

# Antibodies to *Borrelia burgdorferi* in European Populations

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We compared the antibodies to *B. burgdorferi* in three different populations in order to evaluate the diagnostic reliability of Lyme borreliosis serologic analysis. The subjects included 25 patients with Lyme borreliosis (Group 1); 50 patients with diseases of unknown cause, *B. burgdorferi* ELISA-positive in serum and without *B. burgdorferi* infection (Group 2); and 1,251 individuals without Lyme borreliosis (Group 3). All samples were tested for *B. burgdorferi* B31 and *B. afzelii* antigens using ELISA. The positive results of the ELISA *B. burgdorferi* B31 assay were confirmed with Western blot for the same strain. In Group 3,

162 (12.9%) patients were ELISA positive for *B. burgdorferi* B31, while only 6 (0.6%) patients had IgG ELISA antibodies to *B. afzelii*. Bands in WB were detected in 104 (8.4%) of the Group 3 subjects. The bands found to be most reliable for the identification of strain *B. burgdorferi* B31 by IgG WB were those representing the 93, 39, 34, and 23-kDa proteins. Our results show that serologic diagnosis of Lyme borreliosis is far from clearly established. To date, the only reliable criteria are clinical ones correlated with laboratory evidence. *J. Clin. Lab. Anal.* 14:20–26, 2000.  
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**Key words:** *Borrelia burgdorferi*; *Borrelia afzelii*; Lyme borreliosis; serology

## INTRODUCTION

Since description of Lyme borreliosis (LB) in 1977 (1), efforts have been centered on obtaining clinical criteria and laboratory methods that guarantee a correct diagnosis and provide reliable data on the actual prevalence of the disease in different geographical areas (2,3). Because the clinical manifestations of LB (4) are often unspecific or difficult to distinguish from other diseases, its diagnosis depends on laboratory tests that reliably confirm or exclude its presence. Correct diagnosis is problematic due to: (1) the varying degree of reliability of the different techniques now used to detect antibodies for the differential diagnosis of LB (5); (2) different interpretative criteria for confirming these antibody assays as positive (6); and (3) possible cross-reactions with other antigenic structures, such as microorganisms or tissues, in the human body (7,8).

The first diagnosis of LB at University Hospital San Cecilio (Granada, Spain) was made in 1990 (9). Afterwards, sera and cerebrospinal fluid (CSF) from patients with symptoms of LB and antibodies to *B. burgdorferi* were systematically saved. We observed a high number of *B. burgdorferi* ELISA-positive sera in which the clinical evolution, serologic sequential determinations, and/or response to treatment of the patients was not compatible with LB. This observation generated doubts about the reliability of certain serologic techniques.

This study was therefore designed to evaluate the reliabil-

ity of the IgG WB test with *B. burgdorferi* B31 proteins when used to confirm any positive results obtained with previous ELISA testing. We compared the results for three separate populations: 25 LB patients (Group 1), 50 other patients with LB-type symptoms who were *B. burgdorferi*-serum positive yet had not developed LB (recruited longitudinally over a period of five years) (Group 2), and 1,251 subjects without LB suspected who had participated in a cross-sectional survey for one month after finalization of the former studies (Group 3). Results from these three groups were compared in order to determine which bands were most often present in the patients fulfilling criteria for LB, and, on the basis of our findings, to put forth improved criteria for the laboratory diagnosis of LB.

## MATERIALS AND METHODS

### Serum Samples

Serum samples from the 1,326 total subjects were analyzed at the University Hospital San Cecilio and classified into three

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Received 9 February 1999; Accepted 1 October 1999

groups whose characteristics are specified in the following paragraphs. All were first tested for infection by *Treponema pallidum* with an Haemagglutination test, and a Fluorescent Treponema Absorbent (FTA-abs) test to exclude syphilis. Then the subjects were tested for anti-*B. burgdorferi* antibodies using two ELISA methods (tests 1 and 2) and a WB technique (test 3). Analyses for antibodies to *B. burgdorferi* were repeated for those subjects giving a positive initial result.

