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Comparative evaluation of three different ELISA methods for the diagnosis of early culture-confirmed Lyme disease in Italy

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In this study the raising and development of the immune response to *Borrelia burgdorferi* infection in 45 Italian patients suffering from culture-confirmed Lyme borreliosis erythema migrans was investigated. A total of 95 serially collected serum samples were tested by using three different commercial ELISAs: recomWell Borrelia (Mikrogen), Enzygnost Borreliosis (DADE Behring) and Quick ELISA C6 Borrelia (Immunetics). The sensitivities of the ELISAs were as follows: Enzygnost Borreliosis IgM, 70·5 %; Quick ELISA C6 Borrelia, 62·1 %; recomWell Borrelia IgM, 55·7 %; recomWell Borrelia IgG, 57·9 %; and Enzygnost Borreliosis IgG, 36·8 %. In order to compare the specificity values of the three ELISAs, a panel of sera obtained from blood donors (210 samples coming from a non-endemic area and 24 samples from an endemic area) was tested, as well as sera from patients suffering from some of the most common biological conditions that could result in false-positive reactivity in Lyme disease serology ($n = 40$). RecomWell Borrelia IgG and recomWell Borrelia IgM were the most specific (97·1 % and 98·9 %, respectively), followed by Quick ELISA C6 Borrelia (96·7 %). Enzygnost Borreliosis IgG and IgM achieved 90·1 % and 92·3 % specificity, respectively. Sera that gave discrepant results when tested by the three ELISAs were further analysed by Western blotting.

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INTRODUCTION

Lyme disease, caused by the tick-borne spirochaetes belonging to *Borrelia burgdorferi sensu lato*, is a multistage infection that has become the most common vector-borne disease in North America and Europe (Gern *et al.*, 1998). In Europe three different species of *B. burgdorferi sensu lato* (namely *Borrelia burgdorferi sensu stricto*, *Borrelia garinii* and *Borrelia afzelii*) are known to be pathogenic for humans (Baranton *et al.*, 1992; Ciceroni *et al.*, 2001; O'Connell *et al.*, 1998; Strle *et al.*, 1996; Wang *et al.*, 1999), all of them demonstrating both inter- and intraspecies heterogeneity (Baranton *et al.*, 1992; Wilske *et al.*, 1996).

Lyme disease usually begins with a characteristic expanding skin lesion, erythema migrans (EM) (Nadelman *et al.*, 1996; Nadelman & Wormser, 1998; Steere, 1994). Diagnosis is based on clinical and laboratory findings. Serological testing is the most commonly used corroborative laboratory method; however, the occurrence of cross-reacting antibodies may result in false-positive findings (Aguero-Rosenfeld *et al.*, 1996; Magnarelli *et al.*, 1990, 1994, 2000). Furthermore, patients may 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).

A two-step testing strategy for the serodiagnosis of Lyme disease has been recommended both in the USA (Centers for Disease Control and Prevention, 1995; Wormser *et al.*, 2000) and in Europe (Wilske *et al.*, 2000). This strategy consists of the use of an ELISA or immunofluorescence assay, followed by Western blotting (WB) if the results obtained by the screening tests are indeterminate or positive. Several attempts to standardize the serological tests for Lyme disease have been made (Hauser *et al.*, 1997, 1998, 1999; Heikkila *et al.*, 2002; Robertson *et al.*, 2000), but considerable variations in results have been obtained even when using the same strategy (Robertson *et al.*, 2000).

Abbreviations: EM, erythema migrans; WB, Western blotting

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The purpose of the present study was to compare the performance of three different ELISA tests for the serological diagnosis of Lyme disease. A recombinant antigen-based-ELISA, a detergent extract from *B. afzelii* PKo based-ELISA and a synthetic peptide-based ELISA were evaluated. This study was performed with a panel of human sera collected from patients suffering from early Lyme disease in Italy.

METHODS

Study groups. In this study a total of 369 human serum specimens were studied. Ninety-five sera were obtained from 45 culture-confirmed Lyme disease patients (31 males and 14 females) aged between 29 and 65 years (mean age 42·8) suffering from EM following a tick bite. Patients were enrolled in the study after a mean EM duration of 16 days (ranging between 5 and 106). All the patients came from an endemic area in the north-east of Italy. Approximately 70 % of the patients recalled the tick bite, but all of them had occupational or recreational risk of exposure to *Ixodes ricinus* ticks.

