ORIGINAL ARTICLE

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Assessment of the frequency of different Borrelia burgdorferi sensu lato species in patients with Lyme borreliosis from north-east Poland by studying preferential serologic response and DNA isolates

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Abstract

Introduction and objective. Several Borrelia burgdorferi sensu lato species cause Lyme borreliosis throughout Europe and their geographic distribution may influence clinical manifestations of the disease. In Poland, Lyme borreliosis presents mainly with neurologic and cutaneous symptoms, while clinically overt arthritis is rare. The presented study investigates the prevalence of B. burgdorferi s.l. genospecies in a group of patients with different clinical forms and stages of Lyme borreliosis in north-east of Poland. This has not previously been studied.

Material and methods. Preferential reactivity towards different B.burgdorferi s.l. species was investigated with a previously validated immunoblot assay in sera of 33 patients with disseminated Lyme borreliosis: 10 with neuroborreliosis, 6 with acrodermatitis chronica atrophicans and 17 with osteoarticular symptoms. Also typed were B.burgdorferi s.l. DNA isolated from the skin and synovial fluid of 7 patients with erythema migrans, acrodermatitis chronic atrophicans and arthritis.

Results. Preferential reactivity was detected in 30 out of 33 serum samples. Of these, 25 reacted preferentially with B.afzelii, 3 with B. garinii and 2 with B. burgdorferi ss. B.burgdorferi DNA was isolated from all studied samples and typed as B.afzelii in 5. In a patient with acrodermatitis chronica atrophicans studied with both methods simultaneously, B.afzelii was identified by both genotyping and serotyping.

Conclusions. Both methods gave consistent results, indicating B.afzelii as the main agent of all the clinical forms of the Lyme borreliosis in the study area.

Key words

Lyme borreliosis, Borrelia burgdorferi sl, Borrelia afzelii, aetiology, serologic typing, genotyping

INTRODUCTION

As manifestations of Lyme borreliosis differ depending on the causative species of Borrelia burgdorferi sensu lato (B. burgdorferi sl), the geographic distribution of species may be of clinical importance, influencing the clinical presentation of the disease in different areas [1, 2, 3, 4, 5]. Three B. burgdorferi sl species recognized as main agents of Lyme borreliosis in humans (B. burgdorferi sensu stricto, B. garinii and B. afzelii) and several others of less certain pathogenicity (B. valaisiana, B. lusitaniae, B. spielmani) coexist in Europe [6, 7, 8, 9, 10]. Of these, B. garinii is most frequently isolated from ticks throughout most of Europe, followed by B. afzelii, B. burgdorferi ss and B. valaisiana, with high regional and local variability, however [6, 7]. Analysis of B. burgdorferi sl isolates from Lyme borreliosis patients in different European countries have generally confirmed the high prevalence of B. afzelii and B. garinii [4, 5, 11, 12, 13, 14]. In Poland, the etiology of B. burgdorferi sl infections in humans has not been studied so far [15]. Surprisingly, B. burgdorferi ss has been the species most frequently found in infected ticks, with the exception of the areas in the south-east and north-east, where respectively B. garinii and B. afzelii dominate [16, 17, 18, 19].

Antigenic variability between B. burgdorferi sl species is reflected by a humoral response of the host, which offers the possibility of identifying an infecting genospecies on the basis of serologic data [20, 21, 22]. Norman et al. used a panel of antigens from eight B. burgdorferi sl strains, and found that sera from Lyme borreliosis patients in North America reacted more strongly to B. burgdorferi ss, and sera obtained in Europe – to B. afzelii and B. garinii, in accordance with their geographic distribution [20]. The assay based on this principle has also been validated by the direct identification of B. burgdorferi isolated from patients [22, 23]. Accuracy of serologic typing increases with the duration of the infection and with the increasing antibody titre and spectrum; the method is therefore valid mainly in late Lyme borreliosis [23]. Although the technique is indirect and its specificity may be limited by a cross-reactivity between B. burgdorferi sl species, it allows for the detection of the most probable causative

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species based on the serum sample only, and may be applied in the great majority of patients with late infection. On the other hand, isolation of B. burgdorferi sl DNA from tissues or body fluids is best performed in erythema migrans (EM) and becomes problematic or not feasible in disseminated infection, especially in late Lyme borreliosis [2, 24]. As the isolation-based techniques should be most reliable in patients with the early infection and serotyping in late infection, both methods may complement one another in epidemiologic investigations.

OBJECTIVES

The objective of the presented study was to identify a causative species of B. burgdorferi sl in patients with different clinical manifestations of Lyme borreliosis from north-east Poland. The sera of patients with disseminated Lyme borreliosis were studied with a previously described immunoblot protocol and performed B. burgdorferi sl DNA isolation and typing was performed from skin biopsy and synovial fluid samples.