Group 1 consisted of samples from 25 patients with LB, 20 of which were from patients with Lyme arthritis, provided by DadeBehring Laboratories (Marburg, Germany), while the other 5 were from the Microbiology Department of Hospital San Cecilio (Table 1). The LB-case serum samples were collected for use in this study on the basis of: first, characteristic clinical manifestations of the patients, following European Union Concerted Action on Lyme Borreliosis (EUCALB) criteria. Such criteria include *erythema migrans*, lymphadenosis benigna cutis, acrodermatitis chronica atrophicans, neuroborreliosis, arthritis, or acute atrio-ventricular conduction disturbances. Also, exposure to ticks within weeks to years earlier should have been possible, including intrathecal-specific antibody production and pleocytic lymphocytosis in neuroborreliosis CSF (10). ELISA-positive antibodies to *B. burgdorferi* were the second basis for serum sample collection here, and the third requirement was nonreactivity with any other serologic assay to suspected bacteria (enterobacteria, rickettsia, brucella, coxiella, erlichia, chlamydia, mycoplasma, legionella), virus (hepatitis, HIV, herpesvirus, rubella, measles, parotitis, enterovirus, or respiratory), or parasites (toxoplasma, hidatidosis). Serum samples from Lyme arthritis patients were from persons with intermittent attacks of asymmetric oligoarticular swelling and pain, affecting primarily the knee.

Serum samples were contributed by physicians with extensive experience in the clinical diagnosis of this disease. The three Spanish patients with neurological symptoms were tested for antibodies in CSF using ELISA (test 1). When an ELISA-positive result was obtained, the intrathecal antibody production was considered only if the CSF-serum albumin ratio was  $< 7.5-10^{-3}$  (11).

Group 2 consisted of 50 patients without *B. burgdorferi* infection who: (1) exhibited symptoms resembling those of LB; (2) gave ELISA positive to *B. burgdorferi* in serum; and (3) had not, as far as they knew, been bitten by a tick. The clinical diagnoses corresponding to this group were multiple sclerosis (n = 12), sensorimotor polyneuropathy (n = 9), optic neuritis (n = 3), lateral amyotrophic sclerosis (n = 1), other neurological symptoms (n = 3), chronic arthritis (n = 4), arthritis of the coccyx (n = 1), dermatomyositis (n = 1), seronegative spondyloarthropathy (n = 1), scleroderma (n = 10), and fever of unknown origin (n = 5).

Group 3 was comprised of 1,251 samples from patients without *B. burgdorferi* infection seen in Hospital San Cecilio for diagnoses of processes other than LB: *T. pallidum* infection (n = 14), HIV infection (n = 47), acute herpes infection (n = 30), neurological diseases (n = 80), rheumatological diseases (n = 24) and other noninfectious diseases (n = 156). In addition, 100 pregnant women and 800 other healthy subjects were included in this group. The Lyme test was not requested by the respective physicians.

Follow-up analyses of antibodies to *B. burgdorferi* were run on 18 sera from Group 3 (12 subjects), 25 sera from Group 2 (17 patients), and on 27 sera and 2 CSF from Group 1 (5 patients), that had initially shown positive in test 1.

The following assays were performed in our laboratory by a single investigator and according to the manufacturer's instructions.

**TABLE 1. Significant clinical findings in Spanish patients with confirmed Lyme borreliosis**

Pt	Age	Clinical diagnosis	Serology	Response
1	45-year-old man	Insect bite. Sinus block, pacemaker. Meningoencephalitis.	Intrathecal synthesis of antibodies (ELISA result from CSF <sup>a</sup> : 1.45; IgG WB bands: 93, 66 and 41 kDa; IgM WB bands: negative).	Initially responded to ceftriaxone. Recurrence with neurological symptoms that remitted with ceftriaxone.
2	25-year-old woman	Tick bite. Fever of unknown origin. Cardiac insufficiency. Meningoencephalitis.	Intrathecal synthesis of antibodies (ELISA result from CSF <sup>a</sup> : 1; IgG WB bands: 60, 41, 39 and 18 kDa; IgM WB bands: 23 kDa) (reference 15).	Good initial response to ceftriaxone. Recurrences with fever of unknown origin, that responded to ceftriaxone.
3	15-year-old woman	Tick bite. Bilateral symmetrical polyarthritits.	IgG WB bands: 93, 66, 58, 41, 39, 34, 21 and 18 kDa; IgM WB bands: 41 and 39 kDa (reference 9).	Good initial response to treatment with ceftriaxone. Recurrence on arthritis.
4	50-year-old woman	Tick bite. Myeloradiculitis.	Intrathecal synthesis of antibodies (ELISA result from CSF <sup>a</sup> : 1; IgM WB bands: 93, 41 and 34 kDa; IgM WB bands: 93, 41 and 23 kDa).	Good response to ceftriaxone.
5	11-year-old girl	No antecedents of tick bite. <i>Erythema migrans</i> . Grade II auriculoventricular block.	IgG WB bands: absent. IgM WB: 66, 60, 41, 34, 31, 23, kDa (reference 16).	Satisfactory response to doxycycline.