A skin punch biopsy was obtained from each patient at the start of the study and was incubated in Barbour-Stoenner-Kelly medium (BSKII) plus ciprofloxacin (0·4 μg ml^{-1}) and rifampicin (40 μg ml^{-1}); the tubes were examined weekly by dark-field microscopy for motile spirochaetes over a period of at least 30 days, as previously described (Marangoni *et al.*, 1999). All 45 cultures were positive for Lyme disease spirochaetes within 1 month.

At the initial clinical evaluation, each patient was bled and given specific antibiotic therapy for Lyme borreliosis. For the follow-up study, additional serum samples were taken at 30 and 60 days after the enrolment (except 10 patients who were bled only twice, at 0 and 30 days after entering the study, and 15 patients who were bled only once, at time 0).

Two hundred and ten additional sera were obtained from the blood bank of St. Orsola Hospital in Bologna, and 24 samples were obtained from healthy blood donors from an endemic Lyme disease area in north-eastern Italy (Trento). Furthermore, a panel of 40 sera was obtained from patients suffering from some of the common conditions that can result in false-positive reactivity in Lyme disease serology. In this group the following specimens were included: sera from patients suffering from *Streptococcus pyogenes* acute infection [streptolysin O antibody response (ASO) >400 IU ml^{-1}] ($n = 10$), serum samples drawn from subjects with a clinical diagnosis of infectious mononucleosis detected as positive by Paul–Bunnel–Davidsohn agglutination ($n = 10$), sera from patients with Hepatitis A virus acute infection (IgM positive) ($n = 10$) and, finally, sera from syphilis patients (primary and secondary stage) ($n = 10$).

PCR. PCR was performed by using five different sets of primers whose sequences were obtained from the literature (Marconi & Garon, 1992; Picken, 1992): FL6–FL7 amplifies a fragment of the flagellin gene found in all the *B. burgdorferi sensu lato* strains; LD amplifies a 16S rRNA genomic fragment common to the three genospecies; BB, BG and BA each amplify a species-specific 16S rRNA genomic fragment. These primer sets generated amplification products of 276, 357, 574, 574 and 591 bp, respectively.

Spirochaetes were extracted for PCR as previously described (Sambri *et al.*, 2001). PCR reagents from the GeneAmp kit (Perkin-Elmer Cetus) were used. A total of 50 pmol of the appropriate primer set and 25 μl of the spirochaetes boiled suspension were used in each 50 μl reaction mixture. All amplifications were carried out with an automatic Eppendorf Mastercycler Personal DNA thermal cycler. Thirty-nine strains were identified as *B. afzelii* (86·7 %), five as *B. garinii* (11·1 %) and only one strain as *B. burgdorferi sensu stricto* (2·2 %), by PCR assay.

Serological methods

RecomWell Borrelia. The recomWell Borrelia test (Mikrogen) is a quantitative *in vitro* method for the detection of IgG or IgM antibodies against *B. burgdorferi sensu lato* in human serum or plasma samples. This test is based on the principle of an indirect 'sandwich' enzyme immunoassay and is prepared with a recombinant form of the *B. burgdorferi sensu lato* antigens. The IgM assay contains OspC and p41/intern, whereas the following antigens are used to coat IgG recomWell Borrelia plates: p100, OspC, p41/intern and p18. This test was performed following the manufacturer's instructions.

Enzygnost Borreliosis. Enzygnost Borreliosis (DADE Behring) is a method for the qualitative detection and quantitative determination of specific IgG and/or IgM antibodies to *B. burgdorferi sensu lato* in human serum or plasma. The method is based on a detergent extract from *B. afzelii* strain PKo. The assay was processed by an automated instrument (Genesis RSP 200/BEP III) following the instructions of the manufacturer.

Quick ELISA C6 Borrelia. Quick ELISA C6 Borrelia assay (Immunetics) is a quantitative competitive method based on a synthetic peptide antigen (C6 peptide) in a 96-microwell plate ELISA format. The antigen amino acid 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). This test does not discriminate between an IgG and IgM response. The test was performed according to the manufacturer's instructions.

RecomBlot Borrelia. RecomBlot Borrelia (Mikrogen) is an immunoblot test for the detection of IgG or IgM antibodies directed against *B. burgdorferi sensu lato* in human serum or plasma. Each test strip is loaded with recombinant antigens derived from all three genospecies of *B. burgdorferi sensu lato*, as follows: p100, p41, p39, OspA and p18 are derived only from *B. afzelii*; OspC is present in three distinct forms and each one is derived from *B. burgdorferi sensu stricto*, *B. afzelii* and *B. garinii*; finally, the internal part of p41 is derived both from *B. afzelii* (named 41 inta) and *B. garinii* (named 41 intg). Each test was interpreted following the manufacturer's score system.