MATERIAL AND METHODS

Patients. Thirty-three patients with different clinical manifestations of disseminated Lyme borreliosis admitted to the Department of Infectious Diseases and Neuroinfections in Białystok were tested for preferential serologic reactivity (Tab. 1). An attempt was made to select patients with the high probability of an active disseminated infection and with variable clinical manifestations, but preferably with a long-lasting disease, as well as with high serum levels and wide spectrum of the specific IgG antibodies against B. burgdorferi sl by conventional serologic techniques. All patients came from the highly endemic region in Podlaskie and Warmińsko-Mazurskie provinces in north-eastern Poland, and all reported tick bites or exposure to ticks. At the moment of the sample collection, the symptoms had been present for at least two weeks (in two patients with neuroborreliosis) and typically for much longer periods of time, up to a few years. Antibodies against B. burgdorferi sl were detected in serum drawn, together with the sample collected for serotyping or within a preceding month. In some patients with osteoarticular complaints of the large peripheral joints, no objective signs of arthritis were present when seen at the Department (patients described as suffering from artralgia in Tab. 1). Altogether, there were 10 patients with neuroborreliosis (including 9 with central nervous system involvement), 17 with osteoarticular symptoms (including 9 with overt arthritis on admission) and 6 with ACA. One patient diagnosed with ACA had accompanying involvement of the adjacent joint, and another had paresthesia and mild sensory deficit within the cutaneous foci. One patient with arthritis had co-existent mild neurologic symptoms and tested positively for intrathecal anti-B. burgdorferi sl IgG antibody synthesis.

The specific anti-B. burgdorferi sl IgG antibodies were detected with an ELISA kit (Biomedica, Boston, USA) and with at least one of the commercial immunoblot assays (Recomblot, Mikrogen, Germany, or Eco-line, Genzyme Virotech, Germany). Seven patients with central nervous system neuroborreliosis had intrathecal antibody synthesis confirmed by simultaneous immunoblot of serum and cerebrospinal fluid. In one patient, csf tests gave strongly positive results for specific IgG both with ELISA and immunoblot, but without confirmed intrathecal synthesis, and in one patient it was not possible to perform csf examination.

Table 1. Clinical and serologic data of the study patients

No	Sex	Age	Clinical form	Main clinical symptoms	Time since onset	anti-B. burgdorferi antibodies ELISA [a]	cerebrospinal fluid examination	
							pleocytosis	anti B. burgdorferi antibodies[b]
1	M	27	NB	VI cranial nerve paresis	7 months	IgM – 20 BBU/ml (+) IgG - 56 BBU/ml (+++)	270/ µl, 99% lymphocytes	IgM – 80 BBU/ml (+++), IgG – 77 BBU/ml (+++) immunoblot-blot IgG(+) [d]
2	M	45	osteoarticular	bilateral recurrent gonarthritis	2 years	IgM – 17 BBU/ml (+) IgG > 58 BBU/ml (+++)	-	-
3	F	24	NB	tremor, VII cranial nerve paresis	20 months	IgM – 21 BBU/ml (++) IgG - 88 BBU/ml (+++)	160/ µl, 90% lymphocytes	IgM – 6,4 BBU/ml (+) IgG – 98 BBU/ml (+++); immunoblot-blot IgG(+) [d]
4	F	59	osteoarticular	arthralgia	10 months	IgM – 14 BBU/ml (+) IgG - 53 BBU/ml (+++)	-	-
5	M	39	osteoarticular	arthralgia	>1 year	IgM (-) IgG - 59 BBU/ml (+++)	-	-
6	M	51	NB	polineuropathy	1 year	IgM (-) IgG - 55 BBU/ml (+++)	-	-
7	F	64	NB	radicular pain and spastic paresis of lower limbs	1 year	IgM – 23 BBU/ml (++) IgG - 47 BBU/ml (+++)	43/ µl, 90% lymphocytes	IgM (-) IgG – 72 BBU/ml (+++); immunoblot-blot IgG(+) [d]
8	M	33	osteoarticular	arthralgia	> 2 months	IgM (+/-) IgG - 41 BBU/ml (+++)	-	-
9	F	52	NB	mild paresis of the left limbs, VII and IX right cranial nerves	16 months	IgM – 17 BBU/ml (+) IgG - 99 BBU/ml (+++)	12 / µl, 100% lymphocytes	IgM (-), IgG – 33 BBU/ml (+++) immunoblot-blot IgG(+)
10	M	40	osteoarticular	arthralgia	4 years	IgM (-) IgG - 25 BBU/ml (++)	-	-

