

Seroprevalence of Lyme disease and genospecies of *Borrelia burgdorferi* sensu lato in patients diagnosed with borreliosis in the Province of Warmia-Masuria in north-eastern Poland

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Abstract

Between 2009-2010, a group of 259 patients suspected of contracting Lyme disease were examined in the Provincial Sanitary-Epidemiological Station in Olsztyn for the presence of IgM and IgG against specific *Borrelia burgdorferi* sensu lato (s.l.) genospecies antigens by immunoblot. A total of 27.4% and 29.0% of the blood serum samples showed positive and uncertain results for IgM and IgG antibodies. The majority of positive and uncertain results were found in patients aged 30-40 years (30%) for IgM, and people aged 50 and over (35.8%) for IgG. Significantly more positive results for IgG were found in males (40.2%) than females (19.7%). In both groups, similar proportions of positive results for IgM anti-*Borrelia* were recorded (26.1% of females and 29% of males). In 71.5% of patients, IgM against flagellin protein (p41) of *B. burgdorferi* sensu stricto (s.s.) was found. For IgG, the most frequently detected antibodies were found against the p41 protein of *B. burgdorferi* s.s. (64.8%) and the recombinant variable surface antigens (VlsE) (49%). Among all the analysed antigens those of *B. burgdorferi* s.s. were the most frequent cause of immunological reaction, followed by antigens of *B. afzelii* and *B. garinii*. Reaction to antigens of *B. spielmanii* was rarely detected.

Key words

borreliosis, *Borrelia burgdorferi* s.l. genospecies, immunoblot, IgM, IgG, north-eastern Poland

INTRODUCTION

Lyme borreliosis (LB) is a chronic, multi-systemic zoonosis transmitted by ticks of the genus *Ixodes* [1]. The etiological factors of LB are spirochetes of the complex *Borrelia burgdorferi* sensu lato (s.l.) [2, 3]. Based on morphology, antigens profile and DNA analysis, 12 genospecies have been distinguished in the complex *B. burgdorferi* s.l. [2]. In Central and Eastern Europe, LB is most often caused by *B. burgdorferi* s.s., *B. garinii*, *B. afzelii* [4, 5], although the remaining European species, *B. valaisiana*, *B. lusitaniae*, *B. bissettii* and *B. spielmanii*, have also been isolated from body fluids and tissues of patients with symptoms of LB [6, 7, 8, 9, 10].

The diversity of *B. burgdorferi* s.l. strains and the polymorphism of their antigens are taken into consideration in serological diagnostic tests for LB [11]. According to the recommendations of an international group of experts [12, 13] the proper laboratory diagnostics of LB should include 2 stages: 1) detecting the presence of specific IgM and IgG by highly sensitive immunoenzymatic methods (IFA, ELISA); 2) confirming positive or uncertain results

with the high quality immunoblot method. Contrary to the tests which use whole bacterial cells or their sonicates, the newest immunoblot tests use *Borrelia*-specific proteins, obtained by genetic engineering methods, as diagnostic antigens [11]. This type of diagnostic antigens avoids cross-reactions with antibodies produced during infection with other bacteria (e.g. *Treponema*, *Salmonella*, *Bacillus*) and viruses (e.g. *Herpes*) [12, 14]. Moreover, the use of recombinant proteins allows a simultaneous application of high heterogeneous proteins, such as OspC and p18 antigens from different genospecies of *B. burgdorferi* s.l., in quantities sufficient for the correct reading of the results [15, 16]. Tests based on recombinant proteins also facilitate estimation of the degree of advancement of LB through selection of antigens that are crucial for consecutive phases of the disease [14, 15]. It is also possible to use antigens of several genospecies of *B. burgdorferi* s.l. which dominate in infections in the geographic region, and also those that less often cause LB [12, 14, 15].

Using the possibilities offered by the most modern immunoblot tests, the aim of the presented research was to analyze of occurrence of LB and prevalence of genospecies of *Borrelia burgdorferi* s.l., based on the presence of specific antibodies in the serum of patients in Warmia-Masuria province, diagnosed as Lyme disease at the Provincial Sanitary-Epidemiological Station (PSES) in Olsztyn.

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MATERIALS AND METHODS

Examined patients. The study was conducted in 2009-2010 at PSES in Olsztyn on the serum samples from 259 patients suspected of contracting Lyme disease. The group of examined patients comprised 142 (51%) females and 117 (49%) males. The ages of tested persons ranged from 5-81 years, mean age 45 years.

