Original Article

Evaluation of Six Recombinant Proteins for Serological Diagnosis of Lyme Borreliosis in China*

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Abstract

Objective In this study, we evaluated the diagnostic efficiency of six recombinant proteins for the serodiagnosis of Lyme borreliosis (LB) and screened out the appropriate antigens to support the production of a Chinese clinical ELISA (enzyme-linked immunosorbent assay) kit for LB.

Methods Six recombinant antigens, Fla B.g, OspC B.a, OspC B.g, P39 B.g, P83 B.g, and VlsE B.a, were used for ELISA to detect serum antibodies in LB, syphilis, and healthy controls. The ELISA results were used to generate receiver operating characteristic (ROC) curves, and the sensitivity and specificity of each protein was evaluated. All recombinant proteins were evaluated and screened by using logistic regression models.

Results Two IgG (VlsE and OspC B.g) and two IgM (OspC B.g and OspC B.a) antigens were left by the logistic regression model screened. VlsE had the highest specificity for syphilis samples in the IgG test (87.7%, $P<0.05$). OspC B.g had the highest diagnostic value in the IgM test (AUC=0.871). Interactive effects between OspC B.a and Fla B.g could reduce the specificity of the ELISA.

Conclusion Three recombinant antigens, OspC B.g, OspC B.a, and VlsE B.a, were useful for ELISAs of LB. Additionally, the interaction between OspC B.a and Fla B.g should be examined in future research.

Key words: Lyme borreliosis; Recombinant proteins; Serological diagnosis; Logistic regression; ROC

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INTRODUCTION

Lyme borreliosis (LB), a tick-borne zoonotic disease, is caused by *Borrelia burgdorferi* infection[1]. Many studies have demonstrated that *B. burgdorferi* has at least 15 genospecies, four of which are associated with human disease[2-6]. The major pathogen of LB is *B. burgdorferi* sensu stricto in the USA, while *B. afzelii* and *B. garinii* are the most frequently reported pathogens in Europe and China[2-6]. In China, a human LB case was first reported in 1985 in a forest region in Hailin County, Heilongjiang province. LB cases have been observed in 29 Chinese provinces/municipalities in 30 years. The major endemic areas are in the north forests of China. Based on a random sampling survey in 20 provinces, the serological positivity of LB was 5.06% (1.06%-12.8%) and the morbidity was 2.84% (1.16%-4.51%)[7].

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Biographical note of the first author LIU Wei, female, born in 1990, master's degree candidate, majoring in serodiagnosis of Lyme borreliosis.

LB is a multisystem illness. The early disease usually begins with erythema migrans (EM) skin lesions, the most common clinical manifestation. With disease progression, the spirochete might disseminate to other organs, leading to arthritis, neuroborreliosis, carditis, lymphocytoma, etc.[8-9]

Culture is still the gold standard in the laboratory diagnosis of LB, but its low detection rate and long assay time are not suitable for clinical practice[10-13]. Therefore, the diagnosis of LB is often based on the typical clinical manifestation of EM. In the absence of EM, laboratory serodiagnosis should be used. In the United States and parts of Europe, a two-step approach is recommended for LB serodiagnosis. The first step involves ELISA (enzyme-linked immunosorbent assay) or IFA (immunofluorescence antibody assay), which are used for initial screening, and the second step uses western blotting for reactive or equivocal samples obtained in the first step[8,14-15]. In China, a standard detection kit is still lacking. The whole cell proteins of *B. burgdorferi* are often used in the first step, but the whole cell bacterial protein cross-reacts with proteins from other bacteria, such as *Microspironema pallidum* and HGE (Human Granulocytic *Ehrlichia*)[16-18]. Many recombinant proteins have been examined to improve the specificity and sensitivity of Lyme serological tests, including OspC, Fla, P39, VlsE, BBK32, P37, P22, DbpA, P58, P18, OspA, etc.[16-17,19-21].

