

Comparative evaluation of two enzyme linked immunosorbent assay methods and three Western Blot methods for the diagnosis of culture-confirmed early Lyme Borreliosis in Italy

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SUMMARY

This study investigated the onset and development of the immune response to *Borrelia burgdorferi* infection in 30 Italian patients with culture-confirmed Lyme Borreliosis in the stage of erythema migrans (EM). All patients received antimicrobial treatment when entering the study and were prospectively evaluated monthly for up to 30 days after enrolment. A total of 60 serially collected serum samples were tested by using two different commercial enzyme-linked immunosorbent assays (ELISAs): Anti-*Borrelia* plus VlsE ELISA, Euroimmun, and the synthetic peptide-based ELISA, Quick ELISA C6, Immunitics. Sixty-five potentially cross-reacting sera were also tested. Anti-*Borrelia* plus VlsE ELISA IgG was far more sensitive than Quick ELISA C6 (56.6% and 33.3%, respectively). Moreover, considering that 17 additional sera from the first bleeding group of Lyme disease patients were IgM positive when tested by Anti-*Borrelia* plus VlsE IgM, the sensitivity of Anti-*Borrelia* plus VlsE as a whole system rose to 85.0%. Nevertheless, due to the specificity values of Anti-*Borrelia* plus VlsE ELISA identified in this study (98.5% for IgG and 78.5% for IgM), the need of a confirmatory test for the diagnosis of Lyme disease remains. All the sera were also tested by two different commercial Western Blot (WB) assays: Euroline-WB against *Borrelia*, Euroimmun, and Qualicode *B. burgdorferi* WB, Immunitics, in comparison with a multispecies "home made" WB. Performances of the three WB methods for the detection of IgM were very similar. On the contrary, these WBs performed with different values of sensitivity and specificity when IgGs were evaluated. The most sensitive method was the "home-made" WB IgG (71.7%), followed by the Euroline-WB IgG against *Borrelia* (68.3%). Qualicode *B. burgdorferi* WB IgG demonstrated to be only 26.6% sensitive. Both "home-made" WB IgG and Qualicode *B. burgdorferi* WB IgG were 100% specific, whereas Euroline-WB IgG against *Borrelia* scored 12 cross-reacting samples as borderline, showing a specificity value of 80.0%.

KEY WORDS: Lyme, Borreliosis, Enzyme linked immunosorbent assay, recombinant VlsE, C6 peptide, Western Blotting

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INTRODUCTION

Lyme disease, caused by spirochetes belonging to *Borrelia burgdorferi* sensu lato genogroup, is the most common vector-borne disease in North America and Europe (Stanek and Strle, 2003). In the United States, *B. burgdorferi* sensu stric-

to (s. s.) is the causative agent of Lyme borreliosis, whereas in Europe three different species pathogenic for humans (namely: *B. burgdorferi* s. s., *B. garinii*, and *B. afzelii*) of the *B. burgdorferi* sensu lato (s. l.) group (Baranton *et al.*, 1992; Cicceroni *et al.*, 2001; O'Connell *et al.*, 1998; Strle *et al.*, 1996; Wang *et al.*, 1999) are known. The illness usually begins with a characteristic, expanding skin lesion, erythema migrans (EM), that is the best clinical marker for the early disease (Nadelman *et al.*, 1996; Nadelman and Wormser, 1998; Steere *et al.*, 2004). Laboratory diagnosis is mainly based upon serological findings. However, serology harbors several problems. The occurrence of cross-reacting antibodies may result in false-positive findings (Aguero-Rosenfeld *et al.*, 1996; Magnarelli *et al.*, 1990; Magnarelli *et al.*, 2000). Furthermore, patients may still be seronegative in the early stages of the infection and the humoral immune response can be diminished after the early onset of the antibiotic treatment (Aguero-Rosenfeld *et al.*, 1996; Bacon *et al.*, 2003; Peltomaa *et al.*, 2003; Strle *et al.*, 1996). Due to these difficulties, a two-step testing strategy for the serodiagnosis of Lyme disease has been recommended both in the United States by the Centers for Disease Control and Prevention (CDC) since 1995 (Centers for Disease Control and Prevention, 1995; Wormser *et al.*, 2000), and in Europe by the German Society for Hygiene and Microbiology (Wilske *et al.*, 2000). This strategy consists in the use of a screening method, such as enzyme-linked immunosorbent assay (ELISA) or immunofluorescence assay (IFA), followed by the confirmatory Western blotting (WB) method, to be performed when samples were scored as indeterminate or positive by the screening techniques. The purpose of the present study was to evaluate two different new generation ELISAs and three different WBs with a panel of human sera collected from Italian patients with early disease and culture-confirmed EM lesions.

