Original Article

A Study of the Technique of Western Blot for Diagnosis of Lyme Disease caused by *Borrelia afzelii* in China*

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Abstract

Objective To study the technique of Western blot for the diagnosis of Lyme disease caused by *Borrelia afzelii* in China and to establish the standard criteria by operational procedure.

Methods FP1, which is the representative strain of *B. afzelii* in China, was analyzed by SDS-PAGE, electro transfer and immunoblotting assays. The molecular weights of the protein bands of FP1 were analyzed by Gel-Pro analysis software. In a study using 451 serum samples (159 patients with Lyme disease and 292 controls), all observed bands were recorded. The accuracy of the WB as a diagnostic test was established by using the ROC curve and Youden index.

Results Criteria for a positive diagnosis of Lyme disease were established as at least one band of P83/100, P58, P39, OspB, OspA, P30, P28, OspC, P17, and P14 in the IgG test and at least one band of P83/100, P58, P39, OspA, P30, P28, OspC, P17, and P41 in the IgM test. For IgG criteria, the sensitivity, specificity and Youden index were 69.8%, 98.3%, and 0.681, respectively; for IgM criteria, the sensitivity, specificity and Youden index were 47%, 94.2%, and 0.412, respectively.

Conclusion Establishment of WB criteria for *B. afzelii* is important in validating the diagnostic assays for Lyme disease in China.

Key words: Lyme disease; Western blot; Diagnostic method; *Borrelia afzelii*

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INTRODUCTION

Lyme disease, or Lyme borreliosis (LB) caused by the bacterium *Borrelia burgdorferi* (*B. burgdorferi*), is the most frequently reported tick-borne disease in the United States. It is also found in parts of Europe, Asia and other temperate regions of the world[1]. Erythema migrans (EM), the characteristic expanding rash of early localized Lyme disease, is present in a few cases, while joint pain, neurologic, cardiac and the other manifestations are present in later stages of Lyme disease[2]. Diagnosis of the disorder is mainly based on clinical symptoms and serodiagnose.Up

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until now, the enzyme-linked immune sorbent assay (ELISA) and indirect immunofluorescence assay (IFA) have been the most common serological methods used for screening for the disease, while Western blots (WB; immunoblots) have been used to confirm positive cases[3-5]. However, inherent problems of these tests are the occurrence of cross-reacting antibodies[6] leading to false-positive results, while a few patients may still be seronegative in the early stage of the infection. In addition, the sensitivity and specificity of these seroassays vary between different laboratories because of a lack of standardization.

The important *Borrelia* antigens of Lyme disease spirochete, *B. burgdorferi*, such as P83/100[7-8], P66[9], and the outer-surface protein (Osp)[10-11], have been identified and characterized by immunological and molecular biological investigations. *Borrelia* heat shock proteins (Hsp), of the Hsp60 and Hsp70 families (homologs of *Escherichia coli* GroEL and DnaK), have also been studied[12-15].

Different genotypes of Lyme disease spirochetes can result in different disease characteristics. There are also differences between the antigen quality and geographical region. Because of this, in 1995 and in 1997, researchers in the United States and Europe separately established specific criteria for positively identifying different genotypes using WB[16-18]. In China, a diagnostic assay for a standardized WB to identify the predominant species, *Borrelia garinii* (*B. garinii*), was established in 2010[19]. To validate the serological diagnosis of Lyme disease in China, we have in this study established a standard WB method for *Borrelia afzelii* (*B. afzelii*), which is the second most common pathogenic genotype in China.

MATERIALS AND METHODS

Serum Samples

A total of 451 serum samples were used in the study; 159 from patients with various stages of Lyme disease and 292 from controls. Fifty-two serum specimens were from untreated patients with EM provided by a dermatologist. Sixty-five samples were from a neuroborreliosis (NB) group consisting of 25 patients (designated group NB I) from whom *B. burgdorferi sensu stricto* could be grown from culture from cerebrospinal fluid (CSF) specimens and 40 other patients (designated group NB II) with characteristic symptoms of acute NB, CSF/serum antibody indices≥2.0 and negative results from CSF cultures. All serum and CSF samples were obtained on the same day. Forty-two patients (28 with acrodermatitis chronica atrophicans (ACA) diagnosed by a dermatologist and 14 patients with Lyme arthritis) made up a group with late LB. Possible differential diagnoses had been excluded. The control group comprised 105 healthy blood donors, 58 patients with syphilis in stage II or III, 75 patients with Leptospirosis and 54 with Rheumatoid arthritis (RA). The healthy blood donors had no history of frequent tick bites, or related clinical symptoms, such as erythema, neurological symptoms, or joint disorders.

Bacterial Culture and Antigen Preparation

B. afzelii strain FP1, which had been isolated from the blood of a patient with neuroborreliosis in Nanchuan county, Chongqing municipality in the Sichuan Province, China, in 1991[20-21], was used for antigen preparation. FP1 (approximately 20 passages) was grown in Barbour-Stoenner-Kelly (BSK) medium at 33 °C for 4 to 7 days, until a cell density of 10^7/mL was reached. Cells were harvested by centrifugation at 12 000 revolutions per minute (rpm) for 30 min at 4 °C and were washed four times with phosphate buffered saline (PBS) at 0.01 mol/L pH 7.4. The final pellet was suspended again in PBS and protein determinations were performed using the Bradford protein assay (Bio-Rad, Munich, Germany). The antigen preparations were stored at -20 °C until use. Samples of antigen of the PD91 strain of *B. burgdorferi* were used for comparison with strain FP1.