In this study, the diagnostic efficiency of six recombinant antigens of Chinese *B. burgdorferi* isolates were evaluated to identify the most appropriate antigen for LB serodiagnosis.

MATERIALS AND METHODS

Serum Samples

Three panels of serum samples were used in this study. The first panel of serum samples was obtained from LB patients at MuDanjiang Linye Hospital (Heilongjiang Province, China, 2012-2013). Clinical diagnosis of LB was based on the following conditions: 1, history of a tick bite; 2, EM, expanding red ≥5 cm in diameter, with or without central clearing; if <5 cm in diameter, a delay in appearance after the tick bite of at least 2 days, and an expanding rash at the site of the tick bite; 3, IFA or ELISA (positive, +); 4, western blot (positive, +); 5, other symptoms of LB, such as arthritis, carditis, and neuroborreliosis[8,22]. All participants who met conditions 1, 2, and 3 or conditions 1, 3, 4, and 5 were diagnosed as LB. Sample characteristics are summarized in Table 1. A total of 139 serum samples from LB patients, including 67 IgM and 72 IgG seropositive samples were used for ELISA. The second panel included 90 serum samples from healthy donors as controls. The third panel had 89 specimens obtained from syphilis patients at Urumqi First People's Hospital (Xinjiang Uygur Autonomous Region, China). These specimens were diagnosed as syphilis based on clinical manifestations and TPHA (*Treponema pallidum* hemagglutination) tests. All serum samples were stored at -80 °C before tests and each sample was divided into 3 Eppendorf tubes to minimize the loss of antibodies during freezing and thawing.

This study was approved by the Ethical Review Committee of the National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention (China CDC). Participants also provided written informed consent to participate in the study.

Antigens

Six proteins were examined in this study, four of which (P39 B.g, P83 B.g, OspC B.g, and Fla B.g) were cloned and expressed as described previously[23-26]. Primers for these antigens were described in previous studies[23-26] with slight modifications. In addition, a conserved region of VlsE from *B. afzelii* strain GDsh1 was expressed. This conserved region was identified in all VlsE sequences of *B. afzelii* strains in PubMed and was expressed for the first time in our study. Another protein, OspC B.a, and two *B. burgdorferi* strains used in this study were provided by the Department of Lyme borreliosis, National Institute for Communicable Disease Control

Table 1. Characterization of Lyme Borreliosis Serum Samples Used in the Study

Main Clinical Features	IFA IgM (+)/*n*	IFA IgG (+)/*n*
Erythema migrans (EM)	33	23
Erythema with general malaise	29	36
Neuropathy with EM	3	5
Lyme arthritis	1	8
Reinfected	1	0
Total	67	72

and Prevention, Chinese Center for Disease Control and Prevention (China CDC). *B. burgdorferi* strain PD91 was isolated from LB patients and GDsh1 was isolated from Rattus norvegicus. These strains were genotyped by a multilocus sequence analysis (MLSA). A whole cell ELISA kit was constructed using the PD91 strain. All primers and strains for candidate antigens are described in supplementary material Table S1.

ELISAs with Recombinant Antigens

Recombinant antigens and whole cells of *B. burgdorferi* were used to detect serum IgG and IgM antibodies by class-specific ELISAs. The optimal concentrations of all proteins were determined by checkerboard titrations[17]. The most appropriate serum dilution was 1:50, except for OspC at 1:100. The γ-chain-specific affinity-purified horseradish peroxidase-labeled goat anti-human IgG antibodies (Sigma, St. Louis, MO, USA) were diluted at 1:8000, while the μ-chain-specific affinity-purified horseradish peroxidase-labeled goat anti-human IgM antibodies (Sigma) were diluted at 1:10,000. Details of the blocking, incubation, and washing procedures, the substrate, and the measurement of absorbance values have been reported previously[17].