Serological tests. The presence of IgM and IgG antibodies against specific antigens of *B. burgdorferi* s.l. was determined with immunoblot test using commercial kits *recomLine Borrelia IgM* and *recomLine Borrelia IgG* (Mikrogen, Germany). In both kits, recombinant proteins specific for 4 genospecies: *B. burgdorferi* s.s. (p41, OspC, p18), *B. afzelii* (p100, p39, OspA, OspC, p18), *B. garinii* (p58, OspC, p18) and *B. spielmanii* (OspC, p18), as well as VlsE (conservative protein fragment for different genospecies of *B. burgdorferi* s.l.), were used as diagnostic antigens. The tests were conducted in accordance with the procedure recommended by the manufacturer. Positive or negative results were determined on the base of the sum of point values assigned to each individual bands corresponding to reaction of IgM and IgG antibodies with particular antigens of *B. burgdorferi* s.l. (Tab. 1). Results with the value ≤ 5 were assumed to be negative, those equal to 6 were classified as borderline, and those ≥ 7 were assumed to be positive

Table 1. Number of points for bands obtained by the reaction of IgM and IgG antibodies with specific *Borrelia*-antigens in the immunoblot test *recomLine Borrelia* (Mikrogen, Germany)

Antibodies	Antigens of <i>B. burgdorferi</i> s.l.							
	p100	VlsE	p58	p41	p39	OspA	OspC	p18
IgM	5	5	4	1	4	5	8	5
IgG	5	5	4	1	5	5	5	5

Statistical analysis. The data were analyzed using χ^2 test and 2-sample proportion test with 1-tailed hypothesis. The significance level 0-0.05 was assumed in all statistical tests. Both tests were conducted using STATISTICA for Windows v. 7.1 package (StatSoft. Inc, Tulsa, Oklahoma, USA).

RESULTS AND DISCUSSION

Seroepidemiology of borreliosis. In Poland, human LB is becoming increasingly more frequent. According to the National Institute of Public Health, the number of LB cases in Poland in 2010 was 9,011 [17] and had increased more than 10-fold since 1996, the year when the official register of LB was started. For years, the highest incidence of LB has been observed in north-eastern Poland (Provinces of Podlasie and Warmia-Masuria) and in the southern part of the country (Provinces of Małopolska, Podkarpacie, Silesia and Lublin) [17]. During 1993-1995, the screening studies among inhabitants of Podlasie and Warmia-Masuria provinces showed the presence of antibodies specific to *B. burgdorferi* in 23.7% of the examined persons [18], while among blood donors in randomly selected provinces in Poland they were found in 11-13% [19].

Among the 259 patients suspected of contracting LB, diagnosed in 2009-2010 at the PSES in Olsztyn, positive or

Table 2. Presence of IgM and IgG antibodies against *B. burgdorferi* in patients according to district and province

District/ Province	Anti- <i>B. burgdorferi</i> IgM and IgG antibodies n (%)				Total n (%)
	IgM -	IgM+ ^a	IgG -	IgG+ ^b	
Bartoszyce	10 (90.9)	1 (9.1)	6 (54.6)	5 (45.4)	11 (100)
Braniewo	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	2 (100)
Działdowo	2 (40.0)	3 (60.0)	3 (60.0)	2 (40.0)	5 (100)
Elbląg	2 (100)	0	2 (100)	0	2 (100)
Elk	2 (100)	0	1 (50.0)	1 (50.0)	2 (100)
Giżycko	18 (66.7)	9 (33.3)	20 (74.1)	7 (25.9)	27 (100)
Gołdap	3 (33.3)	6 (66.7)	4 (44.4)	5 (55.6)	9 (100)
Iława	4 (100)	0	4 (100)	0	4 (100)
Kętrzyn	22 (88.0)	3 (12.0)	18 (72.0)	7 (28.0)	25 (100)
Lidzbark	3 (60.0)	2 (40.0)	2 (40.0)	3 (60.0)	5 (100)
Mragowo	18 (81.8)	4 (18.2)	17 (77.3)	5 (22.7)	22 (100)
Ostrzica	1 (25.0)	3 (75.0)	2 (50.0)	2 (50.0)	4 (100)
Nw. Miasto	0	1 (100)	1 (100)	0	1 (100)
Olecko	2 (50.0)	2 (50.0)	3 (75.0)	1 (25.0)	4 (100)
Olsztyn	70 (76.1)	22 (23.9)	69 (75.0)	23 (25.0)	92 (100)
Ostróda	9 (69.2)	4 (30.8)	10 (76.9)	3 (23.1)	13 (100)
Pisz	4 (57.1)	3 (42.9)	3 (42.9)	4 (57.1)	7 (100)
Szczytno- Węgorzewo	8 (80.0)	2 (20.0)	9 (90.0)	1 (10.0)	10 (100)
Warmia- Masuria	182 (72.8)	68 (27.2)	177 (70.8)	73 (29.2)	250 (100)
Other	6 (66.7)	3 (33.3)	7 (77.8)	2 (22.2)	9 (100)
Total	188 (72.6)	71 (27.4)	184 (71.0)	75 (29.0)	259 (100)