SDS-PAGE and WBs

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed by standard methods. The prepared antigen was dissolved in an equal volume of lysing buffer (2× protein electrophoresis on sample short-term buffer RT209 TANGEN), and heated for 10 min at 100 °C. Protein was run on polyacrylamide gels (12 acrylamide/bisacrylamide ratio 29:1; 12 cm by 14 cm by 16 mm) at 120 to 150 V for 7 h at room temperature. The proteins were transferred to nitrocellulose membrane (16 cm × 16 cm chemical, factory in Beijing) for 2 h at 24 V and 200 mA. After transfer, the membrane was blocked with nonfat dried milk diluted in PBS (0.01 mol/L, pH 7.0, PBS: Tween 20=2000:1) for 24 h at 4 °C. It was washed with PBS, dried and cut into strips 3 mm in width, and stored at 4 °C until used. Antigen strips were incubated for 4 h at room temperature in sera diluted 1:25 with PBS/Tween 20 (PBST) for both IgG and IgM, washed five times with PBST (0.01 mol/L,

pH 7.0, PBS:Tween 20=2000:1) for no less than 10 min each time and then incubated for 2 h with horseradish peroxidase-conjugated rabbit antihuman IgG (1:8000) and IgM (1:6000) antibodies, respectively (Sigma). After washing for 10 min with PBST, the color indicator of the antigen-antibody reaction was developed by adding 4-Chloro-1- Naphthol and H_2O_2. When the positive control serum sample reached a defined intensity, the reaction was stopped by 90% H_2SO_4. Serum samples from subjects in the different study groups were incubated with strips that were obtained from the same gel to avoid any bias caused by procedural variation. In each test, control samples of each antigen were incubated in parallel with the test samples. The position of control and duplicate strips were always fixed in the same position for documentation and analysis to ensure a high degree of accuracy.

Analysis of WBs

Interpolation between molecular mass (MM) marker lanes was used for calculating the positions of bands, and the molecular weight of protein bands of the strain FP1 were confirmed using the Gel-Pro analysis software, version 1.0 (GAS7001B, Uvi Company, Combrige, British). To further confirm the positions of the protein bands, WB strips were incubated with serum of a rabbit immunized with the FP1 and with an IgG positive control sample that acted as MM markers. After all bands had been identified, blots were also analyzed visually. Band intensities were determined semi-quantitatively by comparison with defined control sera. Data were recorded in a data bank for further analyses.

Statistics

Fisher's exact test (two-tailed) and McNemar's χ^2 test (two-tailed) were used for analysis of the data. Receiver operating characteristic (ROC) curves were obtained by plotting sensitivity against specificity for various cutoff values.

ELISA Assay

One hundred and seventeen cases (52 EM cases and 65 NB cases) were tested for IgM antibody and 159 cases (52 EM cases, 65 NB cases and 42 late-LB cases) were tested for IgG antibody by the ELISA method. Two hundred and ninety-two control group sera were also tested by the ELISA method.

The details of the ELISA assay were as follows: Each sample was diluted 1:500 with PBST (0.01 mol/L pH 7.4), and then added in duplicate into two sets of microtiter plates that had previously been coated with whole cell antigen. Negative, positive and blank control samples were added to each assay. The plates were incubated at 37 °C for 40 min, then washed five times with PBST (0.01 mol/L pH 7.4), and air dried at room temperature. Horseradish perxidase-conjugated goat antihuman IgM and IgG antibody (SIGMA) were added to the two sets of plates, respectively. After 40 min incubation, plates were washed five times with PBST (0.01 mol/L pH 7.4), and air dried at room temperature. The reaction was developed by adding 50 μL color solution (BIOSUBSTRATE, Beijing, China). The color development was stopped by adding 50 μL 2 mol/L H_2SO_4 to each well. The OD values were measured by a microplate reader (Bio-Rad, Model 550) at 495 nm; the result was considered positive when the OD_{495} value was more than 0.13[22].

RESULTS

Identification of Bands

According to our research, the main proteins of FP1 were P83/100, P75, P66, P60, P58, P43, P41, P39, OspB, OspA, P30, P28, OspC, P17, and P14 (Figure 1A). P39 could clearly be differentiated from P41, and OspA could clearly be differentiated from P30, which could clearly be distinguished from P28. There was also a clear distinction between P60 and P58. It was shown that FP1 and PD91 protein band differences focused mainly on proteins below 36 kD on the SDS-PAGE. OspA, OspB and proteins with a small molecular weight, such as the PC protein, showed a high level of protein polymorphism (Figure 1B).

Western Blot Reactivity of Clinically Defined Sera

Specimens were grouped by reported clinical symptoms, and then were tested by the WB method. The results are shown in Figure 2.

The frequency of reactivity of the bands discovered from sera of subjects in the various study groups is summarized in Table 1. To compare with the frequencies of band recognition for each study group with Lyme disease and those for the total control groups (blood donors, patients with syphilis stage II or III, patients with Leptospirosis and patients with RA were analyzed statistically, and bands with highly significant differences between Lyme disease case groups and control groups (P<0.001) are boxed.