For each antigen, receiver operating characteristic (ROC) curves were generated using the results for LB sera and healthy controls, and the area under the ROC curve (AUC) was estimated. The cut-off value for each antigen was determined by the maximum Youden index of the ROC curve evaluated using MedCalc software.

Statistical Analysis

Logistic regression and Chi square tests were used to evaluate the efficiency of the 6 antigens with respect to the serum detection of IgG and IgM antibodies, and all statistical analyses were performed in MedCalc and SAS. *Z*-statistics were obtained from the pairwise comparisons of ROC curves in MedCalc (*Z*=Difference between areas/Standard Error).

RESULTS

Sensitivity and Specificity of the Six Recombinant Antigens

Recombinant proteins were used to detect serum IgG and IgM from three panels. The ROC curves for the antigens are displayed in Figure 1.

IgM Antibody Cutoff values for antigens in the detection of IgM based on the ROC curves were as follows: Fla B.g 0.083, OspC B.a 0.063, OspC B.g 0.087, P39 B.g 0.052, P83 B.g 0.053, VlsE B.a 0.065, and whole cell proteins 0.07. The sensitivity ranged from 61.2% to 95.5% and specificity ranged from 60% to 88% (Table 2). In a comparison of diagnostic values, OspC B.g had a higher diagnostic value than P39 B.g, P83 B.g, and VlsE B.a ($P<0.05$). In addition, P39 had the lowest diagnostic value ($P<0.05$) among all proteins (Table 3). For the 89 syphilis serum samples, VlsE B.a, Fla B.g, and OspC B.g had high specificities, and no significant difference was detected between the three proteins ($P>0.05$).

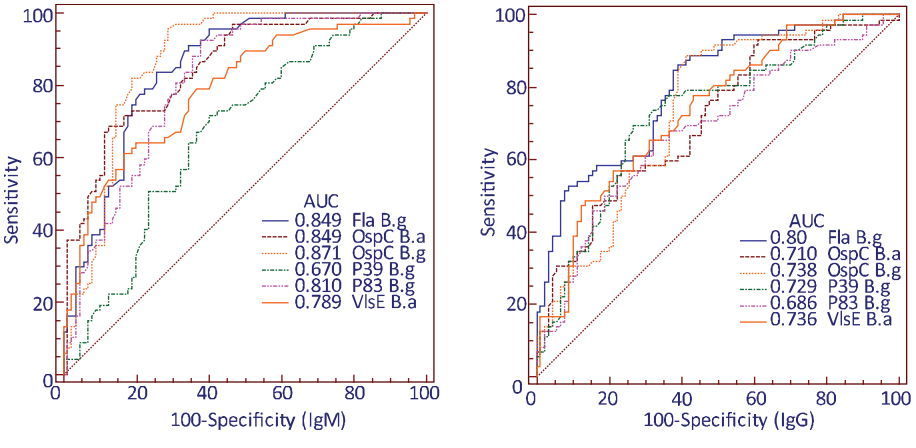

Figure 1. ROC curves for 6 antigens in serum IgM and IgG antibody detection. The diagonal line indicates that there is no difference between LB sera and controls.

IgG Antibody Cutoff values for the detection of IgG using different antigens were as follows: Fla B.g 0.164, OspC B.a 0.37, OspC B.g 0.16, P39 B.g 0.17, P83 B.g 0.2, VlsE B.a 0.16, and whole cell proteins 0.16. The sensitivity ranged from 56.9% to 88.9%, and specificity ranged from 58.9% to 78.9% (Table 4). Based on *Z*-statistics, Fla B.g had a higher diagnostic value than the other proteins ($P<0.05$) (Table 3). For syphilis serum samples, Fla B.g had the highest positive detection rate ($P<0.05$). VlsE B.a and OspC

Table 2. Summary of IgM Antibody Detection in The Serum of *Borrelia burgdorferi* for Various Antigens in Patients with Lyme Borreliosis and Syphilis