MATERIALS AND METHODS

Study groups

This study investigated 125 human serum specimens. 60 sera were obtained from 30 culture-confirmed Lyme disease patients (males and females) aged between 29 and 65 years (mean

age 42.8) suffering from EM following a tick bite. A skin punch biopsy was obtained from each patient when entering the study and cultivated in Barbour-Stoenner-Kelly medium (BSKII) plus ciprofloxacin (0.4 µg/ml) and rifampin (40 µg/ml); the tubes were examined weekly by dark-field microscopy for motile spirochetes over a period of at least 45 days, as previously described (Marangoni *et al.*, 1999). At the initial clinical evaluation, each patient was bled and given specific antibiotic therapy for Lyme borreliosis. The follow-up study was done by taking additional serum samples at 30 days after enrolment. Entry in the study was made after a mean EM lasting time of 16 days (ranging between 5 and 106). An additional panel of 65 sera was obtained from patients with some of the most common biological conditions possibly resulting in false-positive reactivity in Lyme disease serology, such as specimens obtained from patients with *Streptococcus pyogenes* acute infection (streptolysin O antibody response >400 U.I./ml) (n=10), serum samples drawn from subjects with a clinical diagnosis of infectious mononucleosis detected as positive by the Paul-Bunnell-Davidsohn agglutination (n=10), sera from syphilis patients (primary and secondary stage) (n=20), and, finally, sera from patients suffering from other tick-borne infections, like Babesiosis (n=20) or Rickettsiosis (n=15).

Polymerase Chain Reaction (PCR)

PCR was performed by using five different sets of primers whose sequence was obtained from the literature (Marconi and Garon, 1992; Picken 1992). All the PCR reagents, except the primers, were from the GeneAmp kit (Perkin-Elmer Cetus). A total of 50 pmol of the appropriate primer set and 25 µl of the spirochetes boiled suspension were used in each 50 µl reaction mixture. All amplifications were carried out with an automatic Mastercycler Personal DNA thermal cycler (Eppendorf, AG. Hamburg, Germany).

C6 *B. burgdorferi* (Lyme) ELISA kit

Quick ELISA C6 *Borrelia* assay (Immunetics, Cambridge, Mass., USA) is a quantitative and competitive method based on a synthetic peptide antigen (C6 peptide) in a 96 microwell plate ELISA format. The antigen amino acidic sequence is derived from the VlsE protein of *B.*

burgdorferi, which has been shown to elicit an immune response consisting primarily of IgG antibodies (Bacon *et al.*, 2003; Lawrenz *et al.*, 1999; Liang *et al.*, 1999; Magnarelli *et al.*, 2002). Results were scored as negative (ELISA index score, ≤ 0.90), equivocal (0.91 to 1.09), or positive (≥ 1.1), as suggested by the manufacturer.

Anti-Borrelia plus VlsE ELISA

Anti-*Borrelia* plus VlsE ELISA, (Euroimmun, Lübeck, Germany) is a quantitative method based on a mix of whole antigen extracts of *B. burgdorferi* s. s., *B. afzelii* and *B. garinii* and recombinant VlsE lipoprotein of *B. burgdorferi*. Three calibrators, positive and negative controls were used. Results were scored as negative (Ratio < 1.0) or positive (Ratio ≥ 1.0), following the manufacturer's instructions.

Euroline-WB against Borrelia

The Euroline-WB (Euroimmun, Lübeck, Germany) uses proteins from a European isolate of *B. afzelii* plus a membrane strip coated with a recombinant form of the VlsE antigen. According to the product insert, this antigen combination guarantees optimum sensitivity and specificity of the assay. Each test strip has an integrated conjugate reactivity control. Each kit includes a developed positive control strip used as a band locator. Bands were scored as weak (intensity $<$ cutoff), or clear (intensity \geq cutoff). An IgG WB test was considered positive when at least two bands out of p83, p39, p31, p30, OspC, p21, p19 and p17 were present, or when the VlsE antigen band was present even if no additional specific bands were present. An IgM WB test was considered positive when at least OspC antigen band was clearly present or when OspC was weak and one more band among p83, p39, p31, p30, p21, p19 and p17 was clearly recognized.

