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Comparative evaluation of three Western Blot assays for the diagnosis of Lyme borreoliosis in Romania

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ABSTRACT

The diagnosis of Lyme borreliosis (LB) is very complex due to the diversity of clinical manifestations, very few being exclusive for *Borrelia burgdorferi* infection . In this context the diagnosis is based on clinical criteria (symptoms and clinical signs), exposure history and laboratory test results. Generally, the microbiological laboratory data are a major criterium for the clinical diagnosis of LB. The antibody detection methods are the main laboratory tests used to support a clinical diagnosis for most LB stages. In this study we investigated the detection specificity of antibodies against *B. burgdorferi* s.l by using three different Western blot (WB) tests to evaluate possible correlations between the clinical manifestations of LB and infecting *B. burgdorferi* genospecies in 26 Romanian patients with positive ELISA results for LB. Each of WB tests allows detection of specific antibodies against one of three *B. burgdorferi* s.l genospecies responsible for LB cases in Romanian patients, represented by *B. afzelii*, *B. garinii* and *B. burgdorferi* sensu stricto. The results of our study showed that both IgG and especially IgM antibodies cross-react mainly with antigens of *B. garinii* and *B. afzelii*, no specific correlation can be done between genospecies of *B. burgdorferi* s.l involved in infection and clinical manifestations of patients with clinical suspicion of LB. Therefore the WB tests used for the confirmation of ELISA results in the serum and cerebrospinal fluid (CSF) samples of patients suspected of LB must allow the simultaneous detection of the antibodies against antigens of all three *B. burgdorferi* s.l genospecies. It is also recommended the use of the latest generation of WB kits, which contain not only the antigens purified from three strains of *B. burgdorferi* genospecies, but also the antigens that are expressed during *in vivo* infection, obtained by recombinant DNA technology.

Keywords: *Lyme borreliosis, laboratory diagnosis, two-step strategy, Western blot, Borrelia burgdorferi genospecies.*

1. INTRODUCTION

Lyme borreliosis is a multisystemic infection caused by the pathogenic genospecies of the *Borrelia burgdorferi* s.l complex, including *B. burgdorferi* sensu stricto (s.s.), *B. garinii, B. afzelii, B. bavariensis,* and *B. spielmanii*. All pathogenic genospecies can cause erythema migrans (EM), which is the classical target rash that is characteristic of Lyme disease, but distinct genospecies possess diferent organotropism, and may preferentially cause distinct clinical manifestations of LB. *B.*

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burgdorferi s.s. is most commonly associated with arthritis, *B. garinii* with neuroborreliosis, and *B. afzelii* with cutaneous manifestations of LB (EM, lymphocytoma borreliosis, and acrodermatitis chronica atrophicans) [1]. *B. burgdorferi* s.s. and *B. afzelii* may also be associated with neurological manifestations, but at a lower rate than that of *B. garinii* [2-4].

In some geographical regions it has been observed an increase in the rates of disease transmission and in the incidence implicitly [5], so that LB should continue to be regarded as an emerging disease. In Romania, according to data reported by the National Center for Surveillance and Control of Communicable Diseases of the National Institute of Public Health, both institutions under the coordination of the Ministry of Health, the incidence of LB in 2011 was 2/100,000. Recent studies have shown that *B. burgdorferi* s.l genospecies circulating in Romania are: *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* [6].

LB diagnosis should be based primarily on the clinical presentation and an assessment of tick-exposure risk. The appropriate criteria of clinical case definitions underlying standardized diagnosis are described by Stanek in 2011 and presented in detail by EUCALB (European Concerted Action on Lyme borreliosis). In most cases laboratory support is essential because of the nonspecific nature of many clinical manifestations. Serological testing for detection of antibodies to *B. burgdorferi* remains the mainstay of diagnostic testing method, being usually the first and often the only method which supports the clinical diagnosis, because: it is relatively easy to obtain samples, the availability of laboratory facilities necessary for carrying it out, as well as of serological diagnostic kits [7].

Serological diagnosis in Europe relies on a two-step strategy. According to EUCALB, the first step consists of an ELISA screening having a specificity of at least 90% established in a healthy blood donor population. If the ELISA result is positive or equivocal, a confirmatory test based on a blot assay providing at least 95% specificity must be performed. This procedure aims to increase the specificity and the positive predictive value of the serology. Despite the improvements made in recent years concerning developing of appropiate laboratory tests, the serologic diagnosis of Lyme borreliosis remains challenging due to the complexity of the antigenic composition of *B. burgdorferi* s.l and the temporal appearance of antibodies to different antigens at successive time intervals after Borrelia infection [8, 9], but also due to the numerous false-positive results (reported especially with other spirochetes, Epstein–Barr virus, cytomegalovirus, human immunodeficiency virus) and discrepancies in serologic assays, even with the blot techniques [10].