Antigen	Lyme Borreliosis			Syphilis
	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Positive samples (%)
Fla B.g	0.85 (0.78-0.90)	83.6 (72.5-91.5)	74.4 (64.2-83.1)	28 (31.4)
OspC B.a	0.85 (0.78-0.90)	68.7 (56.2-79.4)	87.8 (79.2-93.7)	36 (40.4)
OspC B.g	0.87 (0.81-0.92)	95.5 (87.5-99.1)	71.1 (60.6-80.2)	30 (33.7)
P39 B.g	0.67 (0.59-0.74)	71.6 (59.3-82.0)	60.0 (49.1-70.2)	47 (52.8)
P83 B.g	0.81 (0.74-0.87)	92.5 (83.4-97.5)	62.2 (51.4-72.2)	51 (57.3)
VlsE B.a	0.79 (0.72-0.85)	61.2 (48.5-72.9)	83.3 (74.0-90.4)	21 (23.6)
Whole cell	0.84 (0.77-0.89)	88.1 (77.8-94.7)	68.9 (58.3-78.2)	50 (56.2)

Note. AUC=Area under the ROC curve. CI=Confidence interval.

Table 3. Pairwise Comparison of ELISA Results for 6 Recombinant Proteins in LB Serum Samples and Healthy Controls

Recombinant Proteins	IgM		IgG	
	Z-statistic	*P*-value	*Z*-statistic	*P*-value
Fla B.g - OspC B.a	0.01	0.993	2.41	0.016
Fla B.g - OspC B.g	0.96	0.337	1.97	0.049
Fla B.g - P39 B.g	4.73	<0.0001	2.24	0.025
Fla B.g - P83 B.g	1.31	0.191	4.39	<0.0001
Fla B.g - VlsE B.a	1.73	0.083	1.96	0.05
OspC B.a-OspC B.g	0.58	0.560	0.70	0.482
OspC B.a - P39 B.g	4.37	<0.0001	0.64	0.524
OspC B.a - P83 B.g	1.06	0.288	0.76	0.447
OspC B.a - VlsE B.a	1.67	0.095	0.61	0.540
OspC B.g - P39 B.g	5.14	<0.0001	0.22	0.827
OspC B.g - P83 B.g	2.28	0.022	1.49	0.137
OspC B.g - VlsE B.a	2.41	0.016	0.05	0.958
P39 B.g - P83 B.g	4.28	<0.0001	1.43	0.151
P39 B.g - VlsE B.a	2.84	0.004	0.17	0.863
P83 B.g - VlsE B.a	0.64	0.524	1.39	0.164

Note. *Z*-statistics were obtained from pairwise comparisons of ROC curves analyzed using MedCalc software.

Table 4. Summary of IgG Antibody Detection of *Borrelia burgdorferi* using Various Antigens in Patients with Lyme Borreliosis and Syphilis

Antigen	Lyme Borreliosis			Syphilis
	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Positive samples (%)
Fla B.g	0.80 (0.73-0.86)	86.1 (75.9-93.1)	61.1 (50.3-71.2)	80 (89.9)
OspC B.a	0.71 (0.63-0.78)	56.9 (44.7-68.6)	76.7 (66.6-84.9)	14 (15.7)
OspC B.g	0.74 (0.66-0.80)	88.9 (79.3-95.1)	58.9 (48.0-69.2)	46 (51.7)
P39 B.g	0.73 (0.65-0.80)	69.4 (57.5-79.8)	73.3 (63.0-82.1)	42 (47.2)
P83 B.g	0.69 (0.61-0.76)	65.3 (53.1-76.1)	67.8 (57.1-77.2)	51 (57.3)
VlsE B.a	0.74 (0.66-0.80)	56.9 (44.7-68.6)	78.9 (69.0-86.8)	11 (12.3)
Whole cell	0.81 (0.75-0.87)	79.17 (68.0-87.8)	73.3 (63.0-82.1)	70 (78.7)

Note. AUC=Area under the ROC curve. CI=Confidence interval.