***Borrelia burgdorferi* EcoBlot.** *Borrelia burgdorferi* EcoBlot (Genzyme-Virotech) is a native *B. burgdorferi sensu stricto* (strain 2591) antigen-based immunoblot for the qualitative detection of *B. burgdorferi sensu lato* IgG or IgM antibodies in human serum samples. The test was performed and interpreted in accordance with the manufacturer's instructions.

Strain cultivation and preparation of antigens. *B. burgdorferi sensu stricto* strain IRS, *B. garinii* strain P/Bi and *B. afzelii* strain vs461 were cultivated in standard BSKII medium without the addition of antibiotics; the spirochaetes were harvested and the antigen preparations were made and stored as previously reported (Cevenini *et al.*, 1992; Marangoni *et al.*, 1999; Sambri *et al.*, 2002).

SDS-PAGE and Western blotting (WB). Separation of polypeptides was performed with a Laemmli buffer system by using a 12 % w/v acrylamide gel (Laemmli, 1970; Sambri *et al.*, 1999). The WB procedure was performed according to 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 % v/v Tween 20.

To enable 'blind' interpretation of the results, the WB strips were coded so that the source of the serum samples was not apparent. The identity of each antigen was inferred by using a panel of monoclonal antibodies kindly provided by K. Davis (Centers for Disease Control and Prevention, Atlanta, USA). Each serum sample was evaluated by using a three-lane strip. Each single lane was loaded with *B. burgdorferi sensu lato* strains IRS, P/Bi or vs461, respectively. An IgG WB test was considered positive when at least two bands of p83/100, p58, p39, OspA, OspB, p28, OspC, p22 or p18 were present, whereas an IgM WB test was considered positive when at least one band of p39, OspC or p18 was clearly recognized, as previously described (Marangoni *et al.*, 1999).

RESULTS

In an attempt to improve Lyme disease diagnosis in Europe, the sensitivities and specificities of three different antigen-based ELISAs were compared. These were the Quick ELISA C6 Borrelia, a synthetic peptide antigen-based ELISA, the recomWell Borrelia, a recombinant antigen-based ELISA, and Enzygnost Borreliosis, a traditional ELISA kit prepared with a detergent extract of *B. afzelii* PKo.

Two different panels of sera were evaluated: the first consisted of samples obtained from Italian patients suffering from culture-confirmed EM, whereas the second was composed of sera collected from blood donors and patients with infections that could interfere with Lyme disease serology. The culture-confirmed samples were subdivided into three different groups: group 1 consisted of the sera obtained at enrolment, group 2 included the samples collected 30 days after entering the study and group 3 was made up of the specimens obtained 2 months after enrolment.

Sensitivities of the ELISAs

The results of the tests on samples from patients with Lyme disease are presented in Table 1. With the culture-confirmed group 1 sera (45 samples), the sensitivity of recomWell Borrelia IgG was 40·0 %, whereas that of Enzygnost Borreliosis IgG was only 33·3 %. recomWell Borrelia IgM showed a sensitivity of 46·7 %, whereas Enzygnost Borreliosis IgM was 66·7 % sensitive. Taken as a whole, the sensitivity of recomWell Borrelia was 57·8 %, that of Enzygnost Borreliosis was 71·1 %, while Quick ELISA C6 Borrelia showed a sensitivity of 55·6 %. The 16 sera that gave discrepant results when tested by the three ELISA kits were further analysed by all WB methods (i.e. recomBlot Borrelia, EcoBlot Borrelia and the in-house WB method). The results are presented in Table 2. The in-house WB method was more sensitive than the two commercial WB kits, both of which gave similar results.

With the culture-confirmed group 2 sera (30 samples), the sensitivity of recomWell Borrelia IgG was 66·7 %, whereas that of Enzygnost Borreliosis IgG was only 36·7 %. RecomWell Borrelia IgM and Enzygnost Borreliosis IgM achieved a sensitivity of 70 % and 73·2 %, respectively. Taken as a whole, the sensitivity of recomWell Borrelia and Enzygnost Borreliosis was the same (83·3 %), whereas that of Quick ELISA C6 Borrelia was much lower (60 %). Ten sera showed discrepant results by the three ELISA kits and consequently these specimens were tested by WB (see Table 2 for WB results). The WB method developed in-house was again the most sensitive method.