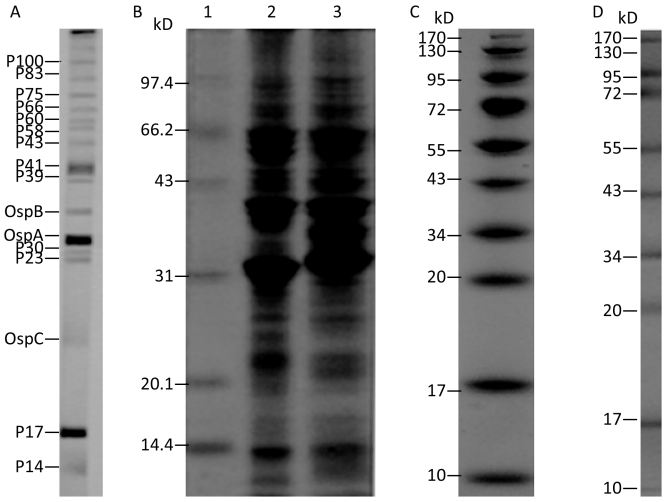

Figure 1. Antigen Identification of FP1 with different assays. A: Immunoblots of FP1 strain with positive rabbit sera. B: Coomassie brilliant blue-stained SDS-polyacrylamide gel of the antigen preparations used for this study. Lane 1, Molecular weight markers for proteins; Lane 2, strain PD91; Lane 3, strain FP1. C: Coomassie brilliant blue-stained SDS-polyacrylamide gel of Prestained Marker. D: Prestained Marker, which transferred to NC membrane.

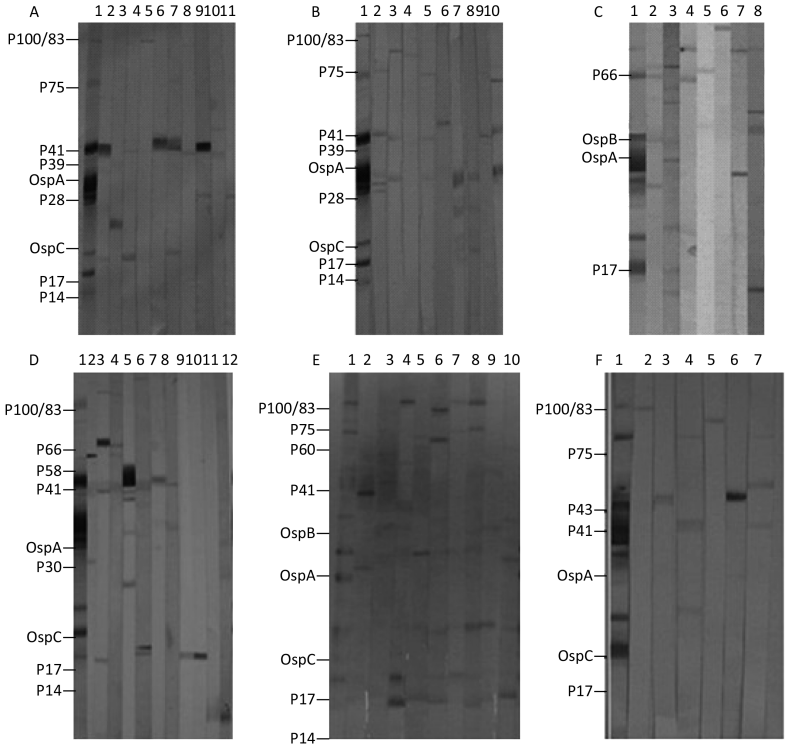

Figure 2. Representative WBs of strain FP1 with sera from various groups of Lyme disease patients and controls. A: Lane 1, the positive control (the serum of rabbit immunized against strain FP1); Lane 2-11, WB IgG of strain FP1 with sera from EM patients. B: Lane 1, the positive control; Lane 2-10, IgM WB with sera from NB patients. C: Lane 1, positive control; Lane 2-8, IgG WB with sera of EM patients. D: Lane 1, the positive control; Lane 2-12, IgG WB from NB patients. E: Lane 1, the positive control; Lane2-10, IgG WB with sera from late-stage Lyme disease, including Lane 2-5, IgG WB with sera from Lyme disease arthritis and lane 6-10 WB with ACA patients. F: Lane 1, the positive control; WB with patients' sera from throughout the country. National Institute for Communicable Disease Control and Prevention, Chinese Center for Disease Control and Prevention provided WB with patients' sera from throughout the country. Lane 2-5, IgG WB; Lane 6-7, IgM WB.

Table 1. Frequency of Recognition of Protein of Strain FP1

Protein Band	Molecular Weight (KD)	%Sera with IgG Reactivity to Protein Band[a]							%Sera with IgM Reactivity to Protein Band[a]					
		LB Sera			Control Group Sera				LB Sera		Control Group Sera			
		EM	NB	Late	Syphilis	Leptospirosis	Health	RA	EM	NB	Syphilis	Leptospirosis	Health	RA
		n=52	*n*=65	*n*=42[b]	*n*=58	*n*=75	*n*=105	*n*=54	*n*=52	*n*=65	*n*=58	*n*=75	*n*=105	*n*=54
P83/100	100/83	6*	11*	18*					2*	4*				
P75	75	3	2	7*	4		3			3	3	6	1	
P66	66	4	5	7*			2	2						
P60	60	3	2	6	3	6	3	3	1	1	6	1	2	1
P58	58	5*	5*	11*					2*	3*				
P43	43	2	2	5*	4		1	3			3			
P41	41	15*	20*	25*	8	10	7	7	11*	15*	5	8	2	2
P39	39	5*	13*	10*					4*	4*				
OspB	35	2*	5*	9*										
OspA	32	12*	12*	17*			4		3*	6*				1
P30	30	4*	3*	4*					2*	1				
P28	28	2*	2*	7*						1				
OspC	22	2*	5*	12*					5*	5*				
P17	17	3*	7*	7*			1		1	3*				
P14	14	6*	3*	18*			1							

Note. [a]*:* Frequencies of band recognition of sera from patients with LB versus those of sera from total control group (*n*=292) were analyzed by Fisher's exact test. If *P*<0.001, *frequencies were showed in Table1. Zeros were not showed to improve clarity. [b]*:* Sera from patients with late-stage LB were only tested for IgG.

