *J Clin Microbiol.* 1992; 30: 370-376.
28. Steere AC, Sikand VK, Schoen RT, Nowakowski J. Asymptomatic infection with *Borrelia burgdorferi*. *Clin Infect Dis.* 2003; 37: 528-532.
29. Steere AC, Sikand VK, Meurice F, Parenti DL, Fikrig E, Schoen RT, et al. Vaccination against Lyme disease with recombinant *Borrelia burgdorferi* outer-surface lipoprotein A with adjuvant. Lyme Disease Vaccine Study Group. *N Engl J Med.* 1998; 339: 209-215.
27. Ma B, Christen B, Leung D, Vigo-Pelfrey C. Serodiagnosis of Lyme borreliosis