^a - sum of positive results (IgM+) and borderline results (IgM+/-);

^b - sum of positive results (IgG+) and borderline results (IgG+/-).

uncertain results for IgM class were obtained for 27.4% (n=71) of the patients, whereas for the the IgG class the respective proportion was 29.0% (n=75) (Tab. 2). The largest group of patients came from Olsztyn district (n=92), followed by the districts of Giżycko (n=27), Kętrzyn (n=25) and Mragowo (n=22) (Tab. 2). In these districts, positive or uncertain results for the IgM class ranged from 12.0%-33.3% of the patients, while for IgG class - from 22.7%-28.0%. The percentage of patients positively diagnosed with LB at the PSES in Olsztyn, based on the immunoblot test, was comparable with the results obtained for professional groups at high risk of LB. In north-eastern Poland, IgM and IgG anti-*Borrelia* were found in 23.8% of forestry workers [20]. Among foresters from south-eastern Poland, positive results were obtained for 12.8% and 25% for IgM and IgG, respectively [21]. Whereas in more than 35% of forestry workers from Lower Silesia [22], Kuyavian-Pomeranian province [23] and West Pomerania [24], anti-*Borrelia* antigens were found. Cisak *et al.* [25] detected positive results for LB in 33% of farmers from the Lublin region.

The division of patients diagnosed at the PSES in Olsztyn into 4 age groups showed no significant differences between the groups (Tab. 3). Positive and uncertain results for IgM class were the most frequent in the group of patients aged 30-40 (30%), and for the IgG class - among patients above the age of 50 (35.8%). The lowest level of IgM were among patients aged between 40-50 (23.9%), IgG - among those below the age of 30 (18.0%). Differences between females and males were significant only for the frequency of positive results for the IgG class antibodies (Tab. 4). The percentage of patients with positive results was greater among males (40.2%), compared to females (19.7%). The percentage of patients with IgM anti-*Borrelia* were similar in both groups (26.1% of females and 29% of males).

Table 3. Presence of IgM and IgG antibodies against *B. burgdorferi* s.l. according to age of patients

Age interval	Anti- <i>B. burgdorferi</i> IgM and IgG antibodies n (%)				Total n (%)
	IgM ^a	IgM ^b	IgG ^a	IgG ^b	
<30 years	36 (72.0)	14 (28.0)	41 (82.0)	9 (18.0)	50 (100)
30-40 years	28 (70.0)	12 (30.0)	31 (77.5)	9 (22.5)	40 (100)
40-50 years	35 (76.1)	11 (23.9)	34 (73.9)	12 (26.1)	46 (100)
>50 years	89 (72.4)	34 (27.6)	78 (63.4)	45 (36.6)	123 (100)
Total	188 (72.6)	71 (27.4)	184 (71.0)	75 (29.0)	259 (100)

^a – sum of positive results (IgM+) and borderline results (IgM+/-);^b – sum of positive results (IgG+) and borderline results (IgG+/-).**Table 4.** Presence of IgM and IgG antibodies against *B. burgdorferi* s.l. according to gender of patients