The aim of the study was to evaluate the detection specificity of three Western blots (Euroimmun) tests of IgM and IgG antibodies against *B. burgdorferi* s.l for the diagnosis of early and late stage of Lyme borreliosis. Each of this kit allows detection of antibodies against one of three genospecies of *B. burgdorferi* s.l complex involved in LB in Romanian patients. We parallel tested 26 LB suspected patients with positive ELISA results for *B. burgdorferi* by using three WB kits. At the end of these testing we performed a comparative evaluation of detection specificity of *B. burgdorferi* antibodies by each kit and of the possibility of establishing of correlations between clinical manifestations of LB and infecting *B. burgdorferi* genospecies.

2. EXPERIMENTAL SECTION

In this study a total of 26 patients suspected of LB have been investigated. From all patients were collected serum samples that were tested by a commercial enzyme-linked immunosorbent assay (ELISA): ″*recom*Well Borrelia″ (Mikrogen).

″Anti-Borrelia afzelii WESTERNBLOT IgG″ and ″Anti-Borrelia afzelii WESTERNBLOT IgM″ kits from Euroimmun are containing strips with antigenic extracts of *B. afzelii* strain electrophoretically separated and are allowing detection of IgG, and IgM, respectively against *B. afzelii* from human

serum and plasma samples. Likewise the WB kits: Anti-*Borrelia garinii* WESTERNBLOT IgG″, ″Anti-*Borrelia garinii* WESTERNBLOT IgM″ Anti-*Borrelia burgdorferi* WESTERNBLOT IgG″ and ″Anti-*Borrelia burgdorferi* WESTERNBLOT IgM″ contain strips with antigenic extracts of *B. garinii*, and *B. burgdorferi*, respectively, which are allowing the detection of IgG and IgM antibodies against *B. garinii* and *B. burgdorferi* s.s, respectively from human serum and plasma samples. The specificity of antigens from IgM bands are the same as those of IgG strips for all three types ok kits. Depending on their specificity the antigens of *B. afzelii, B. garinii* and *B. burgdorferi* were generally divided into three categories (table 1).

Table 1: The categories of *B. burgdorferi* antigens used in the three WB kits used.

Category	Antigens
B. afzelii	
(1)	Cross-reacting antigens and antigens with undefined specificity with molecular weight of 16 kDa, 28 kDa, 35 kDa, 43 kDa, 50 kDa, 60 kDa and 75 kDa
(2)	The gen specific antigen with molecular weight of 41 kDa (flagelline)
(3)	Species specific antigens and high specific antigens with molecular weight of 17 kDa, 19 kDa, 21 kDa, 25 kDa, 30 kDa, 31 kDa, 39 kDa and 83 kDa.
B. garinii	
(1)	Cross-reacting antigens and antigens with undefined specificity with molecular weight of 28 kDa, 37 kDa, 43 kDa, 50 kDa, 57 kDa, 59 kDA, 62 kDa and 75 kDa
(2)	The gen specific antigen with molecular weight of 41 kDa (flagelline)
(3)	Species specific antigens and high specific antigens with molecular weight of 19 kDa, 21/22 kDa, 25 kDa, 29 kDa,30 kDa, 39 kDa and 83 kDa
B. burgdorferi	
(1)	Cross-reacting antigens and antigens with undefined specificity with molecular weight of17 kDa, 28 kDa, 32 kDa, 36 kDa, 43 kDa, 47 kDa, 50 kDa, 57 kDa, 59 kDa, 62 kDa and75 kDa
(2)	The gen specific antigen with molecular weight of 41 kDa (flagelline)
(3)	Species specific antigens and high specific antigens with molecular weight of 18 kDa, 21/22 kDa, 25 kDa, 31 kDa, 34 kDa, 39 kDa and 83 kDa

The results interpretation of IgM and IgG WBs was performed according with specifications of kits manufacturer. Thus only bands from (3) and (2) categories were taken into account for assigning of a results as being equivocal or positive.

3. RESULTS SECTION

The clinical manifestations and the results of WB tests performed on 26 analyzed patients are presented in tables 2 and 3. The patients tested for both types of antibodies (IgM and IgG) by all three WB kits are noted from 1a to 10 a, whereas the patients tested only for IgM were noted from 1 to 10. The patients tested only for IgG by three WB kits were noted from 1b to 6b.