<sup>a</sup>The result (test 1) expresses the ratio between absorbance of sample and the cut-off value.

### Haemagglutination Test

The haemagglutination test for *T. pallidum* was obtained from Celloagnost Syphilis H, DadeBehring. The samples were diluted in sorbent to remove nonspecific antibodies and then incubated with erythrocytes sensitized with *T. pallidum* antigen. The results were expressed in qualitative form.

### FTA-abs Test

The FTA-abs test for *T. pallidum* was obtained from Fluorokit, Incstar, Inc., Stillwater, MN. The samples were diluted in sorbent to remove nonspecific antibodies and were then incubated with *T. pallidum* antigen. Results were expressed in qualitative form.

### ELISA anti-*B. burgdorferi* Tests

#### Platelia Lyme

The Platelia Lyme assay (test 1; Sanofi Pasteur, France) is an indirect ELISA with sonicated *B. burgdorferi sensu stricto* (strain B31) antigens and goat antiserum conjugated with peroxidase to detect IgG plus IgM. Dilution of samples was 1:100 using a protein solution for absorption. The automated system ELISA Processor III (DadeBehring) was used. Results are expressed in AU/ml. Figures higher than the cut-off value were considered positive results.

#### Enzygnost borreliosis

This indirect ELISA (test 2; DadeBehring) uses purified proteins of 100 kDa, 41 kDa, OspA, OspC, and 17 kDa from *B. afzelii* strain PKo and caprine antiserum conjugated with peroxidase to detect IgG or IgM (IgM was tested in Groups 1 and 2). It was performed using dilutions of sera with pre-absorption solution at 1:231 for IgG and at 1:42 for IgM. The samples for IgM were pre-absorbed using ovine anti-IgG antibodies (DadeBehring). Testing was done with the ELISA Processor III system (DadeBehring). The Alpha-Method system was used for the exact determination of IgG titer (12). Results were expressed in AU/ml for IgG, and in qualitative form for IgM.

#### Western blot anti-*B. burgdorferi* test

This WB test (test 3; MarDx, USA) was used to confirm the positive results from test 1. This test for IgG and IgM (IgM was tested in Groups 1 and 2) is based on *B. burgdorferi sensu stricto* strain B31 antigen from a low-passage strain and separated in the presence of sodium dodecyl sulfate by polyacrylamide gel electrophoresis. The resolved protein bands are transferred onto nitrocellulose membranes by means of electrophoresis. The procedure was an indirect enzyme-immunoassay with antiserum conjugated with alkaline phosphatase to detect IgG or IgM. A dilution of samples at 1:100 with protein solution was used. Testing was done with an

automated system (Autoblot, Biokit, Spain), and results were expressed in qualitative form. The blind-code immunoblots were scored visually by a single reader. The presence of a band was confirmed when its intensity was greater than the band corresponding to a low-positive control.

### Analysis of Results

Diagnostic reliability (sensitivity and specificity) was calculated for the interpretation of the bands identified in the IgG WB. Three different criteria were used:

1. Tilton criteria (13)—to be considered positive if any combination of these bands is present: (a) 31 or 34 + 41 or 39; (b) 23 + 31, 34, 39 or 41; or (c) 31 + 34 kDa;
2. Dressler et al. (14)—considered positive if 5 of the following 10 bands were present: 18, 21 or 23, 28, 30, 39, 41, 45, 58, 66, 93 kDa.
3. New criteria—The presence of the bands that we had selected by means of the procedure was based on: (a) exclusion of the nonspecific bands (7 out of 15 bands from WB) that appeared most frequently in the healthy subjects of Group 3 (100 pregnant females and 800 other healthy subjects); and (b) determination of the presence of 93 or 39 or 34 or 23-kDa band.