With the culture-confirmed group 3 sera (20 samples), the sensitivity of recomWell Borrelia IgG was 85·0 %, whereas that of Enzygnost Borreliosis IgG was much lower (45·0 %). RecomWell Borrelia IgM performed with a sensitivity of 55 %, whereas Enzygnost Borreliosis IgM was 75 % sensitive. Taken as a whole, the sensitivity of recomWell Borrelia was 95·0 %, that of Enzygnost Borreliosis was 85·0 %, and, finally, that of Quick ELISA C6 Borrelia was 80·0 %. The six sera that gave discrepant results when tested by the three ELISA kits were further studied by WB (see Table 2 for WB results). The in-house-developed WB method was confirmed as the most sensitive method.

Specificities of the ELISAs

Table 3 gives details of the findings obtained by testing samples from blood donors and patients suffering from pathological conditions that can interfere with Lyme disease serology. All the sera that gave a positive ELISA result were analysed by the three WB methods but none were confirmed as being positive.

Table 1. Number of positive samples detected by different serological methods used in the laboratory diagnosis of Lyme borreliosis on serial serum samples of patients with culture-confirmed EM

Group 1 serum samples obtained at enrolment, group 2 at 30 days and group 3 at 60 days after entering the study. The results were obtained by testing the sera once.

Group (no. of serum samples)	No. of positive samples detected (%)						
	RecomWell Borrelia IgG	RecomWell Borrelia IgM	RecomWell Borrelia	Enzygnost Borreliosis IgG	Enzygnost Borreliosis IgM	Enzygnost Borreliosis	Quick ELISA C6 Borrelia
1 (45)	18 (40·0)	21 (46·7)	26 (57·8)	15 (33·3)	30 (66·7)	32 (71·1)	25 (55·6)
2 (30)	20 (66·7)	21 (70·0)	25 (83·3)	11 (36·7)	22 (73·2)	25 (83·3)	18 (60·0)
3 (20)	17 (85·0)	11 (55·0)	19 (95·0)	9 (45·0)	15 (75·0)	17 (85·0)	16 (80·0)
Total (95)	55 (57·9)	53 (55·7)	70 (73·7)	35 (36·8)	67 (70·5)	74 (77·9)	59 (62·1)

Table 2. Sera with discrepant findings when analysed by the three ELISAs were further examined by three WB methods

1, RecomWell Borrelia (IgG and/or IgM); 2, Enzygnost Borreliosis (IgG and/or IgM); 3, Quick ELISA C6 Borrelia; 4, Ecoblot Borrelia IgG; 5, Ecoblot Borrelia IgM; 6, recomBlot Borrelia IgG; 7, recomBlot Borrelia IgM; 8, in-house-developed WB IgG; 9, in-house-developed WB IgM. RecomWell Borrelia and Enzygnost Borreliosis results are shown with all the samples that were IgG- or IgM-reactive as positive; in other words, these two assays were studied as whole systems, in order to compare their results with those obtained by Quick ELISA C6 Borrelia.

Sample no.	Group	1	2	3	4	5	6	7	8	9
1	1	−	+	−	−	−	−	−	−	+
4	1	−	+	−	−	−	−	−	−	−
9	1	+	+	−	−	−	−	−	−	+
12	1	−	+	−	−	−	−	−	−	+
13	1	+	−	+	−	−	+	−	+	−
29	1	−	+	+	+	−	+	−	+	−
36	1	−	+	+	−	−	−	−	−	+
47	1	−	+	−	−	−	−	−	−	+
63	1	+	−	−	−	−	−	+	−	+
65	1	−	+	−	−	−	−	−	−	−
66	1	+	+	−	−	−	−	−	+	−
74	1	−	+	+	−	−	−	−	+	−
81	1	−	+	+	+	−	+	−	+	−
85	1	+	−	+	+	−	+	−	+	+
86	1	+	+	−	+	+	+	+	+	+
90	1	+	+	−	−	−	−	−	−	+
6	2	+	+	−	−	−	−	−	−	+
23	2	−	+	−	−	−	−	−	−	−
24	2	+	+	−	+	−	+	−	+	+
27	2	+	+	−	−	−	−	+	−	+
34	2	−	+	+	+	+	+	+	+	−
40	2	+	+	−	+	+	+	+	+	+
68	2	+	−	−	−	−	+	−	+	−
69	2	+	+	−	+	−	−	−	+	+
76	2	+	−	−	−	−	−	−	+	−
83	2	+	+	−	−	+	−	+	−	+
5	3	+	−	+	−	−	−	−	+	−
20	3	−	+	−	−	−	−	−	−	−
62	3	+	−	+	+	−	+	−	+	−
67	3	+	+	−	+	−	+	−	+	+
79	3	+	−	−	−	−	−	−	+	−
82	3	+	+	−	+	−	+	−	+	−