Table 1 (Continuation). Clinical and serologic data of the study patients

No	Sex	Age	Clinical form	Main clinical symptoms	Time since onset	anti-B. burgdorferi antibodies ELISA [a]	cerebrospinal fluid examination	
							pleocytosis	anti B. burgdorferi antibodies[b]
11	F	47	NB	VI and VII cranial nerves paresis	6 weeks	IgM – 21 BBU/ml (++) IgG – 14 BBU/ml (+)	243/ μl, lymphocyte predominance[c]	IgM (-) IgG (-)[c]
12	F	63	NB	mild hemiparesis and paresthesia	20 months	IgM – 23 BBU/ml (++) IgG – 83 BBU/ml (+++)	13/ μl	IgM 7 BBU/ml (+), IgG – 77 BBU/ml (+++); immunoblot-blot IgG(+)[d]
13	M	27	ACA, osteoarticular	elbow inflammation with local skin and subcutaneous induration	1 year	IgM (-) IgG – 94 BBU/ml (+++)	-	-
14	F	41	osteoarticular, NB	inflammation of the left ankle, arthralgia, tinnitus, paresthesia,	18 months	IgM (-) IgG – 73 BBU/ml (+++)	normal	immunoblot-blot IgG(+)[d]
15	M	49	osteoarticular	inflammation of the right wrist, recurrent artralgia	4 years	IgM (-) IgG > 79 BBU/ml (+++)	-	-
16	M	76	ACA	inflammatory/atrophic lesions on the dorsal side of both hands	> 6 months	IgG – 58 BBU/ml (+++)	-	-
17	F	76	osteoarticular	arthralgia, myalgia	9 months	IgM (-) IgG – 47 BBU/ml (+++)	-	-
18	M	46	osteoarticular	arthralgia, myalgia	11 years	IgM (-) IgG > 56 BBU/ml (+++)	-	-
19	M	69	ACA	typicall cutaneous lesion on the right hand and forearm	4 years	IgG – 17 BBU/ml (+)	-	-
20	F	62	ACA	typical cutaneous lesion 30 cm in diameter, on the thigh	2 years	IgM (-) IgG > 62 BBU/ml (+++)	-	-
21	M	73	osteoarticular	arthralgia	5 years	IgM (-) IgG – 87 BBU/ml (+++)	-	-
22	F	35	osteoarticular	recurrent gonarthritis	> 10 years	IgM (-) IgG – 80 BBU/ml (+++)	-	-
23	M	49	NB	meningitis, VII nerve paresis	5 months	IgM – 15 BBU/ml (+)	133/ μl, lymphocyte predominance	immunoblot-blot IgG(+)[d]
24	M	60	osteoarticular	mild bilateral gonarthritis, recurrent artralgia	4 years	IgG – 74 BBU/ml (+++)	-	-
25	M	60	osteoarticular	recurrent artralgia, tendonitis of Achilles tendon	4 years	IgG (+++)	-	-
26	M	54	osteoarticular	artralgia	2 years	IgM (-) IgG > 72 BBU/ml (+++)	-	-
27	F	27	osteoarticular	severe bilateral gonarthritis (assymetrical), arthritis/artralgia of other large joints (wrists, ankles temporomandibular joint)	5 months	IgM – 23 BBU/ml (++) IgG – 67 BBU/ml (+++)	-	-
28	F	53	NB	VII nerve paresis	6 weeks	IgM - 17 BBU/ml (+) IgG – 32 BBU/ml (+++)	906/ μl, 94% lymphocytes	immunoblot-blot IgG(+)[d]
29	M	60	osteoarticular, NB[f]	cutaneous lesions on the both tights, with local sensory deficit and paresthesia	2 years	IgM (-) IgG - 58 BBU/ml (+++)	-	-
30	F	55	osteoarticular[e]	recurrent gonarthritis	10 years	IgM (-) IgG - 44 BBU/ml (+++)	-	-
31	M	52	ACA[f]	typical lesion on the elbow	2 years	IgM (-) IgG > 69 BBU/ml (+++)	-	-
32	M	66	NB	distal polineuropathy	3 years	IgM (-) IgG - 25 BBU/ml (+++)	1 / μl, increased protein concentration	immunoblot-blot IgG(+)[d]
33	M	43	osteoarticular[e]	mild gonarthritis, artralgia	6 months	IgM (-) IgG - 72 BBU/ml (+++)	-	-

[a] expressed in BBU/ml (Borrelia Biomedica Units/ml, cutoff – 11 BBU/ml for serum, 5 BBU/ml for cerebrospinal fluid, according to manufacturer instruction); (-) negative, (+) positive, (++) high positive, (+++) very high positive; all positive ELISA IgG results were confirmed with immunoblot: in patient 20 – with Recomblot assay from Mikrogen (Germany), in all the other patients – EcoLine from Genzyme Virotech (Germany)
[b] simulateneously with the serum antibodies examination and with the same ELISA assay, except for patients 11 and 14
[c] cerebrospinal fluid general and serologic examination performed in a different centre, before transfer of the patient
[d] reactivity stronger/ wider than in serum, suggesting intrathecal synthesis
[e] diagnosis confirmed with the synovial fluid PCR (untypable isolate)
[f] diagnosis confirmed with the skin biopsy PCR (B. afzelii)
B.b. – Borrelia burgdorferi sensu lato
NB – neuroborreliosis
ACA – acrodermatitis chronica atrophicans
LC – Lyme carditis
M – male
F – female

Patients with atypical or rare presentations (artralgia, peripheral nervous system involvement, central neuroborreliosis without proven intrathecal antibody synthesis, or without confirmed pleocytosis) were enrolled only if all the available data strongly supported the diagnosis and allowed for the recognition of probable Lyme borreliosis and antibiotic treatment. All patients came from highly endemic areas, reported temporal relation of the symptoms to tick bites, were unequivocally seropositive to B. burgdorferi sl in the repeated serologic tests, had no co-existent pathology which could explain their symptoms and responded to the antibiotic treatment. Serum samples were obtained on admission before the antibiotic treatment, together with venous blood for diagnostic purposes.

In four patients, in whom material from the infection site was obtained for diagnostic reasons, DNA isolation and genotyping was attempted. These included patients 29 and 31 with ACA (skin biopsy for histopathologic confirmation) and patients 30 and 33 with arthritis (synovial fluid). Three more patients with erythema migrans (EM) seen at the Department were enrolled for DNA isolation and typing. Punch biopsies were taken from the edge of EM lesions before the start of antibiotic treatment. In one of these patients, simultaneously obtained venous blood sample was also tested.