Table 2: The IgM results obtained using three WB kits produced by Euroimmun

Pacient no.	Diagnostic	WB – B. burgdorferi	WB – B. garinii	WB – B. afzelii
1a	Cutaneous rash	Negative	Negative	Negative
2a	Without diagnostic	Negative	**Positive** (OspC, p41)	**Positive** (OspC, p41)
3a	Erythema migrans	**Positive** (p21, p41)	**Positive** (OspC)	**Positive** (OspC, p31, p41)
4a	Neurological syndrome	Negative	**Positive** (OspC)	**Positive** (OspC, p41)
5a	Facial palsy	Negative	**Positive** (OspC)	**Positive** (OspC, BmpA, p60)
6a	Lyme borreliosis	Negative	**Positive**	**Positive**

			(OspC)	(OspC, p41)
7a	Erythema migrans	**Positive** (p21, p41)	**Positive** (OspC, p41)	**Positive** (OspC)
8a	Lyme borreliosis	Negative	**Positive** (OspC)	**Positive** (OspC, p41)
9a	Erythema migrans	**Positive** (p21, p41)	**Positive** (OspC, p41)	**Positive** (OspC)
10a	Cutaneous rash	Negative	Negative	Negative
1	Polineuropathy	Negative	**Positive** (OspC)	**Positive** (OspC, p41)
2	Arthritis	Negative	Negative	Negative
3	Myocarditis	**Positive** (OspC)	**Positive** (OspC)	**Positive** (OspC, p41)
4	Arthritis	Negative	Negative	Negative
5	Arthritis	**Positive** (BmpA, p50)	Negative	Negative
6	Polyarthritis	Negative (p36, p50)	Negative	**Positive** (OspC, BmpA, p62)
7	Tick-bite	Negative	**Positive** (OspC)	**Positive** (OspC, p41, p60)
8	Without diagnostic	Negative	Negative	Negative
9	Tick-bite	Negative	**Positive** (OspC)	**Positive** (OspC, p41, p60)
10	Tick exposure	Negative	Negative	Negative

Table 3: The IgG results obtained using three WB kits from Euroimmun

Pacient no.	**Diagnostic**	**WB - B. burgdorferi**	**WB - B. garinii**	**WB - B. afzelii**
1a	Cutaneous rash	Negative	Negative (p41, p57)	Negative
2a	Without diagnostic	Negative	**Positive** (OspC, p30, p39, p41, p75)	**Positive** (OspC, p30, BmpA, p41, p43, p75)
3a	Erythema migrans	Negative	**Equivocal** (OspC, p41, p57)	**Positive** (p17, OspC, p41, p43, p75)
4a	Neurological syndrome	Negative	**Positive** (p17, p30, BmpA, p41, p43, p60, p75)	**Equivocal** (p30, p39, p41, p75)
5a	Facial palsy	Negative (p43)	**Positive** (BmpA, p30, p41, p43, p60)	**Positive** (p37, BmpA, p41, p57)
6a	Lyme borreliosis	Negative	**Equivocal** (BmpA, p41)	**Equivocal** (OspC, p41, p43)
7a	Erythema migrans	Negative (p43)	**Equivocal** (OspC, p41, p57)	**Positive** (OspC, BmpA, p41, p43, p60)
8a	Lyme borreliosis	Negative	**Equivocal** (BmpA, p41)	**Equivocal** (OspC, p41)
9a	Erythema migrans	Negative (p43)	**Equivocal** (OspC, p41, p57)	**Positive** (OspC, BmpA, p41, p43, p60)
10a	Cutaneous rash	Negative	Negative (p41)	Negative
1b	Arthritis	Negative	**Equivocal** (OspC, p41, p57)	**Equivocal** (OspC, p41, p43, p50, p60)
2b	Cutaneous rash	Negative (p57/p59)	**Equivocal** (OspC, p41, p57)	**Equivocal** (OspC, p41, p43, p50)
3b	Neurological syndrome	Negative	**Positive** (OspC, p30, p41, p50, p57, p75)	Negative (p41, p60, p75)

4b	Palsy	Negative (p43)	**Positive** (OspC, p37, BmpA, p41, p50, p75)	**Positive** (OspC, BmpA, p41, p43, p60)
5b	Erythema migrans	Negative	**Positive** (p19, BmpA, p41, p75, p83)	**Positive** (p17, BmpA, p19, OspC, p31, p41, p43, p83, p75)
6b	Cutaneous rash	Negative	Negative	Negative