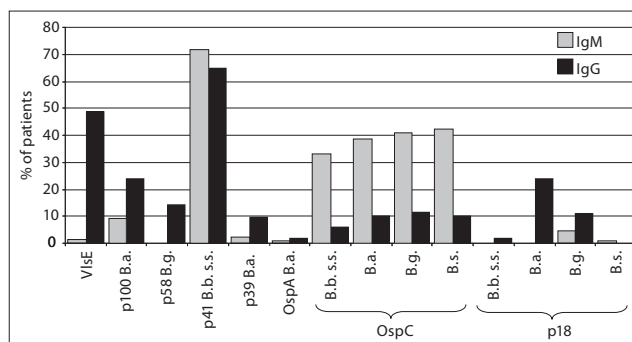
Sex	Anti- <i>B. burgdorferi</i> IgM and IgG antibodies n (%)				Total n (%)
	IgM ^a	IgM ^b	IgG ^a	IgG ^b	
Males	83 (70.9)	34 (29.0)	70 (59.8)	47 (40.2)	117 (100)
Females	105 (73.9)	37 (26.1)	114 (80.3)	28 (19.7)	142 (100)
Total	188 (72.6)	71 (27.4)	184 (71.0)	75 (29.0)	259 (100)

^a – sum of positive results (IgM+) and borderline results (IgM+/-);^b – sum of positive results (IgG+) and borderline results (IgG+/-); * χ^2 test, p<0.001.

Seroprevalence of genospecies of *B. burgdorferi* s.l.

Identification of IgM and IgG antibodies against *B. burgdorferi* is a basic laboratory test in LB diagnostics [13]. In human serum, the antibodies are produced 2-4 weeks after infection. IgM are the first to appear, followed by IgG as the main immunoglobulins in response to the pathogens [13].

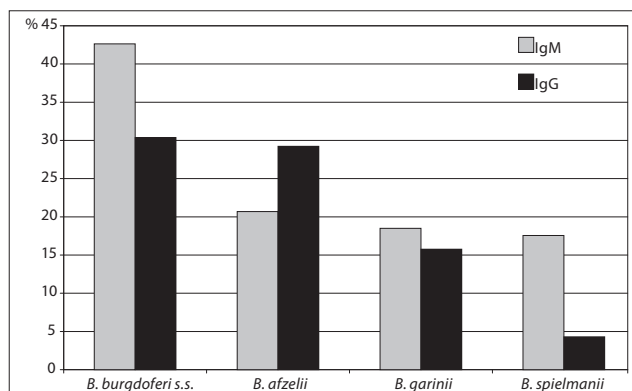
Among the patients diagnosed with the suspicion of LB suspicion in 2009-2010 at the PSES in Olsztyn, at least one fraction of IgM or IgG against antigens of *B. burgdorferi* s.l. was found in more than 77% (n=200). In this group, 27.5% (n=55) of the patients had IgM and 35% (n=70) IgG antibodies; 37.5% of the patients (n=75) had antibodies of both classes. The type of antibodies of each class in the serum of the infected patients depended on the heterogeneity of the species of the *B. burgdorferi* s.l. complex causing the infection, and on the polymorphism of their antigens. The recombinant proteins used in immunoblot as diagnostic antigens are not only characterised by high immunogenic properties, but are also selected in such a way as to indicate the stage of advancement of the disease. In patients with early LB, the most often detected antibodies are IgM against flagellin protein (p41) and outer surface protein C (OspC) of *B. burgdorferi* s.l., which begins to be expressed already in the early stage of tick feeding [15]. In many studies [26, 27, 28], the early immune response to both antigens was the most often detected. In the group of patients with IgM anti-*Borrelia* diagnosed at the PSES in Olsztyn, 71.5% (p<0.001) had antibodies against *B. burgdorferi* s.s. flagellin protein (p41) (Fig. 1). The next antibodies with respect to frequency were antibodies against OspC of *B. spielmanii* (42.3%), *B. garinii* (40.8%), *B. afzelii* (38.5%) and *B. burgdorferi* s.s. (33.1%) (p<0.001). No patients were found with IgM against the decorin-binding protein (p18, syn. DbpA, Osp17) of *B. afzelii* and *B. burgdorferi* s.s., or p58 protein of *B. garinii*. These antigens, together with antigens p83, p39, p14, p30, p43 and VlsE, are included into markers of late immune response for IgG anti-*Borrelia* antibodies [15]. In accordance

**Figure 1.** Percentage of patients with IgM and IgG antibodies against specific antigens of *B. burgdorferi* s.l. genospecies (abbreviations: B.a. – *B. afzelii*, B.g. – *B. garinii*, B.b.s.s. – *B. burgdorferi* s.s., B.s. – *B. spielmanii*).

with this, in the group of patients with IgG antibodies, besides response to the immunodominant flagellin protein (p41) of *B. burgdorferi* s.s., antibodies against VlsE protein were detected in 64.8% (p<0.001) and 49% (p<0.05) positive samples, respectively (Fig. 1). Antibodies against proteins p18 and p100 of *B. afzelii* (24.1% each) were detected twice less frequently. IgG against antigen OspA of *B. afzelii* and p18 of *B. burgdorferi* s.s. were detected only for 2.1% of positive samples. IgG against p18 of *B. spielmanii* were not found in any of the examined patients.