Qualicode B. burgdorferi Western Blot kit

The Qualicode WB kit (Immunetics, Cambridge, Mass., USA) uses sodium dodecyl sulfate-solubilized *B. burgdorferi* proteins separated by gel electrophoresis and transferred to a nitrocellulose membrane. The membranes were processed according to the product instructions. Positive, weakly positive and negative controls were included. Bands were identified using an IgG and

IgM blot reading guide consisting of a previously developed and mounted strip from the same production lot. The intensity of the bands was monitored by comparison to the IgG or IgM weakly reactive control. Only bands of intensity equal to or greater than this minimum were scored. Blots were interpreted following the CDC criteria (Centers for Disease Control and Prevention, 1995).

Strains cultivation and preparation of antigens

B. burgdorferi sensu stricto strain IRS (ATCC 35211), *B. garinii* strain P/Bi (Hauser *et al.*, 1999) and *B. afzelii* strain VS461 (Norman *et al.*, 1996) were cultivated in standard BSK II medium without the addition of antibiotics, as previously reported (Cevenini *et al.*, 1992; Marangoni *et al.*, 1999; Sambri *et al.*, 2002). All the strains were harvested when grown to a cell density of 10^8 /ml. The protein concentration of the final suspension (1 mg/ml in phosphate buffered saline 0.1M, pH 7.2) was estimated by the Bradford protein assay, and the preparations were stored at -80°C until used.

SDS-PAGE and Western Blotting (WB)

Separation of polypeptides was performed with a Laemmli buffer system (Laemmli 1970) using a 12% acrylamide gel (Sambri *et al.*, 1999). The Western Blot procedure was performed according to Towbin (Towbin *et al.*, 1979) as previously described (Marangoni *et al.*, 1999; Sambri *et al.*, 2001). After electrophoretic transfer the blots were incubated overnight at room temperature with sera diluted 1:100 (for IgG detection) or 1:50 (for IgM detection) in PBS containing 0.05% (vol/vol) Tween 20. The reader was blinded to the identity of the serum samples when the homemade WB strips were examined. The identity of each antigen was inferred by using a panel of monoclonal antibodies kindly provided by K. Davis (CDC, Atlanta, USA). Each serum was evaluated using a three-lanes strip, where individual lanes were loaded with *B. burgdorferi* s. l. strains IRS, P/Bi and VS461, respectively. An IgG WB test was considered positive when at least two bands of p83/100, p58, p39, OspA, OspB, p30, p28, OspC, p21, p17 were present, whereas an IgM WB test was considered positive when at least one band of p39, OspC, p17 was clearly recognized, as previously described (Marangoni *et al.*, 1999).

RESULTS

PCR

All 30 cultures from patients showed a positivity for Lyme disease spirochetes within one month: 24 strains were identified as *B. afzelii* (80.0%), five were *B. garinii* (16.7%) and only one strain was identified as *B. burgdorferi* s. (3.3%) by PCR assay.

SEROLOGICAL METHODS

The results obtained with sera from Lyme disease culture confirming patients and cross-reacting samples are summarized in table 1. Lyme disease patients were bled twice: one blood sample was obtained when entering the study (first bleeding group) and a second sample was obtained from each patient one month later (second bleeding group). Anti-*Borrelia* plus VlsE ELISA IgG was more sensitive than Quick ELISA C6 (sensitivity was 56.6% and 33.3%, respectively). Moreover, considering that 17 additional sera from the first bleeding group of Lyme disease patients were IgM positive when tested by Anti-*Borrelia* plus VlsE IgM, the sensitivity of Anti-*Borrelia* plus VlsE as