The antigens cross-reactive between Lyme disease patients and syphilis patients in *B. afzelii* were P75, P60, P43, and P41 and the cross-reactive antigens between Lyme disease patients and Leptospirosis patients were P75, P60, and P41 (Figure 3).

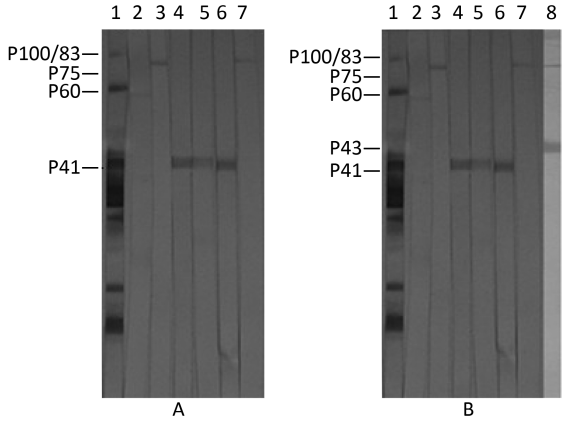

Figure 3. Results of cross-reactivity. A: Cross-reaction of Lyme disease and Leptospirosis. Lane 1, positive control (the serum of rabbit immunized against strain FP1); Lane 2-5, WB IgG of strain FP1 with sera from Leptospirosis patients; Lane 6 and 7, WB IgM of strain FP1 with sera from Leptospirosis patients. B: Cross-reaction of Lyme disease and syphilis. Lane 1, the positive control (the serum of rabbit immunized against strain FP1); Lane 2-5, WB IgG of train FP1 with sera from syphilis patients; Lane 6 and 7, WB IgM of strain FP1 with sera from syphilis patients.

Definition of Diagnostic Method for Positive WB Results

To establish the criteria of WB for diagnosis of Lyme disease infected with *B. afzelii*, two questions needed to be addressed: (i) What protein combination from the 15 major protein bands should be used in the measurement of the test? (ii) What optimum development time would achieve the best diagnostic performance in the WB to allow the desired protein band combinations to be scored? The ROC curve is a helpful method of measuring these requirements.

There were too many combinations of the 15 major bands to analyze all conceivable combinations, therefore the following strategies were established. Combinations of bands showing no false-positive reactions were chosen first. Then, significant bands ($P<0.05$) obtained by testing the result among the LB group versus the results among the whole control group were added stepwise to the putative criteria. More than 50 band combinations were assessed for IgG tests and more than 10 band combinations were assessed for IgM tests.

Table 2 shows areas under the ROC curves of certain band combinations for the IgG test (Areas under ROC curves >0.800). Table 3 shows areas under ROC curve of band combinations for the IgM test (Areas under ROC curves >0.700).

Using these optimal ROC curves, we selected the optimal combination of IgG or IgM bands that would give a good differential diagnosis. For IgG blots, the best ROC results occurred with the combination of the 10 most common bands including P83/100, P58, P39, OspB, OspA, P30, P28, OspC, P17, and P14. When at least one of these protein bands was positive, the sero-antibody IgG was considered positive. For IgM blots, the best combination was that of the nine most common bands including P83/100, P58, P39, OspA, P30, P28, OspC, P17, and P41. When at least one of these protein bands was positive, the seroantibody IgM was considered positive. This diagnostic method is important for verifying the statistical significance in WBs for LB. However, if P41 is the only reactive band for IgM, then syphilis and Leptospirosis infection or other related diseases should be excluded. An unacceptably high level of cross-reactivity occurred between Lyme disease and these diseases in P41 that could lead to a high number of false-positives.

For IgG positive results, the sensitivity, specificity and Youden index were 69.8%, 98.3%, and 0.681, respectively; for IgM positive results the sensitivity, specificity and Youden index were 47%, 94.2%, and 0.412, respectively (Table 4).

Sensitivity and specificity of diagnosis for the infection of *B. garinii* was higher than that of *B. afzelii* in the WB IgG result, while for the IgM result, sensitivity for diagnosis of *B. afzelii* is less than that of *B. garinii* and specificity for diagnosis of *B. afzelii* is higher than that of *B. garinii*. Comparison with WB diagnosis methods in Europe shows that the sensitivity and specificity of our results for *B. afzelii* genotype in WB was higher for IgG but lower for IgM (Table 5).