The specificity of recomWell Borrelia IgG was 97·1 %, whereas that of recomWell Borrelia IgM was 98·9 %. Enzygnost Borreliosis IgG was 90·1 % specific, whereas Enzygnost Borreliosis IgM achieved 92·3 % specificity. Taken as a whole, recomWell Borrelia was 96·0 % specific, whereas Enzygnost Borreliosis was only 82·5 % specific. Finally, Quick ELISA C6 Borrelia achieved 96·7 % specificity.

DISCUSSION

The sensitivity of Enzygnost Borreliosis IgM (70·5 %) was higher than that of recomWell Borrelia IgM (55·7 %), whereas recomWell Borrelia IgG was far more sensitive (57·9 %) than Enzygnost Borreliosis IgG (36·8 %), in particular when samples from group 3 (i.e. specimens obtained from patients suffering from infections lasting for at least 60 days) were tested.

It is important to underline that 86·7 % of the Lyme disease patients were infected by *B. afzelii*, as determined by PCR assay performed with strains isolated from individual patients. Enzygnost Borreliosis is based on an antigen obtained by detergent extraction of *B. afzelii* PKo strain and this could explain its high sensitivity with respect to IgM. On the other

Table 3. Number of negative specimens detected in the panel of control sera

The results were obtained by testing the sera once.

Source of sera (no. of samples)	No. of negative specimens detected (%)						
	recomWell Borrelia IgG	**recomWell Borrelia IgM**	**recomWell Borrelia**	**Enzygnost Borreliosis IgG**	**Enzygnost Borreliosis IgM**	**Enzygnost Borreliosis**	**Quick ELISA C6 Borrelia**
Blood donors from the St. Orsola Hospital Blood bank in Bologna (210)	205 (97·6)	210 (100·0)	205 (97·6)	189 (90·0)	203 (96·7)	182 (86·7)	205 (97·6)
Blood donors from the Blood bank in Trento (24)	22 (91·6)	24 (100·0)	22 (91·6)	18 (75·0)	22 (91·6)	16 (66·7)	21 (87·5)
ASO* (10)	10 (100·0)	10 (100·0)	10 (100·0)	10 (100·0)	8 (80·0)	8 (80·0)	10 (100·0)
HAV† (10)	10 (100·0)	10 (100·0)	10 (100·0)	10 (100·0)	8 (80·0)	8 (80·0)	10 (100·0)
EBV‡ (10)	10 (100·0)	7 (70·0)	7 (70·0)	10 (100·0)	4 (40·0)	4 (40·0)	9 (90·0)
Syphilis (10)	9 (90·0)	10 (100·0)	9 (90·0)	10 (100·0)	8 (80·0)	8 (80·0)	10 (100·0)
Specificity value§	266/274 (97·1)	271/274 (98·9)	263/274 (96·0)	247/274 (90·1)	253/274 (92·3)	226/274 (82·5)	265/274 (96·7)

*Patients with streptolysin O antibody titre (ASO) >400 IU ml^{-1}.

†Patients with Hepatitis A virus acute infection (IgM-positive).

‡Paul–Bunnel–Davidsohn agglutination-positive patients.

§Number of negative sera/total number of controls.

hand, the low sensitivity detected in this study for the Enzygnost Borreliosis IgG test is difficult to interpret, since one could expect a sensitivity as high as that found for the IgM.

It is possible to speculate that the antibiotic therapy given to all patients on enrolment to the study may have interfered with the development of the IgG immune response. It has been previously reported that early antibiotic treatment modulates the IgG antibody response during the course of the infection (Aguero-Rosenfeld *et al.*, 1996) and that therapy may influence the immune response to some antigens more than others (Aguero-Rosenfeld *et al.*, 1996, Peltomaa *et al.*, 2003; Strle *et al.*, 1996). Nevertheless, since Enzygnost Borreliosis IgG is a screening test, its lack of sensitivity could be a problem in the routine serological diagnosis of Lyme disease, producing a large number of potentially false-negative results. Moreover, the low IgG detection rate seen in this study by Enzygnost Borreliosis IgG is surprising considering the very high level of background positives identified in healthy subjects coming from an endemic area of north-eastern Italy (25 % of blood donors from Trento scored positive). Data from our laboratory, obtained using a commercially available, native antigen-based enzyme immunoassay (Euroimmun, Lubeck, Germany). indicates that the IgG prevalence in healthy north-eastern Italians is about 10 % (unpublished). Therefore detection of IgG with the Enzygnost Borreliosis kit is unlikely to be the result of a true positive for Lyme borreliosis. On the other hand, the recombinant antigen-based methods detected IgG prevalences among blood donors from north-eastern Italy of 8·4 % (recomWell) and 12·5 % (Quick C6 Borrelia).