a whole system rose to 85%. Both Quick ELISA C6 and Anti-*Borrelia* plus VlsE ELISA IgG were specific (98.5% and 95.4%, respectively), whereas Anti-*Borrelia* plus VlsE IgM performed with a specificity of 78.5%. Regarding WBs IgG results, the most sensitive method was the multispecies “home-made” WB (71.7%), followed by Euroline-WB against *Borrelia* (68.3%). Qualicode *B. burgdorferi* WB was the least sensitive method (26.7%). The value of specificity of Euroline-WB against *Borrelia* IgG was 80.0%, due to 12 sera of the group of potentially cross-reactive samples that were scored as borderline, whereas both Qualicode *B. burgdorferi* WB IgG and “home-made” WB IgG were 100% specific. Performances of the three IgM WBs were very similar: all of them were 100% specific, Euroline-WB against *Borrelia* IgM was 13.3% sensitive, “home-made” WB IgM was 11.7% sensitive and the value of sensitivity of Qualicode *B. burgdorferi* WB IgM was 8.3%.

DISCUSSION

In an attempt to increase the accuracy of Lyme disease diagnosis in Europe, the performances

TABLE 1 - Number of positive specimens out of the total samples obtained from culture confirmed Lyme disease patients and cross-reacting samples. Numbers of specimens are indicated in brackets. Lyme disease patients were bled twice: a first blood sample was obtained when entering the study (first bleeding group) and a second blood sample was obtained from each patient one month later (second bleeding group)

Group (total number)	Quick ELISA C6	Anti- <i>Borrelia</i> plus VlsE ELISA IgG	Anti- <i>Borrelia</i> plus VlsE ELISA IgM	Qualicode <i>B.</i> <i>burgdorferi</i> WB IgG	Qualicode <i>B.</i> <i>burgdorferi</i> WB IgM	Euroline-WB against <i>Borrelia</i> IgG	Euroline-WB against <i>Borrelia</i> IgM	“home made” WB IgG	“home made” WB IgM
Lyme 1st bleeding (30)	0/30	10/30	17/30	0/30	5/30	15/30	8/30	17/30	7/30
Lyme 2nd bleeding (30)	20/30	24/30	0/30	16/30	0/30	26/30	0/30	26/30	0/30
Syphilis (20)	0/20	3/20	8/20	0/20	0/20	2*/20	0/20	0/20	0/20
Babesiosis (10)	1/10	0/10	0/10	0/10	0/15	5*/10	0/15	0/10	0/15
Rickettsiosis (15)	0/15	0/15	0/15	0/15	0/15	5*/15	0/15	0/15	0/15
Mononucleosis (10)	0/10	0/10	3/10	0/10	0/10	0/10	0/10	0/10	0/10
<i>Streptococcus pyogenes</i> infection (10)	0/10	0/10	3/10	0/10	0/10	0/10	0/10	0/10	0/10

*These sera were scored as borderline, since they showed a weak reaction against the VlsE antigen band

of two ELISA methods based on different antigens and three different WBs were compared, using as a source of sera a panel of 60 samples obtained from 30 Italian patients with culture-confirmed Lyme Borreliosis erythema migrans. At the initial clinical evaluation, each patient was bled and given specific antibiotic therapy for Lyme borreliosis. These 30 sera composed the first bleeding group of Lyme disease patients. The follow-up study was done by taking additional serum samples one month after enrolment: these 30 samples constituted the second bleeding group of Lyme disease patients. An additional panel of 65 potentially cross-reacting samples was also included in the study to investigate specificity values of the different methods.

First of all, the performances of Anti-*Borrelia* plus VlsE ELISA and Quick ELISA C6 *Borrelia* were compared. Both ELISAs are new generation immunoenzymatic methods. The former contains a mix of whole antigen extracts of the three genospecies of *B. burgdorferi* s. l. plus a recombinant VlsE (Vmp-like sequence, expressed) lipoprotein of *B. burgdorferi* (Zhang *et al.*, 1997). The latter uses as antigen a 26-mer synthetic peptide (the C6 peptide) based on the invariable region 6 (IR6) of the VlsE. IR6 is a highly immunogenic peptide, that has been shown to remain unchanged during antigenic variation and is both structurally and antigenically conserved among pathogenic *B. burgdorferi* s. l. strains and genospecies (Liang *et al.*, 1999). Anti-*Borrelia* plus VlsE ELISA IgG was more sensitive than Quick ELISA C6 (56.6% and 33.3%, respectively). Both values are too low to be used as a unique screening test. Lack of sensitivity can be a real problem in the routine serologic diagnosis of Lyme disease, producing a large number of potentially false negative results. However, considering that 17 additional sera from the first bleeding group of Lyme disease patients were IgM positive when tested by Anti-*Borrelia* plus VlsE IgM, the sensitivity of Anti-*Borrelia* plus VlsE as a whole system rose to a much higher and completely acceptable value of 85.0%.