Comparison of IgM Tests for 117 LB Cases and 292 Control Group Sera with ELISA and WB

The ELISA-positive rates of EM, NB and control group sera were 57.69% (30/52), 61.54% (40/65), and

Table 2. Areas under the ROC Curves of Band Combinations for the IgG Test

Band Combination	Area under ROC Curve	SE	95% Confidence Interval
P83/100, P58, P39, OspB, P30, P28, OspC, OspA	0.806	0.025	0.758-0.855
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P41	0.801	0.024	0.753-0.848
P83/100, P58, P39, OspB, P30, P28, OspC, P75, OspA	0.807	0.025	0.759-0.855
P83/100, P58, P39, OspB, P30, P28, OspC, P66, P41	0.812	0.024	0.765-0.858
P83/100, P58, P39, OspB, P30, P28, OspC, P66, OspA	0.819	0.024	0.772-0.866
P83/100, P58, P39, OspB, P30, P28, OspC, P66, P14	0.801	0.025	0.752-0.850
P83/100, P58, P39, OspB, P30, P28, OspC, P60, OspA	0.802	0.025	0.754-0.850
P83/100, P58, P39, OspB, P30, P28, OspC, P43, OspA	0.803	0.025	0.754-0.851
P83/100, P58, P39, OspB, P30, P28, OspC, P41, OspA	0.833	0.023	0.788-0.877
P83/100, P58, P39, OspB, P30, P28, OspC, P41, P17	0.815	0.024	0.768-0.861
P83/100, P58, P39, OspB, P30, P28, OspC, P41, P14	0.820	0.023	0.774-0.866
P83/100, P58, P39, OspB, P30, P28, OspC, OspA, P17	0.828	0.024	0.782-0.875
P83/100, P58, P39, OspB, P30, P28, OspC, OspA, P14	0.829	0.024	0.782-0.875
P83/100, P58, P39, OspB, P30, P28, OspC, P17, P14	0.806	0.025	0.757-0.854
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P41	0.804	0.024	0.757-0.851
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, OspA	0.818	0.024	0.771-0.864
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P14	0.802	0.025	0.754-0.851
P83/100, P58, P39, OspB, P30, P28, OspC, P66, P60, P41	0.817	0.023	0.772-0.863
P83/100, P58, P39, OspB, P30, P28, OspC, P66, P60, OspA	0.811	0.024	0.764-0.858
P83/100, P58, P39, OspB, P30, P28, OspC, P43, P41, OspA	0.823	0.023	0.778-0.868
P83/100, P58, P39, OspB, P30, P28, OspC, P43, OspA, P17	0.825	0.024	0.779-0.871
P83/100, P58, P39, OspB, P30, P28, OspC, P43, OspA, P14	0.827	0.023	0.781-0.873
P83/100, P58, P39, OspB, P30, P28, OspC, P41, OspA, P17	0.851	0.021	0.809-0.893
P83/100, P58, P39, OspB, P30, P28, OspC, P41, OspA, P14	0.853	0.021	0.811-0.895
P83/100, P58, P39, OspB, P30, P28, OspC, OspA, P17, P14 *	0.843	0.023	0.798-0.888
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P41	0.809	0.023	0.764-0.855
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, OspA	0.811	0.024	0.764-0.858
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41	0.809	0.023	0.763-0.854
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41, OspA	0.836	0.022	0.794-0.878
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41, P17	0.832	0.022	0.789-0.875
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41, P14	0.836	0.022	0.793-0.878
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41, OspA, p17	0.869	0.019	0.832-0.906
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41, OspA, p14	0.868	0.019	0.830-0.905
P83/100, P58, P39, OspB, P30, P28, OspC, P75, P66, P60, P43, P41, OspA, p17, P14	0.857	0.021	0.816-0.897

Note. *The area under ROC curve shows the optimal band combination in IgG test.

Table 3. Areas under the ROC Curves of Band Combinations for the IgM Test

Band Combination	Area under ROC Curve	SE	95% Confidence Interval
P83/100, P58, P39, OspA, P30, P28, OspC, P17, P41*	0.709	0.032	0.647-0.771
P83/100, P58, P39, OspA, P30, P28, OspC, P17, P75, P41	0.704	0.031	0.643-0.766
P83/100, P58, P39, OspA, P30, P28, OspC, P17, P43, P41	0.707	0.032	0.645-0.769

Note. *The area under ROC curve shows the optimal band combination in IgM test.

Table 4. Evaluation of Various Interpretation Criteria for a Positive WB Result

Ig Class	Band Required	Sensitivity (%)	Specificity (%)	Youden Index
IgG	≥1 of P83/100, P58, P39, OspB, P30, P28, OspC	51.6	100.0	0.516
	≥1 of P83/100, P58, P39, OspB, P30, P28, OspC, P43, OspA, P17	67.9	95.9	0.638
	≥1 of P83/100, P58, P39, OspB, P30, P28, OspC, P43, OspA, P14	68.6	95.5	0.641
	≥1 of P83/100, P58, P39, OspB, P30, P28, OspC, OspA, P17, P14*	69.8	98.3	0.681
IgM	≥1 of P83/100, P58, P39, OspA, P30, P28, OspC, P17	33.3	100.0	0.333
	≥1 of P83/100, P58, P39, OspA, P30, P28, OspC, P17, P75	35.0	96.6	0.316
	≥1 of P83/100, P58, P39, OspA, P30, P28, OspC, P17, P41*	47.0	94.2	0.412
	≥1 of P83/100, P58, P39, OspA, P30, P28, OspC, P17, P75, P41	48.7	91.4	0.401
	≥1 of P83/100, P58, P39, OspA, P30, P28, OspC, P17, P43, P41	47.0	93.8	0.408
	≥1 of P83/100, P58, P39, OspA, P30, P28, OspC, P17, P60, P43, P41	47.0	91.1	0.381

Note. *The interpretation criteria were recommended for IgG and IgM.