RecomWell Borrelia IgG was much more specific than Enzygnost Borreliosis IgG (97·1 % and 90·1 %, respectively). Also recomWell Borrelia IgM was more specific than Enzygnost Borreliosis IgM (98·9 % and 92·3 %, respectively). It is interesting to note that 12 sera obtained from patients with infections other than Lyme disease scored positive with Enzygnost Borreliosis IgM. This lack of specificity makes it difficult to correctly interpret the positive results when investigating the IgM response.

Quick ELISA C6 Borrelia is a new generation immunoassay, with a 26-mer synthetic peptide (the C6 peptide) antigen based on the invariable region 6 (IR6) of the VlsE (Vmp-like sequence, expressed) lipoprotein of *B. burgdorferi* (Zhang *et al.*, 1997). 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 sensu lato* strains and genospecies (Liang *et al.*, 1999). When the C6 peptide was used in a diagnostic ELISA test with serum samples obtained from patients from the USA, the assay performed with good sensitivity and specificity (Liang *et al.*, 1999; Magnarelli *et al.*, 2002; Peltomaa *et al.*, 2003).

Since Quick ELISA C6 Borrelia is not able to discriminate between IgG and IgM responses, in order to compare the results obtained by this method with those of the other two ELISA tests it was necessary to record as positive all the serum samples that were IgG-reactive or IgM-reactive when tested by recomWell Borrelia and Enzygnost Borreliosis. Enzygnost Borreliosis was the most sensitive method (77·9 %), followed by recomWell Borrelia (73·7 %); Quick ELISA C6 Borrelia was the least sensitive (62·1 %), but was the most specific (96·7 %), followed by recomWell Borrelia (96·0 %). Enzygnost Borreliosis was the least specific (82·5 %).

Sera that gave discrepant results when tested by the three ELISAs were further analysed by WB. The most sensitive WB test was the in-house-developed method, prepared with native antigens from three different genospecies of *B. burgdorferi sensu lato*. Ecoblot Borrelia (using native antigens of *B. burgdorferi sensu stricto*) and recomBlot Borrelia (prepared with recombinant antigens of the three genospecies) gave similar results.

Interestingly, when sampled for the first time all 12 patients with an EM lasting more than 3 weeks were both IgG- and IgM-positive when tested by recomWell Borrelia, whereas six of them were positive with Enzygnost Borreliosis IgG, 11 were positive with Enzygnost Borreliosis and, finally, 10 were positive with Quick ELISA C6 Borrelia. None was seronegative using the in-house developed WB IgG test. On the other hand, the first serum samples of all eight patients with an EM lasting less than a week were seronegative with all the methods. Further serum samples were only available for two of these patients: seroconversion was only apparent in the third serum sample when tested by recomWell IgG and the in-house-developed WB IgG method, whereas the samples were negative with all the other methods.

The current recommendation by the CDC and the German Society for Hygiene and Microbiology (DGHM) (Centers for Disease Control and Prevention, 1995; Wilske *et al.*, 2000) involves the use of a second-tier, confirmatory test for Lyme disease when the first test yields a positive or equivocal result. A comparison by Bacon *et al.*, (2003) between a classic two tiered-testing and a VlsE-based ELISA, however, gave higher values of sensitivity for the latter, with good maintenance of specificity. In our opinion, the diagnostic performances of recomWell Borrelia and Quick ELISA C6 Borrelia were both acceptable from the clinical point of view. The former method has the advantage of discriminating between the IgG and the IgM response, and this could be a useful support for clinical diagnosis. Moreover, in this study recomWell Borrelia was more sensitive, especially with sera obtained at enrolment. In Europe the diagnosis of Lyme disease is complicated by the presence of more than one pathogenic genospecies (Baranton *et al.*, 1992; O'Connell *et al.*, 1998; Robertson *et al.*, 2000; Stanek & Strle, 2003). As a confirmatory test we therefore suggest the use of a multispecies Western blot, since this method showed the highest sensitivity.

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