### RESULTS

The Platelia and Enzygnost EIA results are shown in Table 2. In Group 3, 162 subjects (12.9%) tested positive for Platelia EIA and 8 (0.6%) for Enzygnost EIA, 5 of whom (0.4%) had antibodies only for this genospecies. In Group 2, Enzygnost EIA IgM alone was detected in 3 patients.

Tables 1 and 2 summarize the clinical manifestations (9,15,16) in subjects from groups who initially gave ELISA positive. In follow-up analyses for antibodies to *B. burgdorferi*, seroreversion was detected with the first ELISA test in: 1 of 5 patients with LB (a subject in the *erythema migrans* stage); 3 of 17 patients from Group 2 (1 scleroderma and 2 sensorimotor polyneuropathy patients); and 6 of 12 patients in Group 3 (all 6 with acute herpes virus infection).

When the serologic results for both *T. pallidum* and *B. burgdorferi* infections in Group 3 were compared and contrasted, we observed that seven of the 14 subjects with syphilis (50%) were positive in *B. burgdorferi* ELISA test 1. Moreover, a positive finding for the *B. burgdorferi* ELISA test was associated with positive serology for *T. pallidum* infection three times more frequently (4.8%) than was a negative finding (1.4%).

Of the 162 samples from Group 3 that gave positive for Platelia EIA, 104 showed at least one IgG band in WB (64.2%). Of the 50 Group 2 patients, WB revealed bands for IgG in 44 (88%), and for IgM in 28 (56%) cases. Interestingly, serum from one of the five Spanish patients with LB did not present WB IgG bands; however, this patient (who

**TABLE 2. Relation between clinical manifestations and results of ELISA for *B. burgdorferi***

ELISA results <sup>a</sup>	Group 3 (N: 1,251)			Group 2 (N: 50)				Lyme borreliosis (N: 25)	
	+/+	+/-	-/+	+/-/+	+/-/-	+/+/-	-/-/+	+/+/-	+/-/-
Herpes infection		6							
Pregnancy		12	1						
HIV		11							
Syphilis		6	1						
Rheumatological dis.		6		1	6				1
Neurological dis.		15			24	1	3	20	3
Healthy subjects	2	95	3						
Dermatological dis.					10				1
Other conditions	1	8			5				
Total	3	159	5	1	45	1	3	20	5
%	0.2	12.7	0.4	2	90	2	6	80	20

<sup>a</sup>In each column the signs refer to Platelia EIA results (first) and Enzygnost EIA (second symbol for IgG and third for IgM), respectively.

had *erythema migrans*) did yield bands for IgM. The 20 Germanic patients with LB had WB bands only for IgG.

Tables 3, 4, and 5 give the frequencies with which bands were present in each study group. The seven found most frequently in Group 3 (Table 5) were the 18-, 21-, 31-, 41-, 58-, 60-, and 66-kDa bands.

When we studied each single band, the IgG 93-, 39-, 34-, and 23-kDa bands were determined to be the most reliable (Table 6), and the 28-, 30-, 37-, 45-, and 75-kDa bands were the least reliable for distinguishing LB. Finally, Table 7 gives the diagnostic effectiveness of the three criteria used to interpret the IgG WB findings with respect to LB. The sensitivity and specificity of diagnosis was shown to improve when either the 93-, 39-, 34-, or 23-kDa band was present.