Table 5. Comparison of Different Interpretation Criteria

Interpretation Criteria	Strain	IgG		IgM	
		Sensitivity(%)	Specificity(%)	Sensitivity(%)	Specificity(%)
China	PD91	73.2	99.4	50.6	93.1
	FP1	69.8	98.3	47.0	94.2
Europe	PKo	56.1	97.9	42.3	98.6

19.86% (58/292), respectively; the WB-positive rates of EM, NB and control group sera were 46.15% (24/52), 49.23% (32/65), and 5.82% (17/292), respectively. The positive results of 117 LB patients of two methods were analyzed by χ^2 test, which showed statistical difference (χ^2=5.63, P<0.05). The positive results of 292 control group sera of two methods were analyzed by χ^2 test, which also showed statistical difference (χ^2=35.77, P<0.01) (Table 6).

Comparison of IgG Tests for 159 LB Cases and 292 Control Sera with ELISA and WB

The ELISA-positive rates of EM, NB, late LB and control group sera were 78.85% (41/52), 84.62% (55/65), 85.71% (36/42), and 22.95% (67/292), respectively; the WB-positive of EM, NB, late-LB and control group sera were 67.31% (35/52), 69.23% (45/65), 71.43% (30/42), and 1.71% (5/292), respectively. The positive results of 159 LB patients of two methods were analyzed by χ^2 test, which showed statistical difference (χ^2=8.00, P<0.01). The positive results of 292 control group sera of two methods were analyzed by χ^2 test, which also showed statistical difference (χ^2=60.06, P<0.01) (Table 7).

DISCUSSION

Three pathogenic genotypes causing Lyme disease exist in China. *B. garinii* is the predominant species, *B. afzelii* is the second most common species and *Borrelia burgdorferi sensu stricto* is rare[21]. Jiang et al.[19] reported the interpretation criteria of WB for the diagnosis of *B. garinii* infection in 2010. According to the relevant literature, protein

Table 6. Comparison of IgM Tests with ELISA and WB

Clinical Symptom	No. of Tested	ELISA		WB	
		No. of Positive	Positive Rate(%)	No. of Positive	Positive Rate(%)
EM	52	30	57.69	24	46.15
NB	65	40	61.54	32	49.23
Control Group Sera	292	58	19.86	17	5.82
Total	409	128	31.30	73	17.85

Table 7. Comparison of IgG Tests with ELISA and WB

Clinical Symptom	No. Tested	ELISA		WB	
		No. of Positive	Positive Rate(%)	No. of Positive	Positive Rate(%)
EM	52	41	78.85	35	67.31
NB	65	55	84.62	45	69.23
Late-LB	42	36	85.71	30	71.43
Control Group Sera	292	67	22.95	5	1.71
Total	451	199	44.12	115	25.50

profiles, plasmid profiles and monoclonal antibody responses between genomic species of *Borrelia bugdorferi* are different. Therefore, the aim of the study was to validate the WB assay for the diagnosis of Lyme disease in China. We chose the representative strain FP1 of *B. afzelii* as the antigen for the assays. It was isolated from the blood of a patient with facial paralysis in Sichuan Province in 1991[20-21]. Later, a series of studies confirmed that strain FP1 is stable in passage, has a low mutation rate and has relatively clear and complete protein profiles, conducive to serological diagnosis and statistical analysis.

Currently, researchers have indicated that there are more than 30 different *Borrelia* antigens in the *Borrelia burgdorferi* species, but that the protein map of various *Borrelia burgdorferi sensu lato* from different regions and genospecies may vary. The investigations in China have also demonstrated that genomic species found in China have highly polymorphic and unique patterns of protein. In our study, Gel-Pro Analyzer was used to analyze the FP1 SDS-PAGE results, followed by comparison of controls of strain FP1 reactive with positive FPI-immunized rabbit sera. The main antigens identified were P83/100, P75, P66, P60, P58, P43, P41, P39, OspB, OspA, P30, P28, OspC, P17, and P14. The 97 kD and the 83 kD protein were attributed to the protein P83/100. The molecular weights of, OspA, OspB, and OspC were 32 kD, 36 kD, and 22 kD, respectively.

It is important to establish the antigenic composition of *B. burgdorferi sensu lato* in relation to immunodiagnosis. Numerous early studies illustrated the importance of P83/100 as an immunogen for diagnosis of Lyme disease, especially for the stage III of Lyme borreliosis[23]. The recombinant P83/100 protein can induce to produce both IgG and IgM antibodies and can be used as one of the specific antigens in the serodiagnostic tests[24]. In the early stages, one of the most immunodominant antigens in infection with *B. burgdorferi sensu lato* is the OspC protein[25-27], which begins to be expressed during tick feeding when the spirochete is still in the tick midgut. OspC is not expressed during high-passage in the vitro-cultured *B. burgdorferi sensu lato*, which explains why the importance of this antigen was not recognized earlier because of the use of high-passage *B. burgdorferi* as the source of antigen[28-30]. Many studies have shown that OspA is a strong immunogenic antigen, which stimulates the body to produce a high-titer of immunoprotective antibody and can therefore be used both for serodiagnosis and vaccine development[31]. P75 and P60 are heat shock proteins of the HSP60 and HSP70 families. They exist widely in prokaryotic and eukaryotic cells and possess high homologies. Some databases have illustrated that they also exist in syphilis and Leptospirosis, so there is some cross-reactivity in their use for diagnosis[32-33]. Numerous early studies recognized the importance