The study was approved by the Ethical Board of the Medical University in Białystok and the patients gave informed consent for participation.

Serotyping. Serum was isolated within 30 min. after sample collection and frozen at -80 °C until further study. The laboratory procedure and calculation of the results were conducted strictly as described by Ryffel et al., with the use of the same strains (B. burgdorferi ss VS215, B. afzelii VS461, B. garinii VS102 and B. valaisiana VS116) as a protein source for Western-blotting, and performed in the same laboratory (Central Institute of Valais Hospital, Infectious Diseases Department, Sion, Switzerland), where the procedure is used routinely for diagnostic purposes [22]. Identification of the bands with B. burgdorferi sl proteins was performed and the reproducibility of the results obtained with the procedure confirmed as described previously by Ryffel et al. [22].

The blots were read by a single person who was unaware of the patients' clinical data. The intensity of each band was scored for 0-3 points and responses to each genospecies' antigens were summed up. In the first step, the response towards p12, p16, p18, OspA and p66 was estimated, and the reactivity by ≥ 2 points higher with any strain than with the others was considered preferential (method I). If this criterion was not fulfilled, another 14 antigens (p14, p20, OspC, OspD, p30, p35, p39, p41, p45, p55, p58, p60, p73 and p93) were included in the analysis with the response of ≥ 3 points above the others interpreted as preferential (method II); if such were lacking, the result was considered undetermined. The validity of this scoring system was assessed previously by Ryffel et al. [22].

DNA isolation, amplification and typing. DNA isolation was performed with a QIAamp DNA Mini Kit, strictly according to the manufacturer's instructions and directly after obtaining the sample. Real-time PCR for the gene encoding OspA was performed, as described previously by Rauter et al., using a LightCycler rapid thermal cycler system (Roche Diagnostics GmbH, Mannheim, Germany) and a reverse primer, published previously by Demaerschalck et al. [12, 25]. The genotyping of the amplified DNA was performed with the melting curve technique as described by Rauter et al. [25]. Briefly, the LC Red 640 fluorescence was monitored continuously while the temperature was raised from 54 to 95 °C, and a sharp decrease in fluorescence was observed in the temperature at which the probe dissociates from the template. When the strand and the probe are perfectly matched, strong hybridization results and the melting temperature (T_m) is high. Any mismatch results in a lower T_m, due to the decreased hybridization stability that is reflected in the peak of the first derivative of the melting curve. The probe was designed to match the sequence of B. afzelii, while the sequences of B. garinii and B. burgdorferi ss contain two and three mismatches with the probe, respectively, allowing for a characteristic melting profile for each genotype. The average T_m of the reference strains of B. burgdorferi ss (N40), B. garinii (PSth), and B. afzelii (VS461), were previously set at 63, 68, and 72.5 °C, respectively [25].

The LightCycler PCR assay based on the FRET technology targeting the flagellin gene, developed in order to increase sensitivity of molecular diagnostic for Borrelia, was performed simultaneously on the same set of samples as the ospA assay. For this assay, primers and probes were designed using the programme 'Light Cycler probe design Software, version 1.0'. Primers and probes were adapted to amplify all the Borrelia burgdorferi sensu lato isolates. Final concentration of primers and probes were 0.5μM and 0.2 μM, respectively (Tab. 2). Both PCR were run simultaneously in individual capillaries. The flagellin PCR was not primarily designed for typing of amplicons. Temperatures for the melting curves extended from 56 °C, 57 °C, 58 °C and 62 °C for B. garinii (isolate VS BP), B. valaisiana (reference strain VS 116), B burgdorferi (isolate VS 215) and B. afzelii (reference strain VS 461), respectively.

Table 2. Nucleotide sequences of primers and probes for flagellin encoding gene used in this study

Name	Nucleotide sequences (5'-3')	conc (μM)
Bbfla1	RGG TGG AGT TAA TTC TCC T	0.5
Bbfla 2	CAA GTC TAT TTT GGA AAG CAC CT	0.5
Bbfla 640	X CTC TTG CTA AAA TAG AAA ATG CTA TTA GAA TGA TAA GT	0.2
Bbflaflu	ATG TTA CAA CCA CAG TTG ATG CTA ATA CZ	0.2

X = LCRed640- labeled base
Z = fluorescein-labeled base

Validation of the flagellin PCR was achieved with a selection of 27 typed B. burgdorferi sensu lato isolates belonging to B. burgdorferi, B. afzelii, B. garinii, B. valaisiana [25], bacteria (E.coli, Treponema pallidum, Proteus mirabilis, Enterobacter sp., Salmonella sp., Campylobacter sp. Listeria monocytogenes) and 20 quality control specimens (INSTAND 2007-2011). Both specificity and sensitivity were perfectly demonstrated. Melting curve analysis allows to type reliably only typical B. afzelii isolates (melting temperature 62 °C), since one mismatch may induce false interpretation with this PCR assay.