B.a had high specificity values for syphilis serum, and no significant difference was detected between the two proteins (P>0.05).

Evaluation of the Effectiveness of Recombinant Antigens

Two logistic regression models were established for IgM and IgG. The effectiveness of each protein was evaluated for the detection of two antibodies. OspC B.a and OspC B.g had high diagnostic values for IgM, while OspC B.g and VlsE B.a were effective for the diagnosis of IgG (Table 5). The specificity of VlsE B.a in the test of syphilis IgG was much higher than that of OspC B.g (Table 4). Therefore, VlsE B.a was the most effective antigen for the diagnosis of IgG.

Interactions between antigens were analyzed by logistic regression models. Interestingly, there was no significant interaction in the IgG test. However, for IgM, three groups (Fla B.g and OspC B.a, Fla B.g and OspC B.g, and OspC B.g and P39 B.g) were found had interactions in model. The pairs of proteins were mixed and used for ELISA with the three serum panels. The results are shown in Table 6. There was no difference between the mixed proteins and OspC B.g (P>0.05), and the specificity of OspC B.g was higher than that of the mixed proteins in the syphilis test (P<0.05). The diagnostic value of Fla B.g with OspC B.a was lower than those of the single antigens.

Comparison between Diagnoses Using the Screened Recombinant Antigens and The Whole Cell Proteins of Borrelia burgdorferi

The whole cell proteins of B.g strain PD91 was used to detect IgG and IgM in the three serum panels. In terms of OspC B.g IgM, OspC B.g IgG, OspC B.a IgM, and VlsE B.a IgG, there were no differences in the ELISA results between screened antigens and whole cell proteins for LB serum samples and healthy controls (in Figures S1 and S2). However, using syphilis serum samples, the specificity values for the single screened proteins were higher than that of the whole cell (Chi-square tests, P<0.05, data shown in Table 7). In particular, OspC and VlsE were better than the whole cell proteins for the diagnosis of LB.

Table 5. Results of Reduced Logistic Regression Models

Antigen	OR	95% Confidence Limit	*P*-value
OspC B.a (IgM)	10.492	1.096-100.461	0.0414
OspC B.g (IgM)	38.095	2.068-701.937	0.0143
OspC B.g (IgG)	8.788	3.682-20.977	<0.0001
VlsE B.a (IgG)	3.216	1.505-6.872	0.0026

Table 6. IgM ELISA Results Using Mixed Antigens for Various Serum Panels

Antigen	Lyme Borreliosis			Syphilis
	AUC (95% CI)	Sensitivity (95% CI)	Specificity (95% CI)	Positive Rate (%)
Fla B.g & OspC B.a	0.72 (0.73-0.86)	86.1 (75.9-93.1)	61.1 (50.3-71.2)	70.0
Fla B.g & OspC B.g	0.83 (0.63-0.78)	56.9 (44.7-68.6)	76.7 (66.6-84.9)	40.0
P39 B.g & OspC B.g	0.85 (0.66-0.80)	56.9 (44.7-68.6)	78.9 (69.0-86.8)	55.0

Note. AUC=Area under the ROC curve. CI=Confidence interval.

Table 7. Pairwise Comparison of Screened Proteins and Whole Cell Proteins in Syphilis Serum Samples

Proteins	χ^2	*P-value*
Whole cell - OspC B.a (IgM)	4.41	0.036
OspC B.a - OspC B.g (IgM)	0.87	0.352
Whole cell - OspC B.g (IgM)	9.08	0.03
Whole cell - OspC B.g (IgG)	14.26	<0.01
Whole cell - VlsE B.a (IgG)	78.83	<0.01
OspC B.g - VlsE B.a (IgG)	31.62	<0.01