**DISCUSSION**

Although antibody detection is the microbiological method used most widely to diagnose LB, the very existence of the many different serological tests and the different criteria for their interpretation suggests that no single approach is entirely reliable. The cut-off point for indirect immunofluorescence results has not been clearly defined (17–19). ELISA-based methods are also problematic in this sense; one study recently proposed that 80% of the previously accepted cut-off value should be used (20). In addition, the results are difficult to quantify, and questions remain regarding the choice of antigen, and whether recombinant antigens should be used, depending on the dominant genospecies in the geographic area. Our study was

**TABLE 3. Percentage of bands present in IgG WB for *B. burgdorferi* B31 from Germanic Lyme borreliosis patient sera**

Samples	B31-specific protein (kDa) detected in IgG WB (N: 20)															
	93	75	66	60	58	45	41	39	37	34	31	30	28	23	21	18
978				X			X				X		X			
1080				X								X				
1520	X	X		X	X	X	X	X			X	X	X	X	X	X
1352	X			X	X		X	X								X
1368			X	X									X			
1195	X			X	X						X		X			X
1429	X			X	X			X				X	X			
994	X			X	X		X	X				X			X	
1136	X			X	X		X					X	X			
1509	X	X		X			X						X			
1460				X			X					X				
997				X	X		X	X				X			X	X
1218	X			X	X		X				X		X			X
990	X	X	X	X	X		X	X			X				X	
1293	X			X	X		X	X				X			X	
1111	X			X		X		X			X	X		X	X	X
1000		X		X												X
998				X												
1506	X			X	X			X				X				
1510	X			X	X		X					X				X
% Total	65	20	10	100	60	10	60	45	0	0	30	55	40	10	30	40

**TABLE 4. Percentage of positive results in WB tests for *B. burgdorferi* B31 antibodies in serum from Group 2**

	WB IgG (N: 44) <sup>a</sup>															WB IgM (N: 28)								
	93	66	60	58	45	41	39	34	31	30	23	21	18	93	66	60	41	39	37	34	31	23	21	18
<sup>b</sup> A: 25	5	16	14	1		19	5	3	8	4	2	3	3	2	4	3	13	3	1	1	2	2	1	4
B: 10	1	7	3	1	1	9	3	1	1		2	1	3	3	4	3	6	4		1	1	1	4	
C: 7	2	3	3	1		6	2	1		1		2	3				3	1			1			
D: 5	2	3	4			5	1	1			1		3		2	2	4	1		1	1			
Total	10	29	24	3	1	39	11	6	9	5	5	6	12	5	10	8	26	9	1	3	5	3	5	4
%	22.7	65.9	54.5	6.8	2.2	88.6	25	13.6	20.4	11.3	11.3	13.6	42.8	17.8	35.7	28.5	92.8	32.1	3.5	10.7	17.8	10.7	17.8	14.2

<sup>a</sup>Number of subjects with bands in WB.

<sup>b</sup>A, neurological disease; B, dermatological diseases; C, rheumatological diseases; D, other. WB did not reveal antibodies to 75-, 37-, or 28-kDa proteins for IgG, or 75-, 58-, 45-, or 30-kDa proteins for IgM.

based on the diagnostic recommendations of EUCALB (10): screening by EIA or IFA, then confirmation by Western blot to reduce the number of false positives.

Two strains of *B. burgdorferi* have been isolated in Spain (21,22). *Borrelia* has a number of shared antigens, which is why sera may be detected as positive even when a different borrelial genospecies is used. However, our results in patients with LB showed the ELISA based on the *B. afzelii* antigen to perform poorly in the patients of Spanish origin, possibly because of the lower rate of infection by this bacteria in Spain (23) and the purified proteins used. The lack of specificity of the ELISAs currently in use has even led some physicians to diagnose LB in patients with compatible clinical symptoms and positive ELISA in the absence of confirmation by means of other analytical techniques (6,10,24–26).

The presence of particular bands in the WB (qualitative criterion) (13), together with a certain number of bands corresponding to antibodies (quantitative criterion) (14), can be used to offer a diagnosis of LB. One French study found that on the basis of quantitative criteria alone, WB was neither sensitive nor specific in detecting LB in areas where the disease is endemic (27).

The criteria used by Tilton (13) and by Dressler et al. (14) to interpret WBs were not found to be precise in detecting LB in our setting, as indicated by the number of false nega-

tives obtained when attempting to confirm LB (Table 7). Because only one patient in our study had *erythema migrans*, we did not apply the criterion from Engstrom et al. (28) for the serodiagnosis of early LB.

The seroreversion in patients from Groups 2 and 3, with the exception of the patient with *erythema migrans*, suggested that the antibodies detected in the assays were not specific to *B. burgdorferi*. Nonspecific antibodies may appear for epitopes (shared antigen communities) common with human organic structures (especially of the nervous system) and microbial antigens (corresponding, for example, to *T. pallidum* and *Borrelia no burgdorferi*) (7, 29–32). They may also be present as a result of the polyclonal stimulation of the B lymphocytes in the course of other infections (33).