RESULTS

Serotyping. The preferential reactivity was detected with method I in 21 of 33 patients (64%) and with method II in a further eight (24%), allowing for identification of the preferential reactivity according to the pre-established criteria in 29 patients (88%). In one other patient the score could not be counted because of the extremely strong response towards one of the species (B. burgdorferi ss), with coalescent bands throughout the stripe – the reactivity towards this species was considered preferential, although the bands could not be reliably differentiated and thus the reactivity score counted. Undetermined results were obtained in two patients with neuroborreliosis, both with the shortest duration of the disease in the study group (less than 1 month). In one patient with ACA the response was too weak to classify as positive and the score was not counted.

In 25 out of 30 patients, preferential reactivity towards B. afzelii was observed, irrespective of the clinical form of the disease (osteoarticular symptoms, central and peripheral neuroborreliosis, ACA). The representative immunoblot with preferential response to B. afzelii in patient 22 with Lyme arthritis is presented in Figure 1 and Table 3.

Three patients had preferential response towards B. garinii. Two of them presented with osteoarticular symptoms, one with gonarthrits and the other (Tab. 4) with recurrent artralgia without proven arthritis. The third patient with preferential reactivity towards B. garinii had typical subacute meningitis with facial paresis.

Sera of two patients with arthritis reacted preferentially with B. burgdorferi ss. One of them presented with severe arthritis which resolved slowly after antibiotic treatment. No preferential reactivity with B. valaisiana was detected. The scores of reactivity with the four B. burgdorferi sl genospecies for all the study patients are shown in Tab. 5 and the relation between the clinical manifestation and the result of immunoblot is summed up in Tab. 6).

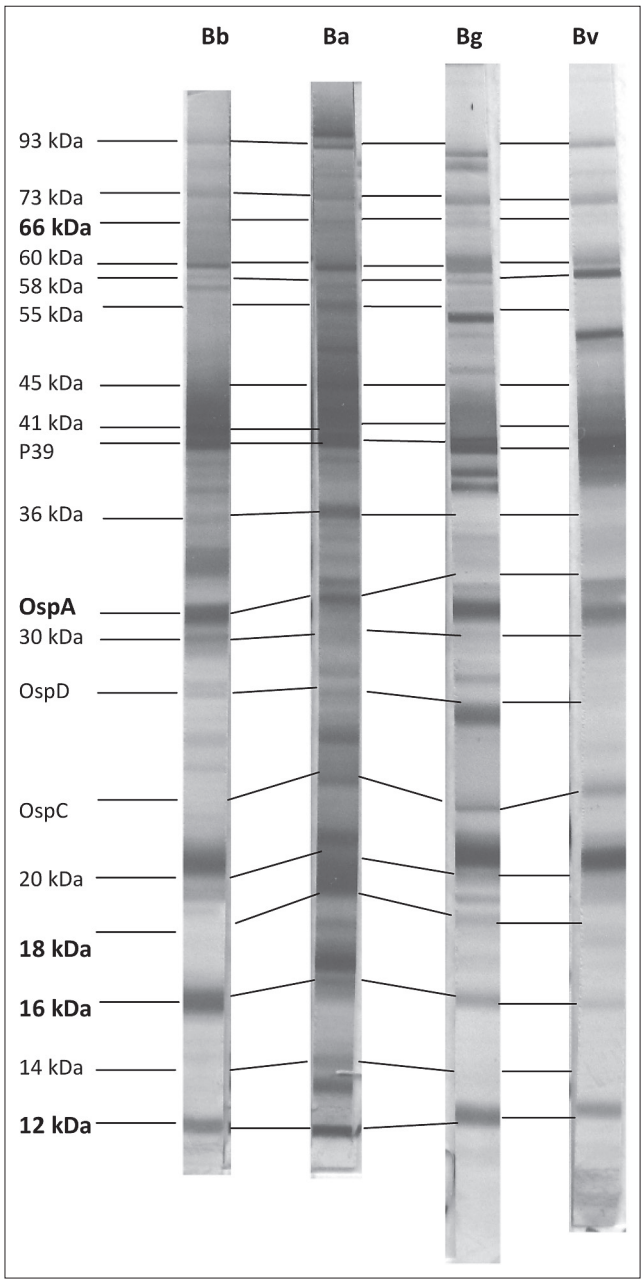

Figure 1. Example of an immunoblot with a preferential reactivity for B. afzelii. Result obtained with serum of patient 22 diagnosed with Lyme arthritis. The bands corresponding to proteins included in the assay are identified at left. Boldface indicates the discriminative proteins used in method I. Scores of reactivity for each band of the presented immunoblot are listed in Table 3.
B.b.ss – B. burgdorferi sensu stricto VS215; B.a. – B. afzelii VS461; B.g. – B. garinii VS102; B.v. – B. valaisiana VS116.