DISCUSSION

B. garinii and *B. afzelii* are the main pathogenic species of *Borrelia burgdorferi* in China. These pathogens have mainly been found in Liaoning Province, Jilin Province, Heilongjiang province, and the Inner Mongolia Autonomous Region[4]. In this study, we obtained patient serum samples from Heilongjiang province and used strains isolated from the endemic area of *B. burgdorferi* as templates to clone and express 6 antigens for serological evaluations. VlsE and the VlsE IR6 peptide are frequently applied in serological tests to detect LB antibodies. The high sensitivity and specificity in serum diagnosis can distinguish between LB and syphilis[6,16,27]. However, VlsE is a variant protein of the Vmp (variable major protein) system, and its high variation among strains makes *in vivo* expression difficult[28]. In this study, we used a Chinese strain as the template and cloned and expressed a conserved segment of VlsE for ELISA. Fla is an effective antigen for the serodiagnosis of early LB using ELISA based on previous analyses[29]. OspC (23 kD) is a molecule with antigenic diversity; it is expressed in the early stage of infection and is associated with the escape of host immunity[30-31]. As a kind of immune-dominant antigen, it is often used to detect IgM for early LB[30-33]. P83/100 and P39 are also commonly used in studies of late LB[34-35]. The P83/100 protein is an immunodominant protein associated with the immune escape mechanism of *B. burgdorferi*[36-37]. This protein exists in 20% of patients with early LB and almost all advanced and late-stage patients[38]. The E4 region of P83 is specifically used for the serodiagnosis of LB[39] and was used in our study. *Borrelia* membrane protein A (BmpA), also called P39, is a specific diagnostic antigen for LB[40]. These antigens are detected at different stages of *B. burgdorferi* infection, and have been studied for use in tests. However, a comprehensive evaluation of recombinant proteins for LB serodiagnosis in China is lacking. We used these antigens to detect antibodies in serum samples at different stages of LB in Chinese patients.

Using the three serum panels for IgG detection, Fla B.g had the best diagnostic value for LB patients and healthy controls, but it also had the highest positive rate using syphilis sera, consistent with the results of a previous report[18]. Fla had poor specificity in this study, consistent with a previous study indicating that it has high cross-reactivity in serologic tests with similar antigens expressed by other bacteria. The specificity of VlsE in the syphilis test was much higher than that of other proteins. According to the reduced logistic regression model, three proteins, OspC B.g, OspC B.a, and VlsE B.a, were left. Combined with the results obtained using syphilis sera, VlsE B.a and OspC B.g for the IgG test and OspC B.g and OspC B.a for the IgM test were optimal for LB diagnosis. In this study, there was no difference between single and mixed proteins. Interactions between the proteins are not clear. The sensitivity and specificity of the whole cell proteins were similar to those of the single proteins for ELISA using LB sera and healthy controls, but the specificity of single proteins was much higher in syphilis serum samples.

In conclusion, we screened three recombinant antigens for LB serum diagnosis, OspC B.g, OspC B.a, and VlsE B.a. An unexpected discovery was that the interaction between Fla B.g and OspC B.a might decrease the diagnostic value in the LB test. This research provides a strong basis for the production of a recombinant antigen ELISA kit for LB serum antibody detection in China.

This study had some limitations. Most of the LB serum samples were obtained from EM patients, but the proportion of advanced and late-stage LB serum samples was small. Evaluations of the efficiency of antigens at different disease stages will improve accuracy. In addition, additional antigens or specific peptides should be considered for the detection of LB serum antibodies in the future.