The presence of bands among certain subjects in Groups 2 and 3 suggests that a positive WB cannot be taken as indisputable evidence of LB. In fact, a significant percentage of subjects from Group 2 (25%) exhibited 93- or 39-kDa bands in the IgG WB without clinical manifestation of the LB; yet these bands did not appear in later determinations. Such findings point to the need for new diagnostic criteria based on the selection of bands representing IgG. The IgG bands—in particular, the 93-, 39-, 34-, and 23-kDa bands—were considered more useful for two reasons: they appeared less frequently in Group 3 patients, and their global sensitivity and specific-

**TABLE 5. Percentage of positive results in WB tests for *B. burgdorferi* B31 protein antibodies in serum from Group 3**

Clinical manifestations <sup>a</sup>	IgG WB (N: 104)															
	93	66	60	58	45	41	39	37	34	31	30	28	23	21	18	
A: 6	1	3	4	3	1	5	1		2	4		1	3	3	1	
B: 12				1		1								1		
C: 11				1		1	1									
D: 6				2	3		2									
E: 6						3	1		1	1	1				1	
F: 15	3	3	4	4	2	12	3	1	3	3		1	3	2	5	
G: 97	3	21	16	21	4	41	4	4	1	12	4	4	1	6	5	
H: 9		2	4	2	2	5	1	1	2					4	2	
Total	10	33	34	35	9	70	11	5	8	22	5	6	7	16	14	
%	9.6	31.7	32.7	33.6	8.6	67.3	10.6	4.8	7.7	21	4.8	5.7	6.7	15.4	13.5	

<sup>a</sup>A, acute herpes virus infection; B, pregnancy; C, HIV; D, syphilis; E, rheumatological diseases; F, neurological diseases; G, healthy subjects; H, other process. WB did not reveal antibodies to the 75-kDa protein.

**TABLE 6. Diagnostic reliability of each single band of 93, 39, 34, or 23 kDa in IgG WB test for *B. burgdorferi* B31**

Reliability <sup>a</sup>	IgG WB Band kDa n°			
	93	39	34	23
Sensitivity	75%	66.6%	66.6%	50%
Specificity	97%	96.1%	98%	98%

<sup>a</sup>Reliability was calculated using 25 patients with LB vs. 100 pregnant females and 800 other healthy subjects.

ity for the diagnosis of LB were greater. Similar results were reported by Zoller et al. (34) and Schwan et al. (35). WB sensitivity and specificity in our experience failed to reach 100%, but might be enhanced with complementary serological studies aimed at demonstrating the persistence of positive results and the appearance of significant bands. These suggestions will need to be tested in other settings. We did not consider IgM WB bands as evidence of LB, because they may appear as a result of the clonal stimulation of B-lymphocytes or the presence of the rheumatoid factor (false positives), and/or be absent when there is no IgM response (false negatives).

In conclusion, because the diagnosis of LB through antibody detection is complicated in cases of previous cured infection, post-infection syndrome, immunomediated diseases, antibodies generated in the context of neuronal destruction, infections by spirochetes or other infections involving the clonal stimulation of B lymphocytes, LB must be confirmed through the serological testing of those patients who fulfill the clinical symptoms. The persistence of antibodies should be demonstrated using criteria such as the ones proposed here in addition to those described by Tilton (13) and Dressler et al. (14). However, even the appearance of suspicious bands in WB analyses does not guarantee an accurate diagnosis of LB.

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**TABLE 7. Reliability of different criteria for IgG WB interpretation with respect to Lyme borreliosis**

Reliability <sup>a</sup>	IgG WB interpretation criteria		
	Tilton	Dressler et al.	93 or 39 or 34 or 23 kDa band <sup>b</sup>
Sensitivity	61.5%	53.8%	73%
Specificity	97.9%	99.6%	97.9%

<sup>a</sup>Reliability was calculated using 25 patients with LB vs. 100 pregnant females and 800 other healthy subjects.

<sup>b</sup>Presence of any band.

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