of the flagellar protein flagellin (41 kD) as an immunodominant antigen. However, this is highly conserved (96%-97%) among other spirochetes and microorganisms of distant evolutionary relationship with which there is significant homology. Strong IgG and IgM responses to this protein are developed within a few days of infection with *B. burgdorferi* sensu lato, but the antigen is highly cross-reactive with antigens found in other spirochetes and some mammalian tissues. In this study, cross-reaction rates of P41 with sera from syphilis, and Leptospirosis patients were 8.62% and 0.67%, respectively, which is similar to other national and international reports[19,34]. The chromosomally encoded P39 is also an important immunogen. Bingnan and other researchers[35] have reported that there are similarities in structure between P39 and the flagellin protein, but their immunogenetic profiles are completely different. P39 has a strong immunogenicity and can be used for serological diagnosis in early Lyme disease[36-37].

In this study, 12% polyacrylamide gels of 14 cm length increased the resolution and the ROC curve was used to analyze different protein combinations. This avoided the problem experienced in those methods where at least two bands were positive, leading to considerable loss of sensitivity. Therefore, the following diagnostic methods are recommended for patients with *Borrelia afzelii* infection in China. For IgG blots, the combination of P83/100, within which are P58, P39, OspB, OspA, P30, P28, OspC, P17, and P14, should be used and a positive recorded when at least one protein band is positive with the IgG seroantibody. For IgM, the combination of P83/100, with P58, P39, OspA, P30, P28, OspC, P17, and P41, should be used and a positive recorded when at least one protein band is positive with the IgM seroantibody IgM. However, if P41 is the only reactive band for IgM, then syphilis and Leptospirosis infection should be excluded. The areas under the ROC curves were 0.843 and 0.709, indicating that the accuracy of the diagnostic method was moderate. At the same time, sensitivity, specificity and the Youden index for this method showed that it was optimal between the various combinations. When using this method to diagnose Lyme disease, its sensitivity is relatively lower (with 69.8% for IgG and 47% for IgM), while its specificity is higher (with 98.3% for IgG and 94.2% for IgM) than the ELISA test. The requirement of a WB as a confirmation test in a two-step protocol was that it should be able to distinguish true-positive and false-positive results, with high specificity. This method just meets this requirement, so it may be well suited to diagnose patients with infectious *Borrerlia afzelii* in China.

Comparison of the results of ELISA and WB methods showed that the sensitivity of the ELISA technique was higher than WB, but the specificity was lower. These results were similar to reports by other workers[2,24].

The recommendations of this study on the optimization of the WB technique for diagnosis of Lyme disease caused by *Borrelia afzelii* are a significant contribution to the epidemiology of this disease in China.

REFERENCES

1. Norman GL, Antig JM , Bigaignon WR, et al. Serodiagnosis of Lyme Borreliosis by *Borrelia burgdorferi* Sensu Stricto, *B. garinii*, and *B. afzelii* Western Blots (Immunoblots). J Clin. Microbiol, 1996; 7(34), 1732-8.
2. Steere AC, Lyme Disease. N Engl J Med, 2001; 345, 115-25.
3. Craft JE, Fischer DK, Shimamoto GT, et al. Antigens of Borrelia burgdorferi recognized during Lyme disease: appearance of a new immunoglobulin M response and expansion of the immunoglobulin G response late in the illness. J Clin. Invest, 1986; 78, 934-9.
4. Craft JE, Grodzicki RL, Steere AC. Antibody response in Lyme disease: evaluation of diagnostic tests. J Infect Dis, 1984; 149, 789-95.
5. Hensen K, Hindersson P, Pedersen NS. Measurement of antibodies to Borrelia burgdorferi flagellum improves serodiagnosis in Lyme disease. J. Clin. Microbiol, 1988; 26, 338-46.
6. Bruckbauer H, Preac-Mursic V, Wilske B. Crossreactive proteins of Borrelia burgdorferi. Eur. J Clin. Microbiol. J Infect. Dis, 1992; 11, 1-9.
7. Jauris-Heipke S, Fuchs R, Lottspeich F, et al. Molecular characterization of the p100 gene of Borrelia burgdorferi strain PKo. J FEMS Microbiol. Lett, 1993; 114, 235-42.
8. LeFebvre RB, Perng GC, Johnson RC. The 83-kilodalton antigen of *Borrelia burgdorferi* which stimulates immunoglobulin M (IgM) and IgG responses in infected hosts is expressed by a chromosomal gene. J Clin. Microbiol, 1990; 28, 1673-6.
9. Bunikis J, Noppa L, Bergstro¨m S. Molecular analysis of a 66-kDa protein associated with the outer membrane of Lyme disease Borrelia. J FEMS Microbiol. Lett, 1995; 131, 139-45.
10. Barbour AG, Tessier SL, Todd WJ. Lyme disease spirochetes and ixodid tick spirochetes share a common surface antigenic determinant defined by a monoclonal antibody. J Infect. Immun, 1983; 41, 795-804.
11. Wilske B, Göbel V, Graf UBB, et al. An OspA serotyping system for *Borrelia burgdorferi* based on reactivity with monoclonal

antibodies and OspA sequence analysis. J Clin. Microbiol, 1993; 31, 340-50.