Serological tests used in LB diagnostics in Europe consider the diversity of *B. burgdorferi* s.l. strains and their participation in causing LB. In these tests, besides the antigens specific for *B. burgdorferi* s.s., *B. afzelii* and *B. garinii* – the genospecies which dominate in infections in Europe – there are also genospecies which less often cause LB (e.g. *B. spielmanii*). The origin of antigens is essential because it may suggest clinical symptoms of LB and the kind of antibiotic to use in the therapy. It has been shown that *B. burgdorferi* s.s. most often produces arthritic symptoms, while *B. afzelii* is most frequent among patients with skin symptoms of LB – erythema migrans (EM) and acrodermatitis chronica atrophicans (ACA) [4]. Studies on the antibiotic sensitivity of individual genospecies have revealed that *B. burgdorferi* s.s. is the most sensitive to amoxicillin, cephalosporin, tetracycline and ciprofloxacin, as well as to erythromycin, and *B. garinii*, to azithromycin and penicillin, while *B. afzelii* is particularly sensitive to ceftioxone [8, 29].

In patients diagnosed with LB at the PSES in Olsztyn by immunoblot, the most often identified bands corresponded with antibodies reacting with *B. burgdorferi* s.s. antigens (Fig. 2). In the IgM class, these constituted 42.6% (p<0.001)

**Figure 2.** Percentage of bands identified by immunoblot corresponding to the reaction of IgM and IgG antibodies with specific antigens of *B. burgdorferi* s.l. genospecies

of all identified bands, and in IgG – 30.4% ($p < 0.001$). Another group of antibodies of high frequency were immunoglobulins against *B. afzelii* antigens (20.7% in IgM and 29.2% ($p < 0.001$) in IgG). Bands corresponding to antibodies against *B. garinii* antigens formed 18.5% and 15.8% of all identified bands in IgM and IgG, respectively. Reaction to antigens of *B. spielmanii* was the least frequent. Bands corresponding to antibodies for antigens of this species constituted 17.6% in IgM and 4.3% in IgG. The participation of particular genospecies of *B. burgdorferi* in immune response observed in this study do not correspond to the results of other authors. According to Biesiada *et al.* [26], among patients diagnosed for LB at the Clinic of Infectious Diseases of the University Hospital in Kraków, the most frequent was immune response to antigens of *B. garinii* (77.5%) and *B. burgdorferi* s.s. (69%) in IgM and IgG, respectively. The least frequent were antibodies against antigens of *B. spielmanii*: 11% in the IgM and 8% in IgG classes. The differences may be associated with different level of *Ixodes ricinus* infection by various *B. burgdorferi* genospecies, depending on the geographic region. Stańczak *et al.* [30] indicated that in *I. ricinus* populations from north-eastern and central Poland, *B. afzelii* was the most prevalent genospecies, whereas in the tick populations from north-western and south-western parts of Poland, *B. burgdorferi* s.s. predominated [31, 32]. The presence of antibodies against *B. spielmanii* antigens in the serum of the patients diagnosed at PSES in Olsztyn, and also in Kraków [26] and Lublin [27], is surprising. This suggests the occurrence of this genospecies in local populations of *I. ricinus*. To date, there have been no reports of the prevalence of *B. spielmanii* in Poland, although it was identified in tick populations in adjacent countries – the Czech Republic [33] and Germany [34, 35]. Current data on the zoonotic sources of *B. spielmanii* indicate that the only confirmed reservoir species for this pathogen is the garden dormouse (*Eliomys quercinus*) [35], noted only in southern Poland [36].

In summary, it should be noted that the detection of antibodies against *B. burgdorferi* s.s., *B. afzelii*, *B. garinii*, and especially *B. spielmanii*, is only an indirect evidence of the participation of these genospecies in causing clinical symptoms of LB in humans, or their presence in *I. ricinus* in Province of Warmia-Masuria. Final confirmation would require ascertaining the presence and identifying genospecies of *B. burgdorferi* s.l. in the material taken directly from the patient and in the local populations of *I. ricinus*, using molecular biology methods. This has been conducted previously only to a limited extent in the Province of Warmia-Masuria [30, 37, 38].

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