The analysis of the WB results for IgM and IgG performed in parallel for patients noted starting from 1a to 10a shows some important aspects: (1) the 1a patient has negative results to both antibodies by all WB kits, indicating that ELISA results are false-positive, and the diagnosis of LB is not confirmed in this patient; (2) at patient 2a the clinical data are lacking, and WB results are positive for IgM and IgG against *B. garinii* and *B. afzelii*; (3) the 3a, 7a and 9a patients have IgM positive results to all three WB kits, whereas for IgG the positive result was obtained against *B. afzelii*, equivocal against *B. garinii* and negative against *B. burgdorferi*; these results suggesting that IgG antibodies have reacted strongly with antigens of *B. afzelii*, weakly with *B. garinii* antigens and very weakly with *B. burgdorferi* antigens; (4) the 4a and 5a patients have IgM positive results against *B. afzelii* and *B. garinii*, but negative for *B. burgdorferi*, whereas the IgG were positive against *B. garinii*, equivocal against *B. afzelii*, and negative for *B. burgdorferi*, which suggesting that testing both antibodies class is more informative for diagnosis, in particular in this case both IgM and IgG antibodies showed high reactivity to the antigen from *B. garinii* kits, which is also the most commonly genospecies incriminated as being responsible for the neurological manifestations of LB in Europe; (5) the 6a and 8a patients have IgM positive results for *B. afzelii* and *B. garinii*, whereas the IgG results are equivocal against these two *Borrelia* genospecies. Taking into account that important information are unknown like: the clinical manifestations, the time period spent from symptoms onset until serum collection, and the antibiotic treatment, it is necessary to retest on another serum sample from these two patients.

The analysis of WB results for IgM performed for patients noted starting from 1 to 10 shows that: (i) the patient 1 with polyneuropathy has IgM positive result against *B. afzelii* and *B. garinii* antigens, the number of present bands being higher for *B. garinii*; (ii) the patient 3 with myocarditis diagnosis has the positive results against antigens of all three *Borrelia* genospecies; (iii) the patient 5 with arthritis is IgM positive only against *B. burgdorferi*; (iv) the patient 6 with polyarthritis has IgM positive result only against *B. afzelii*; (v) the patients 7 and 9 with tick-bite have IgM against *B. afzelii* and *B. garinii*, the number of positive bands being higher against *B. afzelii* than those against *B. garinii*, (vi) the patients 2 and 4 with arthritis, and 8 without clinical data, respectively, have negative IgM results to all WB kits, showing that ELISA results are false-positive, and also that for these patients the LB diagnosis is not confirmed by WB.

The analysis of WB results performed exclusively for IgG to patients noted from 1b to 6b indicate the following aspects: (1) the patients 1b and 2b with clinical diagnosis of arthritis and cutaneous rash, respectively, have equivocal IgG results against antigens from *B. afzelii* and *B. garinii*, which are indicating the need for WB retesting on a new serum sample collected at intervals of several weeks or even months depending on the onset of clinical symptoms and whether an antibiotic treatment was started; (ii) at 3b patient with neurological syndrome were detected antibodies only against *B. garinii*; (iii) at patient 4b with palsy was detected IgG antibodies against antigens of *B. garinii* and *B. afzelii* (iv) at the patients 5b and 6b were detected IgG against *B. afzelii* (with a higher number of positive bands) and *B. garinii*; (v) the patient 6b with poliarthritis has negative IgG results to all WB kits, indicating that the suspicion of LB was not confirmed by WB.

The results obtained using these kits show that both IgG and especially IgM antibodies cross-react mainly against antigens of *B. garinii* and *B. afzelii*, so no specific correlation between genospecies of *B. burgdorferi* sl involved in infection and clinical manifestations of patients with clinical suspicion of LB cannot be done. Also, this information is of no particular importance for the patient and clinician because the antibiotic treatment is tailored to stages and clinical manifestations of LB without being dependent on a particular genospecies or another. Therefore, for a correct diagnosis of LB it is indicated to use WB tests that detect the antibodies against antigens of all three *B. burgdorferi sensu lato* genospecies in serum or cerebrospinal fluid (CSF) samples of patients suspected of LB. It is also recommended the selection of the latest generation of WB kits, which contain not only the antigens purified from three strains of *B. burgdorferi* genospecies but also the antigens that are expressed during *in vivo* infection, by recombinant DNA technology. These antigens are not expressed by the strains grown in the laboratory, and also represent the antigens towards which first antibodies are synthesized in the host body after infection.

4. CONCLUSIONS

The complexity of the antigenic composition of *B. burgdorferi sensu lato* has raised important challenges for the serodiagnosis of LB [11]. A sizable number of antigens are differentially expressed in the vector and the host [12], and some of them are exclusively expressed *in vivo* in the infected mammalian host. Furthermore, antigenic differences exist among the *B. burgdorferi sensu lato* species causing LB [11]. Western blot is important for the characterization of the immune response against specific proteins of *B. burgdorferi sensu lato*. The use of antigens separated by molecular size in WB assays has contributed to the determination of of *B. burgdorferi sensu lato* immunodominant antigens are in different stages of LB. The results of this study demonstrate that a correct serodiagnosis of LB is performed by using WB tests that allow the detection of the antibodies to antigens specific for all three *B. burgdorferi* s.l genospecies in a single test in serum samples of patients with clinical suspicion of LB.

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