12. Hansen K , Bangsborg J, Pedersen MH, et al. Immunochemical characterization and isolation of the gene for a *Borrelia burgdorferi* immunodominant 60-kilodalton antigen common to a wide range of bacteria. J Infect Immun, 1988; 56, 2047-53.
13. Luft BJ, Jiang PD, Munoz WP, et al. Immunologic and structural characterization of the dominant 66- to 73-kDa antigens of Borrelia burgdorferi. J Immunol, 1991; 146, 2776-82.
14. Stamm LV, Gherardini F, Parrish CEA, et al. Heat shock response of spirochetes. J Infect Immun, 1991; 59, 1572-5.
15. Wallich R, Helmes C, Schaible UE, et al. Evaluation of genetic divergence among *Borrelia burgdorferi* isolates by use of OspA, *fla*, HSP60, and HSP70 gene probes. J Infect Immun, 1992; 60, 4856-66.
16. Engstrum SM, Shoop E, and Johnson RC. Immunoblot interpretation result for serodiagnosis of early Lyme disease. J Clin Microbiol, 1995; 33, 419-27.
17. Dressler F, Whalen JA, Reinhardt BN, et al. Western blotting in the serodiagnosis of Lyme disease. J Infect Dis, 1993; 167(2), 392-400.
18. Hauser U, Lehnert G, Lobentanzer R, et al. Interpretation result for standardized Western blots for three European species of Borrelia burgdorferi sensu lato. J Clin Microbiol, 1997; 35(6), 1433-44.
19. Jiang Y, Hou XX, Geng Z, et al. Interpretation result for standardized Western blot for the predominant species of *Borrelia burgdorferi* Sensu Lato in China. J Biomed Environ Sci, 2010; 239(5), 341-9.
20. Zhang ZF, Wan KL, Li YL, et al. Lyme disease spirochete isolated from the blood of one patient with facial paralysis in Sichuan province. J Vector Biology and Control, 1991; 2(6), 382-3. (In Chinese)
21. Hao Q, Hou XX, Geng Z, et al. Distribution of *Borrelia burgdorferi* Sensu Lato in China, J Clin. Microbiol, 2011; 49(2), 647-50.
22. Zhang ZF, Wan KL, Zhang JS, et al. Studies on epidemiology and etiology of Lyme disease in China. Chin J Epidemiol, 1997; 18(1), 8-11. (In Chinese)
23. Zoller L, Burkard S, Schafer H. Validity of Western immunoblot band patterns in the serodiagnosis of Lyme borreliosis. J Clin Microbiol, 1991; 29, 174-82.
24. Rossler D, Eiffert H, Jauris-Heipke S. Molecular and immunological characterization of the p83/100 protein of various Borrelia burgdorferi sensu lato strains. Med Microbiol Immunol (Berl), 1995; 184(1), 23-32.
25. Aguero-Rosenfeld ME, Nowakowski JD , McKenna F, et al. Serodiagnosis in early Lyme disease. J Clin Microbiol, 1993; 31, 3090-5.
26. Dressler F , Whalen A , Reinhardt N, et al. Western blotting in the serodiagnosis of Lyme disease. J Infect Dis, 1993; 167, 392-400.
27. Engstrom SM, Shoop E, Johnson RC. Immunoblot interpretation criteria for serodiagnosis of early Lyme disease. J Clin Microbiol, 1995; 33, 419-27.
28. Coleman JL , Benach JL. Isolation of antigenic components from the Lyme disease spirochete: their role in early diagnosis. J Infect. Dis, 1987; 155, 756-65.
29. Craft JE , Fischer DK, Shimamoto GT, et al. Antigens of *Borrelia burgdorferi* recognized during Lyme disease. Appearance of a new immunoglobulin M response and expansion of the immunoglobulin G response late in the illness. J Clin Investig, 1986; 78, 934-9.
30. Xu QL, Kristy MS, and Fang TL.Verification and dissection of the *ospC* operator by using *flab* promoter as a reporter in *Borrelia burgdorferi*. J Microb Pathog, 2008; 45(1), 70-8.
31. Langermann S, Palaszynski S, Sadziene A, et al. Systemic andmucosal immunity induced by BCG vector expressing outer-surface protein A of Borrelia burgdorferi. J Nature, 1994; 372(6506), 552-5.
32. Park SH, Ahn BY and Kim MJ. Expression and immunologic characterization of recombinant heat shock protein 58 of Leptospira species: a major target antigen of the humoral immune response. J DNA Cell Biol, 1999; 18(12), 903-10.
33. Polla BS. Heat (shock) and the skin. J Dermatologica, 1990; 180(3), 113-7.
34. Hauser U, Lehnert G, Lobentanzer R, et al. Interpretation result for standardized Western blots for three European species of *Borrelia burgdorferi sensu lato*. J Clin Microbiol, 1997; 35(6), 1433-44.
35. Ma B, Christten.B et al. Serodiaosis of Lyme borreliosis by Western Immunblot: reactivity of various significant antibodies against *Borrelia burgdorferi*. J Clin Microbiol, 1992; 30(2), 370-6.
36. Simpson WJ, Cieplak W, Schrumpf ME, et al. Nucleotide sequence and analysis of the gene in Borrelia burgdorferi encoding the immunogenic P39 antigen. J FEMS Microbiol Lett, 1994; 119(3), 381-7.
37. Anton V, Bryksin 1, Henry P, et al. *Borrelia burgdorferi* BmpA, BmpB, and BmpD Proteins Are Expressed in Human Infection and Contribute to P39 Immunoblot Reactivity in Patients with Lyme Disease. J Clin Diagn Lab Immunol, 2005; 8(12), 935-40.