It is possible to speculate that the antibiotic therapy given to all of the patients following entry in the study could have interfered with the development of the immune response. It is known from literature that an early antibiotic treatment

can modulate the response of the IgG antibodies during the course of the infection (Aguero-Rosenfeld *et al.*, 1996) and that therapy may influence the onset of the immune response against some antigens more than others (Aguero-Rosenfeld *et al.*, 1996; Peltomaa *et al.*, 2003; Strle *et al.*, 1996). The specificity values of both the IgG tests studied showed very good results when the cross-reacting sera were tested. Quick ELISA C6 and Anti-*Borrelia* plus VlsE ELISA IgG were very specific (98.5% and 95.4%, respectively), whereas Anti-*Borrelia* plus VlsE IgM was only 78.5% specific. Considering that Anti-*Borrelia* plus VlsE ELISA is a screening method, this value of specificity for IgM detection is more than acceptable.

The most sensitive WB IgG test was the “home-made” one, prepared with native antigens from the three different genospecies of *B. burgdorferi* s. l. (71.7%). Euroline-WB against *Borrelia* IgG was much more sensitive (68.3%) than Qualicode *B. burgdorferi* WB IgG (26.7%): the addition of VlsE antigen seemed to greatly improve the diagnostic performance of a native antigens-based WB. Nevertheless, some problems of false positive results arose from the interpretation criteria used by Euroline-WB against *Borrelia* IgG. We scored 12 cross-reacting samples as borderline, since a weak VlsE band was present in the strips examined. Considering this problem, the value of specificity of Euroline-WB against *Borrelia* IgG was 80.0%, whereas both the “home-made” WB IgG and Qualicode *B. burgdorferi* WB IgG were 100% specific. Performances of the three IgM WBs were very similar: all of them were 100% specific, Euroline-WB against *Borrelia* IgM was 13.3% sensitive, “home-made” WB IgM was 11.7% sensitive and Qualicode *B. burgdorferi* WB IgM was 8.3% sensitive.

The very low IgM detection rate obtained in this study, especially in the second bleeding group of Lyme disease patients, was probably due to the antibiotic therapy received by all patients. Also the long period of time since the tick bite occurred could have been, in selected cases, a cause of IgM seronegativity in this group of sera tested. In the test, when the C6 peptide was used in a diagnostic ELISA test with serum samples obtained from U.S. patients, the assay performed with good sensitivity and specificity (Liang *et al.*,

1999; Magnarelli *et al.*, 2002; Peltomaa *et al.*, 2003). The current recommendation by the CDC and the German Society for Hygiene and Microbiology (Centers for Disease Control and Prevention, 1995; Wilske *et al.*, 2000) relies on the use of a second-tier, confirmatory test for Lyme disease when the first test yields a positive or equivocal result. Vice versa, in a study published in 2003, a comparison between a classic two steps testing and a VlsE-based ELISA reported higher values of sensitivity for the latter, maintaining very good specificity (Bacon *et al.*, 2003). Moreover, in a recent work by Mogilyansky and co-workers (Mogilyansky *et al.*, 2004) Quick ELISA C6 was considered so sensitive and specific that a confirmation by WB may not be required. In our opinion, the diagnostic performance of Anti-*Borrelia* plus VlsE ELISA was better than Quick ELISA C6, being far more sensitive and having the advantage of discriminating between IgG or IgM response: this could be a useful support for the clinical diagnosis. Nevertheless, due to the specificity value identified in this study, Anti-*Borrelia* plus VlsE ELISA did not prove suitable as a confirmatory test. Therefore the need for a confirmatory test, like the WB, remains a must, at least in Europe, where the diagnosis of Lyme disease is complicated by the presence of more than one pathogenic genospecies.

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