Table 3. Scores for reactivity of a serum sample from patient 22 with a clinical diagnosis of Lyme arthritis [a], with a preferential reactivity to B. afzelii (method I and II) to the 19 proteins from 4 Borrelia burgdorferi sensu lato species. Boldface indicates the discriminative proteins used in the method I

Protein band	Score [b]			
	B. b. ss	B.a.	B.g.	B.v.
93 kDa	1	3	0	2
73 kDa	1	2	2	2
66 kDa	**0**	**2**	**1**	**0**
60 kDa	2	3	2	2
58 kDa	1	2	1	3
55 kDa	0	3	1	0
45 kDa	2	3	0	0
41 kDa	3	3	2	2
p39	3	3	3	3
35 kDa	1	3	0	0
OspA	**3**	**3**	**0**	**0**
30 kDa	2	1	0	0
OspD	1	1	0	0
OspC	0	3	2	2
20 kDa	0	3	1	0
18 kDa	**0**	**3**	**1**	**0**
16 kDa	**3**	**2**	**2**	**1**
14 kDa	0	2	0	0
12 kDa	**2**	**3**	**2**	**2**
Score method I [c]	**9**	**13**	**6**	**3**
Score method II [d]	26	48	20	21

[a] the same patient as in Fig. 1
[b] B.b.ss – B. burgdorferi sensu stricto; B.a. – B. afzelii; B.g. – B. garinii; B.v. – B. valaisiana
[c] score with the proteins 12 kDa, 16 kDa, 18 kDa, OspA and 66 kDa – discriminative, if the scored reactivity to one genospecies was at least two points greater than the reactivity for any other species
[d] score with all the included proteins - discriminative, if the scored reactivity to one genospecies was at least three points greater than the reactivity for any other species

Table 4. Scores for reactivity of a serum sample from patient 4 with a preferential reactivity to B. garinii (method II) to the 19 proteins from 4 Borrelia burgdorferi sensu lato species. Boldface indicates the discriminative proteins used in the method I

Protein band	Score [a]			
	B. b. ss	B.a.	B.g.	B.v.
93 kDa	3	3	3	3
73 kDa	1	1	1	1
66 kDa	**1**	**1**	**1**	**1**
60 kDa	2	1	2	1
58 kDa	3	0	2	1
55 kDa	1	2	3	3
45 kDa	2	3	2	2
41 kDa	3	3	3	3
p39	2	3	3	3
35 kDa	1	1	2	1
OspA	**0**	**1**	**1**	**2**
30 kDa	1	2	1	2
OspD	1	0	2	1
OspC	2	3	3	3
20 kDa	1	2	3	1
18 kDa	**0**	**3**	**3**	**1**
16 kDa	**0**	**0**	**0**	**0**
14 kDa	0	0	0	0
12 kDa [b]	**-**	**-**	**-**	**-**
Score method I [c]	**1**	**5**	**5**	**4**
Score method II [d]	24	29	35	29

[a] B.b.ss – B. burgdorferi sensu stricto; B.a. – B. afzelii; B.g. – B. garinii; B.v. – B. valaisiana
[b] 12 kDa band did not present in the immunoblot of this patient due to the differences in antigen band migration during electrophoresis and could not be scored
[c] score with the proteins 12 kDa, 16 kDa, 18 kDa, OspA and 66 kDa – discriminative, if the scored reactivity to one genospecies was at least two points greater than the reactivity for any other species
[d] score with all the included proteins - discriminative, if the scored reactivity to one genospecies was at least three points greater than the reactivity for any other species

Table 5. Results of the immunoblotting in all the study patients

No	Scores of reactivity with four B. burgdorferi sensu lato genospecies[a]								Preferential reactivity
	method I[b]				method II[c]				
	B.b.ss	B.a.	B.g.	B.v.	B.b.ss	B.a.	B.g.	B.v.	
1	6	8	3	4	22	**32**	27	21	B. afzelii
2	1	8	2	7	17	**33**	25	20	B. afzelii
3	2	**7**	1	2	-	-	-	-	B. afzelii
4	1	5	5	4	23	24	**30**	25	B. garinii
5	1	**5**	2	2	-	-	-	-	B. afzelii
6	1	**5**	1	2	-	-	-	-	B. afzelii
7	3	**10**	6	5	-	-	-	-	B. afzelii
8	3	**8**	2	2	-	-	-	-	B. afzelii
9	4	**9**	4	5	-	-	-	-	B. afzelii
10	5	**10**	4	3	-	-	-	-	B. afzelii
11	3	4	3	3	20	22	22	18	undetermined
12	4	**9**	4	5	-	-	-	-	B. afzelii
13	2	**8**	5	3	-	-	-	-	B. afzelii
14	2	**7**	2	1	-	-	-	-	B. afzelii
15	1	**7**	2	4	-	-	-	-	B. afzelii
16	8	9	6	8	22	**28**	24	25	B. afzelii
17	5	**9**	5	6	-	-	-	-	B. afzelii
18	2	**7**	4	3	-	-	-	-	B. afzelii
19	6	**12**	3	5	-	-	-	-	B. afzelii
20	4	**9**	6	5	-	-	-	-	B. afzelii
21	6	**9**	6	4	-	-	-	-	B. afzelii
22	9	**13**	6	6	26	**48**	20	21	B. afzelii
23	2	3	3	3	21	21	**25**	17	B. garinii
24	3	4	4	4	15	**20**	16	10	B. afzelii
25	2	**6**	3	3	-	-	-	-	B. afzelii
26	2	**4**	2	2	-	-	-	-	B. afzelii
27	4	4	4	4	**14**	8	11	10	B. burgdorferi ss
28	4	4	5	4	17	12	17	12	undetermined
29	-	-	-	-	-	-	-	-	negative [d,e]
30	2	4	6	1	-	-	-	-	B. garinii
31	3	**7**	5	3	-	-	-	-	B. afzelii [d]
32	1	**6**	4	2	-	-	-	-	B. afzelii
33	-	-	-	-	-	-	-	-	B. burgdorferi ss [f]