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Supplementary Materials

Table S1. Oligonucleotides Used of Each Antigen in This Study

Primers (5'-3')	Antigens	Strains
F: CGGGGATCCAAGGGGATAAAGGGGATTGTTGA	VlsE	*B.afzelii* GDsh1
R: CGGAAGCTTAGCAAACTTTCCATCCTTAGCCA		
F: CGCGGATCCTTATCTTGTAGTGGTAA	P39	*B.garinii* PD91
R: CGGAAGCTTAATAAATTCTTTAAGAAAC		
F: GCGCCATGG CTGAGATTGAGAGAGTAAA	P83	*B.garinii* PD91
R: CGCGGATCCCATTTTAGAGTCAACATA		
F: GCGCCATGG CTGCATCTACTAATCCT	OspC	*B.garinii* PD91
R: CGCGGATCC TTTGGAGTTTCTGCCACA		
F: CGCGGATCC TCAAACAAATCTGCTTC TCA	Fla	*B.garinii* PD91
R: CGGAAGCTT ATCACTTATCATTCTAATAG		

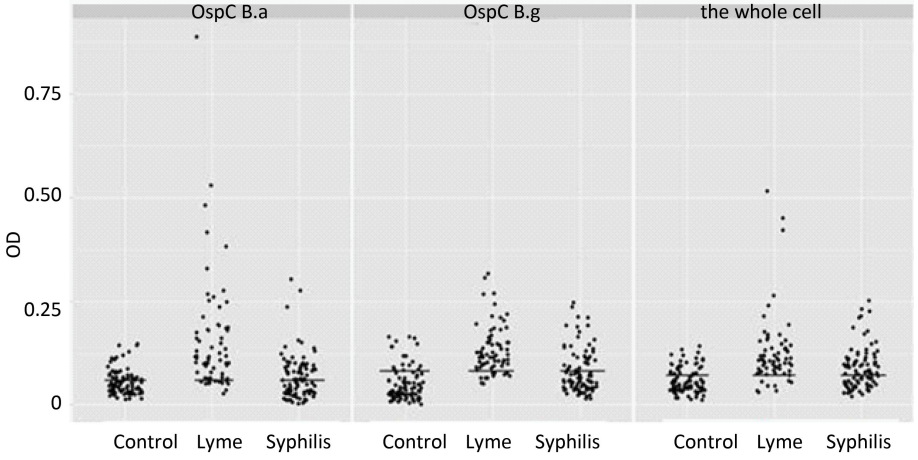

Figure S1. Results of IgM ELISAs with the recombinant proteins OspC B.a. OspC B.g and the whole cell proteins for individual serum samples in different panels. The short horizontal lines showed the cutoff value of each protein.

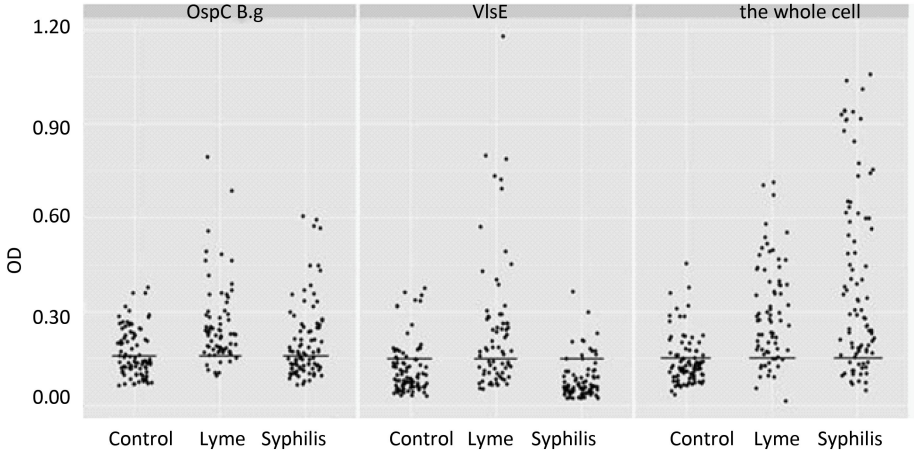

Figure S2. Results of IgG ELISAs with the recombinant proteins VlsE B.a. OspC B.g and the whole cell for proteins individual serum samples in different panels. The short horizontal lines showed the cutoff value of each protein.