[a] the score fulfilling the criteria of the preferential reactivity is marked with bold characters
[b] score with five proteins (12 kDa, 16 kDa, 18 kDa, OspA and 66 kDa) – discriminative, if the scored reactivity to one genospecies was at least two points greater than the reactivity for any other species
[c] score with 19 proteins - discriminative, if the scored reactivity to one genospecies was at least three points greater than the reactivity for any other species
[d] skin biopsy PCR – B. afzelii
[e] not serotyped, because not fulfilling the criteria for positivity towards B. burgdorferi sensu lato
[f] extremely strong response towards B. burgdorferi ss (coalescing bands not allowing for the scoring according to the method)
B.b.ss – B. burgdorferi sensu stricto; B.a. – B. afzelii; B.g. – B. garinii; B.v. – B. valaisiana

Table 6. Preferential reactivity towards B. burgdorferi s.l. genospecies versus clinical form of Lyme borreliosis in the group of 30 patients with the conclusive result (number/percentage)

	Preferential reactivity (number / percentage)		
	B. afzelii	B. garinii	B. burgdorferi ss
osteoarticular (n = 17)	13 / 76%	2 / 12%	2 / 12%
NB (n = 8)	7 / 88%	1 / 12%	0
ACA (n = 5)	5 / 100%	0	0
total LB (n = 30)	25 / 83%	3 / 10%	2 / 7%

LB – Lyme borreliosis
NB – neuroborreliosis
ACA – acrodermatitis chronica atrophicans

DNA isolation and genotyping. B. burgdorferi sl DNA was amplified from all eight studied samples, including detection of the flagellin gene in all the samples and OspA gene in four samples from four patients: two EM biopsies, one ACA biopsy and one synovial fluid sample. Successful typing was possible with all the skin samples, and the species detected in all five was B. afzelii, although only one sample had identical profile with the reference strain VS461 (Tab. 7).

Table 7. Results of the B. burgdorferi DNA isolation and typing from the 7 patients with different clinical manifestations of Lyme borreliosis

Patient	Clinical form	Serum preferential reactivity	Material	DNA amplification		Genotyping result
				OspA gene	Flagellin gene	
Em1[a]	EM	ND	Skin biopsy	(-)	(+)	B. afzelii
			Venous blood	(-)	(+)	untypable
Em2[a]	EM	ND	Skin biopsy	(+)	(+)	B. afzelii
Em3[a]	EM	ND	Skin biopsy	(+)	(+)	B. afzelii
29[b]	ACA	negative	Skin biopsy	(-)	(+)	B. afzelii
30[b]	LA	B. burgdorferi ss	SF	(+)	(+)	untypable
31[b]	ACA	B. afzelii	Skin biopsy	(+)	(+)	B. afzelii[c]
32[b]	LA	B. garinii	SF	(-)	(+)	untypable

[a] patients with erythema migrans – not eligible for serotyping
[b] patients included in the serotyping group, presented in table 1 and table 4
[c] full match with the reference strain B. afzelii VS461
ND – not done
EM – erythema migrans
ACA – acrodermatitis chronic atrophicans
LA – Lyme arthritis
SF – synovial fluid

DISCUSSION

The B. burgdorferi sl species show some level of organ specificity, which is reflected by the most typical symptoms of the infection with each of them. In North America, where B. burgdorferi ss is the only species infecting humans, Lyme arthritis is a typical form of a systemic infection, while neuroborreliosis is less frequent and ACA absent altogether. This contrasts with a more variable spectrum of Lyme borreliosis in Europe, where B. garinii and B. afzelii are more common than B. burgdorferi ss [2, 20]. In European patients, B. garinii has been found to be the most frequent cause of neuroborreliosis and B. afzelii of ACA, while in Lyme arthritis all three genospecies have been identified, with a relatively high proportion of B. burgdorferi ss [1, 11].

A very high rate of preferential reactivity towards B. afzelii was found in the sera of Lyme borreliosis patients from north-east Poland, in the area where, to our knowledge, there have been no previous data on B. burgdorferi sl species in human infections. Preferential reactivity towards B. afzelii was present in patients, irrespective of the clinical manifestation of the disease, including different forms of neurologic and osteoarticular involvement. This result was surprising, both in the light of the clinical investigations linking different Lyme borreliosis presentations to different species, and the studies which have addressed the prevalence of B. burgdorferi sl species in infected ticks in other parts of Poland, and found B. burgdorferi ss to prevail in the most of the country, and B. garinii in the south-eastern area close to the border with Slovakia [16, 17, 18]. However, as those studies were performed in regions several hundred kilometres to the south or west of the site of the presented study, the discrepancy may be due to local geographic variability. In the preliminary studies of the infected ticks collected in north-eastern Poland, B. afzelii was the most frequent species, followed by B. garinii, while B. burgdorferi ss was rare, which is much more similar to, but still not identical with the presented patient data [19]. Of note, Ranka et al. detected B. afzelii in 69% of infected ticks in Latvia (as an only species in 65% and in co-infection with B. garinii in 4%), which is geographically closer to the presented study area than some previously investigated sites in Poland [26]. Also, Galdakaite et al. found B. afzelii to be the predominant genospecies in I. ricinus and I. persulcatus ticks from Lithuania, Lativia and Estonia, with only a small fraction of ticks infected with B. valaisiana or B. garinii [27]. These data may suggest that B. burgdorferi sl species prevalence in the north-east of Poland is more similar to that in the neighbouring Baltic States than to the other parts of Poland. Alternatively, strains of the genospecies other than B. afzelii could be present in ticks and an animal reservoir in the area, as in the other Polish sites, but for some reason could only rarely cause a chronic, symptomatic infection in humans, which would explain their low frequency in the studied patients. The frequency of borrelial strains in a given area may be different in ticks, in erythema migrans isolates, and in disseminated human infections, due to different pathogenecity and invasiveness of particular strains, as shown by Wormser et al. for B. burgdorferi ss ospC genotypes in North America [28].

As the serum of patients with Lyme borreliosis reacts with antigens of all B. burgdorferi species, the difference in reactivity is quantitative and its estimation not straightforward, but requires a careful comparison of immunoblots with a pre-established scoring system. It could be suspected that the unexpectedly high frequency of preferential reactivity towards B. afzelii was an artefact of the method reflecting a tendency of the anti-B. burgdorferi sl antibodies to react unexpectedly strongly with B. afzelii antigens. The method applied here has been originally tested in a group of 96 Swiss patients, in which the fraction of unequivocal results was minimal (6-10%) and re-examination after > 6 months gave identical result in five out of six patients. The results were validated by direct analysis of the isolated B. burgdorferi sl DNA in a subgroup of patients: of the 14 patients in whom DNA was successfully isolated and typed, concordant results were obtained in 11 (79%) [22]. The frequency of particular genospecies differed from the presented study, as a much higher frequency of a preferential reactivity towards B. burgdorferi ss and B. garinii was observed. There was also a strong tendency for the correlation of a species with a clinical manifestation: 84% of patients with ACA had preferential reactivity towards B. afzelii, 62% of patients with arthritis – towards B. burgdorferi ss and 52% of patients with neuroborreliosis – towards B. garinii, while the rate of a preferential reactivity towards B. valaisiana, a species of a dubious pathogenicity, was accordingly very low [22]. These highly different patterns of serologic response in the two groups of patients studied with the identical methodology confirm that these groups had been exposed to B. burgdorferi sl strains with different antigenic properties, and that the method used is able to detect this difference. The most natural interpretation is that B. afzelii is actually much more frequent in north-east Poland as an agent of Lyme borreliosis, and the results of the latest tick studies discussed above support this possibility. As strains within a single B. burgdorferi sl species may also present with a high antigenic variability [20], immunoblot validated in a specific area could theoretically produce misleading results in a different population exposed to antigenically different strains of the same B. burgdorferi sl species, which presents a possible limitation to the presented results. The strong correlation of the serotyping result with the directly identified causative species has been confirmed

in the Swiss population, and for the current study an attempt was made to gather data extending this observation to the presented patient group [22].

For the presented study, B. burgdorferi sl DNA were originally analysed from EM biopsies as the easiest way for isolation, although it did not allow for direct comparison with the serotyping results. All three EM samples yielded a strain of B. afzelii, with the same features in all three, but different from the reference strain VS461. In the next step, four patients were studied in whom both positive serum samples and material from the disease lesion could be obtained. Of these, only two ACA isolates could be typed and both belonged to B. afzelii, with one matching the isolate from EM samples and the other identical to the reference strain. In the first of these patients, the serum sample could not be typed because of a too weak serologic response; therefore, a direct comparison of the results was possible in only one patient in whom the species identified by both methods was B. afzelii. Although a larger group of patients will be necessary to validate the results, the fact that all the human isolates typed were B. afzelii is notably consistent with the serotyping data.

In the presented study group, most of the patients with osteoarticular involvement and a preferential reactivity towards B. afzelii or B. garinii had a mild and unspecific disease, with mild arthritis localised to one joint or with mainly subjective symptoms. On the other hand, the first patient in whom preferential reactivity towards B. burgdorferi ss was detected presented with a relatively severe gonarthritis, and simultaneous involvement of several other joints, slowly resolving after antibiotic treatment, which is a very uncommon presentation in the study location, but reminiscent of the classical Lyme arthritis described in the area of B. burgdorferi ss prevalence in North America [2, 29]. The number of patients in whom preferential reactivity toward B. burgdorferi ss and B. garinii was observed was very small, which made it impossible to analyse them as separate groups. However, it seems that the known tendency of these species to cause specific clinical manifestations was also present in the patients in the presented study, but was simply difficult to document because of the very high predominance of B. afzelii. The low prevalence of B. burgdorferi ss may account for the mild and atypical course of the osteoarticular form of Lyme borreliosis and scarcity of severe Lyme arthritis in the studied area.

CONCLUSIONS

Based on both serologic and genotyping data, B. afzelii seems to be the main etiologic agent of Lyme borreliosis in the endemic area in north-eastern Poland, responsible for the majority of cases of all the manifestations of the disease. The serotyping technique originally validated in a Swiss group of patients seems to produce reliable results in a different patient population from a different geographical area, although further validation with a larger group of patients will be necessary.

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